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From: (b) (6)
To: (b) (6)
Cc: Laing, Eric; (b) (6) ; Christopher Broder
Subject: Re: HJF/USU EID-SEARCH Y4 Scope of Work_draft to edit
Date: Wednesday, August 9, 2023 2:31:37 PM
Attachments: USU HJF EID-SEARCH Y4 Scope of Work_draft v04(b) (6) .docx

---

Hello,

Attached is the updated SOW and budget.

Let me know if you have any questions. And when you will be able to provide the next modification.

Thanks much .... (b) (6)

On Wed, Aug 9, 2023 at 12:10 PM (b) (6) > wrote:

> Hi (b) (6) ,
>
> If you want to reallocate funds within your budget for the travel to Malaysia meeting, please feel free to do it (We expect that (b) (6) could travel to Malaysia for training or troubleshooting in the draft scope of work we shared)
>
> If you're concerned about the available fund for travel within your budget, let me know and we can see how to cover the cost from EHA's budget.
>
> Best,
> (b) (6)
>
> [redacted]
>
> [redacted]
>
> [redacted]
>
> [redacted]
>
> On Wed, Aug 9, 2023 at 11:36 AM Laing, Eric (b) (6) wrote:
>
> > Hi (b) (6) ,
> >
> > I made a couple bullet point changes yesterday. (b) (6) has that version and will be able to provide the update. Also, (b) (6) was invited to a CCM/EHA conference in Oct, can you use EID-SEARCH travel funds for his airfare/lodging?
> >
> > - (b) (6)

(b) (6)

On Fri, Aug 4, 2023 at 4:38 PM (b) (6) >
wrote:

> Here is the updated v3 - it includes revisions to the budget.
>
> Thanks much .... (b) (6)
>
> On Wed, Aug 2, 2023 at 10:03 PM (b) (6) >
> wrote:
>
> > I have two comments for (b) (6) regarding the scope of the work.
> >
> > On Wed, Aug 2, 2023 at 11:54 PM (b) (6)
> > wrote:
> >
> > > FYI - your project ... bringing you into the discussion.
> > >
> > > Attached is the current SOW - while (b) (6) is on vacation, will you take a look and see if anything needs to be updated/changed.
> > >
> > > Thanks much .... (b) (6)
> > >
> > > ---------- Forwarded message ---------
> > > From: (b) (6) >
> > > Date: Wed, Aug 2, 2023 at 12:51 PM
> > > Subject: Re: HJF/USU EID-SEARCH Y4 Scope of Work_draft to edit
> > > To: (b) (6)
> > > Cc: eric.laing (b) (6) Broder, Christopher
> > > (b) (6)
> > >
> > > Sounds good!
> > >
> > > I will get the updated statement of work back to you as soon as possible.
> > >
> > > And we will be looking for the updating reporting requirements on

the next agreement.

Thanks much .... (b) (6)

On Mon, Jul 31, 2023 at 2:58 PM (b) (6) > wrote:

Hi (b) (6),

Thanks for the quick response and update. No problem, we can wait until Dr. (b) (6) is back from vacation.

More changes will be shown in the **body of the contract** that NIH is reviewing now, we'll share them with you once NIH approves it. They are mostly about specifying everything in detail (e.g., we included general languages in the Y3 contract by referring to relevant policies, now we need to elaborate more in writing), and you can see we'll include the whole "NIH Grants Policy Statement" as Attachment F.

In the file I shared, most changes in Attachment A are on #2 and #3 to describe how we manage and oversee the project. So there will be more regular calls at least every month and more regular reporting, including quarterly reporting with specified deadlines (we'll further discuss with the team the most efficient formats for these reportings).

We also included the "Data and Resroucing Sharing Plan" from the originally submitted proposal as Attachment D to demonstrate what we will do for data sharing following the NIH policies.

Please feel free to email or call if anything I can further clarify. And once we get the approved contract template from NIH, it will be helpful if we can get on a Zoom meeting to go through the changes and explain everything as needed.

Thanks,
(b) (6)

[redacted]

On Mon, Jul 31, 2023 at 2:34 PM (b) (6)

(b) (6) wrote:

Hello (b) (6),

Dr (b) (6) is out on vacation right now - Can you highlight what changes or specific aims NIH would like us to address?

Thanks much ... (b) (6)

On Mon, Jul 31, 2023 at 1:33 PM (b) (6) > wrote:

Dear (b) (6) and (b) (6),

I hope you are doing well.

While we are waiting for the Year 4 Notice of Award (U01AI151797), we would like to start discussing with you the scope of work for Year 4.

Please take a look at the draft in the attached file, and **feel free to edit and add details to the planned research activities and update the budget accordingly.**

Please note that the funder specifically required information related to the reporting after they reviewed our Y3 contracts, so we have to include more details and adhere to them.

Please let (b) (6) and me know if you have any questions. We can plan to discuss the work plan at the project all-partner meeting next Wednesday or set up a separate call. Thank you very much!

Best Regards,

(b) (6)

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(b) (6)

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(b) (6) [redacted]

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(b) (6) [redacted]

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(b) (6) [redacted]

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(b) (6) [redacted]

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(b) (6) [redacted]

Confidentiality Notice: This email message, including any attachments, is for the sole use of the intended recipient(s) and may contain confidential and privileged information. Any unauthorized use, disclosure or distribution is prohibited. If you are not the intended recipient, please contact the sender by replying to this e-mail and destroy all copies of the original message.

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From:	(b) (6)
To:	eric.laing_usuhs; Broder, Christopher
Cc:	(b) (6)
Subject:	HJF/USU EID-SEARCH Y4 Scope of Work_draft to edit
Date:	Monday, July 31, 2023 1:34:18 PM
Attachments:	USU HJF EID-SEARCH Y4 Scope of Work_draft v01.docx

---

Dear (b) (6) and (b) (6),

I hope you are doing well.

While we are waiting for the Year 4 Notice of Award (U01AI151797), we would like to start discussing with you the scope of work for Year 4.

Please take a look at the draft in the attached file, and **feel free to edit and add details to the planned research activities and update the budget accordingly.**

Please note that the funder specifically required information related to the reporting after they reviewed our Y3 contracts, so we have to include more details and adhere to them.

Please let (b) (6) and me know if you have any questions. We can plan to discuss the work plan at the project all-partner meeting next Wednesday or set up a separate call. Thank you very much!

Best Regards,

(b) (6)

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From: (b) (6)
To: (b) (6) ; Chris Broder; (b) (6)
eric.laing (b) (6)
Cc: (b) (6)
Subject: EID-SEARCH NoA and Year 3 Report as submitted
Date: Thursday, April 13, 2023 9:42:25 AM
Attachments: NIH_NOA_1U01AI151797-03 with highlights.pdf
5U01AI151797 Y3 Annual Report As Submitted_without budget.pdf

---

Dear All,

Thank you for the call yesterday!

As discussed, I am sharing the Year 3 Notice of Award (NoA) of the EID-SEARCH project and the Year 3 Research Performance Progress Report (RPPR) report as submitted.

The NoA is issued annually, and this Y3 NoA has been attached to the Y3 contracts we signed. You can see these special requirements as highlighted on Pages 6-7.

In addition to the annual RPPR report (dues April 1 every year), we are also required to submit a semi-annual report. The semi-annual report is less structured compared to the RPPR, focusing on research activities mainly without needing to fill in different sections.

In the attached RPPR report, Page 27 G.1 Section is where we report about the special reporting requirements as described in the NoA.

Please feel free to let (b) (6), and me know if you have any questions about these documents. And thank you very much for your understanding to help the project comply with these requirements.

Sincerely,
(b) (6)

On Tue, Apr 11, 2023 at 3:07 PM (b) (6) > wrote:

> Dear All,

Reminder of our EID-SEARCH all partner meeting coming up tomorrow (Wednesday night US time, Thursday morning SE Asian time).

We're going to be doing these every month, with the key goals of 1) identifying important issues from EID-SEARCH HQ, e.g. requests from NIAID etc.; 2) to help each partner to find out what the other partners are doing; 3) brainstorm solutions to problems, and ways to increase efficiency of sampling, testing, characterization, surveys and paper publishing.

At this first meeting, I'll start off with a quick round up of overall EID-SEARCH updates, then we'll just go round the table and get an update from each partner.

Please make sure that at least one or two people from your team will be joining, and get ready to be put on the spot to give a quick update…

Look forward to talking and to having these regularly!

Cheers,

(b) (6)

(b) (6)

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From: (b) (6) [redacted]
Sent: Tuesday, March 21, 2023 11:58 AM
To: (b) (6) [redacted]
'Chris Broder' (b) (6) [redacted]
Cc: (b) (6) [redacted]
eric.laing (b) (6) [redacted]
(b) (6) [redacted]

[redacted]

Subject: EID-SEARCH All-partner calls every month, starting April 12th (April 13th in

Asia)
Importance: High

Hello EID-SEARCH team!

Now that we're near the end of Year 3, and with work really cranking up now in our project, and multiple groups doing all forms of testing and analyses, I'm canceling the 'lab meetings' and setting up a monthly ALL-PARTNER meeting. The first will be on April 12th at 8:30pm Eastern time which is April 13th in the morning in Asia. These will be on the second Wednesday/Thursday of each month and you will receive an automatic calendar invite with Zoom information from (b) (6).

It's very important for each partner to have staff on this call every month. The goal of the meeting is to keep everyone updated on news from NIAID and EcoHealth Alliance re. the project, get updates from each of the partners and brainstorm together to maximize our outputs (science, papers, talks, outreach) towards the grant renewal. So please take one hour per month to join the meeting, and feel free to send me and Hongying any discussion items you have.

I won't be sending out agendas, these meetings are intended to be updates from each of us so that all partners can hear what we're all up to.

Also, please note that this all-partner meeting is in addition to the monthly calls we have with each of the partners individually.

Look forward to seeing you on the 12th…

Cheers,

(b) (6)

(b) (6)

[redacted]

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From: (b) (6)
To: (b) (6)
Cc: (b) (6) ; Christopher Broder; Laing, Eric; (b) (6)
Subject: RE: [EXTERNAL] NIH EID-SEARCH Year 3 Progress Report_by March 24, 2023
Date: Friday, March 24, 2023 3:31:29 PM
Attachments: NIH_EID-SEARCH Y3 Annual Report.pdf
image001.png

Good Afternoon,

Please find the annual progress report attached for the project titled: "Understanding Risk of Zoonotic Virus Emergence in Eid Hotspots of Southeast Asia"
Please let us know if you have any questions.
Thank you,
(b) (6)

Henry M. Jackson Foundation for the
Advancement of Military Medicine
6720A Rockledge Drive, Suite 100
Bethesda, MD 20817

From: (b) (6)
Sent: Wednesday, March 1, 2023 7:41 PM
To: (b) (6)
eric.laing(b) (6)
(b) (6) Broder, Christopher
(b) (6)
Cc: (b) (6)

(b) (6) [redacted]

Subject: [EXTERNAL] NIH EID-SEARCH Year 3 Progress Report_by March 24, 2023

Dear EID-SEARCH Members,

The NIH Research Performance Progress Report (reporting period **06/01/2022-05/31/2023**) for EID-SEARCH (U01AI151797) is due on **April 1, 2023**.

I've attached a template with the different sections we would like to get updates from you as part of the report. All relevant work for any section is welcomed (the more detailed, the better, don't worry about the languages or word limits); I also labeled some sections as **required* where are the most important to concentrate.

And as part of the CREID Network, we also need to highlight the cross-Research Center activities, which are now KPIs to evaluate each Research Center's performance by the funder. So if you have anything relevant to report, please provide information on the Word document and fill in the *Excel file* I've attached.

The information required for this report is majorly focused on the research findings and plans and very minimal on administration/finance unless you have any significant changes on project key/senior personnel or budget.

We would greatly appreciate it if you could send us updates by **Friday, March 24, 2023,** so we can have a few days to consolidate everything to submit.

Please do not hesitate to let me know if you have any questions. Thank you very much!

Sincerely,

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From: (b) (6)
To: (b) (6)
Cc: (b) (6) ; Chris Broder; (b) (6)
Subject: RE: Draft manuscript: Co-circulation dynamics of viruses in a bat population
Date: Saturday, September 10, 2022 9:07:32 AM
Attachments: (b) (6) -et-al_bangladesh-bats-cocirculation-serology_2022-08-11_(b) (6) .docx

---

Hi (b) (6),
I have a few minor editions and comments with others.

Thank you,
(b) (6)

From: (b) (6)
Sent: 10 September 2022 01:08
To: (b) (6)
Cc: (b) (6)
Chris Broder (b) (6)
Subject: RE: Draft manuscript: Co-circulation dynamics of viruses in a bat population

*** This message originated outside MRCG @ LSHTM ***
Sorry to be late to the party – a few more comments attached to add to the insightful suggestions you've already received.

(b) (6) – still think it would be great to link up so we can describe antibody dynamics over time in our recaptures!

Have a great weekend,
(b) (6)

From: (b) (6)
Sent: Friday, September 9, 2022 5:39 PM
To: (b) (6)
Cc: (b) (6)
Chris Broder
(b) (6)
Subject: Re: Draft manuscript: Co-circulation dynamics of viruses in a bat population

(b) (6),

Attached are my edits and comments. I started working on this using the version from Hume and Linfa, so my version here does not include Steve's valuable comments.

Great paper.

Cheers,
(b) (6)

[redacted]

[redacted]

[redacted]

[redacted]

> On Sep 3, 2022, at 2:54 AM, (b) (6) [redacted] wrote:
>
> H(b) (6),
>
> Thanks for sharing this interesting work. Kudos to (b) (6) and the team for pulling all of this together.
>
> Attached are my comments.
>
> To reduce the amount of my time and improve the thoroughness of my review, I use error codes when I identify common errors within a scientific document. A full description of the error and strategies for addressing it can be found in a scientific writing guide written by Dorothy Southern and I (Pathway to Publishing-A Guide to Quantitative Writing in the Health Sciences) published as an Open Access ebook through Springer. It can be downloaded from here: https://link.springer.com/book/10.1007/978-3-030-98175-4
>
> (b) (6)
>
> (b) (6) [redacted] wrote on 8/23/2022 6:40 PM:
>
> > Great to see(b) (6). Reads well, nice graphics. Thanks for co-

authorship.. my edits added to (b) (6) version.

Regards all
(b) (6)

[redacted]

[redacted]

From: (b) (6) [redacted]
Sent: Saturday, 20 August 2022 1:22 AM
To: (b) (6) [redacted] Christopher Broder (b) (6) [redacted]
Cc: (b) (6) [redacted]
Subject: Draft manuscript: Co-circulation dynamics of viruses in a bat population

Colleagues,
I hope you're all doing well. I'm excited to share a draft manuscript, led by (b) (6), that details the serodynamics of henipaviruses, filoviruses and rubulaviruses in a population of *Pteropus medius.* We plan to submit this to *Nature Communications* in September. Please send (b) (6) and I your comments by **September 10th**.

Cheers,
(b) (6)

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[redacted]-et-al_bangladesh-bats-cocirculation-serology_2022-08-11_[redacted].docx>

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From: Broder, Christopher (b) (6)
To: (b) (6)
Subject: Fwd: Draft manuscript: Co-circulation dynamics of viruses in a bat population
Date: Saturday, September 3, 2022 2:11:17 PM
Attachments: (b) (6) et-al_bangladesh-bats-cocirculation-serology_2022-08-11_L (b) (6) _sl.docx

---

hi (b) (6),

Happy Labor day weekend.

What is the story on the screening here? The
paper reads only NiV EBOV and MenV attachment proteins used
so I am assuming all our proteins. NiV-sG / EBO sGp / MeV-sHN ?
What the entire panel of proteins used? Linfa's comment about saying
heniv sero+ and not just NiV only matters if the other sG beads were used
and what the data looks like.

technically, sGp EBO trimer, is attachment+fusion Glycoprotein,
but the sHN if used this was a new project of both Eric and I working with Linda to construct and we
shipped to (b) (6) in early 18'

I think we need to see all the raw data

(b) (6)

---------- Forwarded message ---------
From: (b) (6) >
Date: Fri, Sep 2, 2022 at 3:08 PM
Subject: Re: Draft manuscript: Co-circulation dynamics of viruses in a bat population
To: (b) (6)
Cc: (b) (6)

Christopher Broder (b) (6)

Hi (b) (6),

Thanks for sharing this interesting work. Kudos to (b) (6) and the team for pulling all of this together.

Attached are my comments.

To reduce the amount of my time and improve the thoroughness of my review, I use error codes when I identify common errors within a scientific document. A full description of the error and strategies for addressing it can be found in a scientific writing guide written by Dorothy Southern and I (Pathway to Publishing-A Guide to Quantitative Writing in the Health

Sciences) published as an Open Access ebook through Springer. It can be downloaded from here: https://link.springer.com/book/10.1007/978-3-030-98175-4

(b) (6)

(b) (6) wrote on 8/23/2022 6:40 PM:

> Great to see (b) (6) . Reads well, nice graphics. Thanks for co-authorship.. my edits added to (b) (6) version.
>
> Regards all
>
> (b) (6)

From: (b) (6)
Sent: Saturday, 20 August 2022 1:22 AM
To: (b) (6)
Christopher Broder
(b) (6)
Cc: (b) (6)
Subject: Draft manuscript: Co-circulation dynamics of viruses in a bat population

Colleagues,

I hope you're all doing well. I'm excited to share a draft manuscript, led by [redacted], that details the serodynamics of henipaviruses, filoviruses and rubulaviruses in a population of *Pteropus medius*. We plan to submit this to *Nature Communications* in September. Please send [redacted] and I your comments by **September 10th**.

Cheers,

[redacted]

--

(b) (6) [redacted]

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From: (b) (6)
To: (b) (6) ; Christopher Broder; (b) (6)
Cc: (b) (6)
Subject: RE: Draft manuscript: Co-circulation dynamics of viruses in a bat population
Date: Sunday, August 21, 2022 1:47:19 AM
Attachments: (b) (6) -et-al_bangladesh-bats-cocirculation-serology_2022-08-11_(b) (6).docx

---

Dear (b) (6) and all,

It is great to see this in a ready to go form after so much effort put into this longitudinal study.

See my edits/comments in the attached. My main suggestion is to treat all three groups the same, i.e., at the genus level.

(b) (6) : I finally made my trip to Bangladesh (and icddr,b) working on a BMGF-funded pathogen genomics project. I "challenged" the new ED to host the Nipah@25 in 2024 there and he said he will do his best to make sure it happens. So hopefully we an all meet again in Bangladesh in 2024.

Cheers,

(b) (6)

[redacted]

From: (b) (6)
Sent: Friday, 19 August 2022 11:22 PM
To: (b) (6)
[redacted]
Christopher Broder
(b) (6)
Cc: (b) (6)
Subject: Draft manuscript: Co-circulation dynamics of viruses in a bat population

- External Email -

Colleagues,

I hope you're all doing well. I'm excited to share a draft manuscript, led by (b) (6), that details the serodynamics of henipaviruses, filoviruses and rubulaviruses in a population of *Pteropus medius.* We plan to submit this to *Nature Communications* in September. Please send (b) (6) and I your comments by **September 10th**.

Cheers,

(b) (6)

[redacted]

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From: (b) (6)
To: (b) (6)
Cc: Laing, Eric; (b) (6) ; Broder, Christopher; (b) (6) a
Subject: Re: For you to edit and review by August 15 Monday_CREID EAC report
Date: Monday, August 15, 2022 7:47:58 AM
Attachments: 2022 EAC Report_EID-SEARCH_draft v01-(b) (6) _15Aug22.docx

---

Dear (b) (6) ,

Apologies for the delayed response.
Attached please find the edited report from our side. Here are some changes that we have made, further detail can be found in the report.

- Number of identified viruses
- Number of specimens for MMIA
- Serology testing graphs (I repeated some of the samples that were positive to confirm the results, most of the samples are still positive **except** the two samples that were weakly positive for NiV)
- Number of manuscripts

Please let me know if you require any further information/clarification.

Best regards,
(b) (6)

[redacted]

On 9 Aug BE 2565, at 01:20, (b) (6) wrote:

Thank you so much, (b) (6), for your quick response!

Sending a reminder that please review and edit the document **by August 15 next Monday.** Or if you don't have anything to add, please simply reply to approve the content for submission. Thank you very much!!

Best regards,
(b) (6)

[redacted]

(b) (6)

[redacted]

[redacted].

On Tue, Aug 2, 2022 at 3:36 PM Laing, Eric (b) (6) wrote:

> Hi (b) (6),
>
> Some edits and suggestions are attached.
>
> - (b) (6)
>
> [redacted]
>
> [redacted]
>
> On Tue, Aug 2, 2022 at 1:23 AM (b) (6) >
> wrote:
>
> > Dear All,
> >
> > As mentioned at the EID-SEARCH meeting in July, we'll submit an External Advisory Committee (EAC) report for our Research Center in August, so the EAC can evaluate our work as an individual Research Center and the overall CREID Network to give feedback at the CREID annual meeting with DMID, USG, and other stakeholders in September.
> >
> > Attached please find the very first draft report for your edit and review. We'll greatly appreciate everyone's input on incorporating the EAC recommendations and aligning with NIAID CREID Network Program Priorities as described in the Appendix.
> >
> > For unpublished data, I tried to keep the information general, will also make sure the CREID keeps all information for internal use only (to the EAC), but please let me know if you have any concerns.
> >
> > We hope to submit the report on August 19, and your feedback **by August 15 Monday** will be appreciated! Thank you all very much in advance!

All the best,
(b) (6)

[redacted]

[redacted]

[redacted]

[redacted]

From: (b) (6)
To: (b) (6)
Cc: Laing, Eric; Broder, Christopher; (b) (6)
Subject: Re: CREID 2022 Annual Meeting: Call for Abstracts
Date: Wednesday, May 4, 2022 9:51:09 AM

Thank you, (b) (6), for the information!

Wonderful to hear all these results, we should encourage (b) (6) to submit an abstract and present. I'll take note of this and remind you all to discuss it at the lab meeting next Wednesday.

- (b) (6)

On Tue, May 3, 2022 at 8:11 PM (b) (6) wrote:

> (b) (6)'s guano farmer set has baseline (pre-vaccination, not sure if it's pre-pandemic) and two yearly follow up points. I think it's about 54 farmers, all have been vaccinated. I want to say they are mostly Sinovac but some are Astra, and I don't know if there were any cross-vaccinations.
>
> I recall (b) (6) talking about a separate healthcare worker cohort at one point but I don't know if that is still on the table.
>
> (b) (6)
>
> > On May 3, 2022, at 7:55 PM, Laing, Eric (b) (6) wrote:
> >
> > Hi (b) (6),
> >
> > I can focus on the bat-cov multiplex development, but we only have SARS-CoV-2 validated sera and research studies outside the scope of EID-SEARCH. We'd need some data from (b) (6) (who should be the lead), and it can be data related to guano farmers, pre-COVID-19, or vaccine recipients.
> >
> > There are 3 virus families being screened in the multiplex - there should be at least 2 stories (filo/henipa and sars-2/covid) coming from (b) (6)'s team a Chula. I've been meaning to email (b) (6) about their covid-19 cohort, I have this memory that they have some vaccine recipients ((b) (6), am I remembering this correctly?). My research team has been involved in Pfizer vaccine response research, and head-head comparison with Moderna. Thailand is mostly vaccinated with AZ and Sinovax, a product comparison would be pretty easy to write-up since the assay is a bridge.
> >
> > - (b) (6)

(b) (6)

[redacted]

On Mon, May 2, 2022 at 9:52 PM (b) (6) [redacted] >
wrote:

> Thank you, (b) (6)
>
> Assay development related to EID-SEARCH may fit into the topic of "New techniques, or novel approaches, to sampling, detection, and characterization of pathogen risk" if you would like to present the methodology, but understand it's better to present with sample testing results from the project.
>
> As you suggested, we have shared this with the teams at CM and Chulalongkorn, hope to hear from them soon and work together with you on the abstracts if they're interested to present some preliminary findings from the serological testing. We may check in with (b) (6) about this next Wednesday at the lab meeting (if you and (b) (6) agree).
>
> Thanks,
> (b) (6)
>
> On Mon, May 2, 2022 at 9:09 PM Laing, Eric (b) (6) [redacted] wrote:
>
> > Hi (b) (6) ,
> >
> > Serology testing is underway in Chulalongkorn, but I think that (b) (6) s lead, (b) (6) , would be the appropriate SEARCH researcher to draft an abstract. (b) (6) mentioned that at the previous month's 'lab meeting', (b) (6) had concluded testing some serologies.
> >
> > (b) (6) and I are happy to help with results analysis and drafting abstracts from either Chulalongkorn or CM, LLC/EHA, but we don't have access to any data from our side.
> >
> > - (b) (6)
> >
> > [redacted]

(b) (6)

On Mon, May 2, 2022 at 9:54 AM (b) (6) wrote:

Good Morning, (b) (6),

The CREID Network is holding the annual meeting on September 21-23 in Washington DC, there will be sessions to present scientific findings supported by the CREID, and we need to submit abstracts for the presentations.

Please see the Call for Abstraction information below, it would be great if anyone from your team can submit an abstract for presentation, if you are busy, it will be a terrific opportunity for graduate students or postdocs, too. Please consider this and feel free to contact me if any questions.

The meeting will be hybrid, and we'll let you know once we received the final agenda, so we can discuss travel and meeting in person if you will be available during that time.

Best,
(b) (6)

---------- Forwarded message ---------
From: (b) (6)
Date: Wed, Apr 27, 2022 at 3:12 PM
Subject: CREID 2022 Annual Meeting: Call for Abstracts
To: (b) (6)

Cc: (b) (6)

(b) (6) [redacted]

Dear CREID Network Leadership,

Thanks to your feedback, we will not be placing any limits on the number of allowed abstract submissions. Any submissions not selected for a rapid, virtual poster presentations or longer oral presentations will have the opportunity to create e-posters/slides to be shared with the CREID Network.

Below, please find the updated call for abstracts for the CREID Network's 2022 Annual Meeting. The meeting will include multiple abstract-driven scientific sessions. Each CREID Research Center is requested to submit at least one abstract by May 17, per the guidelines below.

Thank you,

CREID Network 2022 Annual Meeting Organizing Committee

2022 Annual Meeting: Call for Abstracts

The CREID Network 2022 Annual Meeting (September 21-23, in person and online) will include multiple abstract-driven scientific sessions. Each CREID Research Center is requested to submit at least one abstract, per the guidelines below. Abstracts will be assigned for rapid/lightning talks, longer oral presentations, or e-posters/slides that will be shared with the CREID Network. A committee will convene to review the content of the abstracts and determine the assigned format. More information on the review committee process is forthcoming. Submission template is attached.

Due	May 17, 2022
Length	Abstract body (excluding title and authors): 250 words max
Focus	Abstracts should focus on work supported by CREID, including but not limited to: ▪ Innovative research on pathogen discovery and characterization, pathogen/host surveillance, transmission, pathogenesis, host immunological response, natural history, or related assays or reagents; ▪ New techniques, or novel approaches, to sampling, detection, and characterization of pathogen risk; ▪ Preparation for, or results of, research conducted in response to an outbreak; ▪ Novel strategies to translate related findings downstream Selected abstracts will be grouped into sessions based on content/focus.
Format	As the 2022 Meeting will be hybrid, both in-person and virtual presentations are welcome. Abstracts may be selected for a rapid, virtual poster presentation, a full (>10 min) oral presentation, or for e-posters/slides to be shared with the CREID Network. Assigned format and presentation times will be determined based on the content and number of submissions.
Authors/ Presenters	Authors may include investigators and staff from CREID Research Centers, sites, and partners. Women and those from low- and middle-income countries (LMICs) are strongly encouraged to author and present.

	Submissions with authors from multiple Research Centers are welcome.
Submissions	Each Research Center is asked to submit 1-4 abstracts. Each abstracts should include a title, presenters/authors (names, institutional affiliations, emails, and CREID Research Center), and body (250 words max). See basic template, attached. Send to info@creid-network.org by May 17, 2022.

(b) (6)
[redacted]
[redacted]
[redacted]
[redacted]

Disclaimer

This communication, together with any attachment, may contain confidential and/or proprietary information (including without limitation copyright material and/or other intellectual property) and is intended only for the person(s) and/or entity(ies) to whom it is addressed. If you are not the intended recipient of this communication, or if you

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From: (b) (6)
To: (b) (6) ; eric.laing_usuhs; Broder, Christopher; (b) (6)
Cc: (b) (6)
Subject: NIH EID-SEARCH Year 2 Progress Report_submitted
Date: Sunday, April 3, 2022 11:16:43 PM
Attachments: RPPR10427219 Y2 Annual Report As Submitted_short.pdf

---

Dear All,

Attached please find a copy of the submitted Year 2 Progress Report (technical part), thank you very much for your wonderful work, and for sending updates to complete the report!

As we are approaching the end of Year 2 on May 31, we will follow up closely on the Year 2 funding expenses and invoices in the next couple of weeks.

Sincerely,
(b) (6)

[redacted]

On Mon, Feb 7, 2022 at 2:02 PM (b) (6) [redacted] wrote:

> Dear Partners,
>
> The NIH Research Performance Progress Report (reporting period 06/01/2021-05/31/2022) for EID-SEARCH (No. 5U01AI151797) is due on April 1, 2022. I'm sending this email as an initial notification in case your institution needs some time to proceed and prepare a report, but the information required for this report is majorly focused on the research findings and plans, very minimal on administration unless you have any major changes on key/senior personnel.
>
> Attached please find a template with the different sections we would like to get updates from you as part of the report to submit. Any relevant work for each section is welcomed (don't worry about the languages or word limits), but I labeled some sections as *required where are the most important to concentrate.
>
> We would greatly appreciate it if you can send us updates by **March 21, 2022, Monday** so that we can have a few days to edit and combine all things together to submit.
>
> Please feel free to let me know if you have any questions. You will also receive reminders

from me on March 01 and March 14 if it's still too early and you're waiting for more results to include in the report. Thank you very much!!

Sincerely,
(b) (6)
[redacted]

(b) (5)

KEYSTONE SYMPOSIA
on Molecular and Cellular Biology

Lessons from the Pandemic: Responding to Emerging Zoonotic Viral Diseases (D3)

April 10-13, 2022 • Snowbird Resort • Snowbird, UT, USA

Scientific Organizers: Linfa Wang, Sarah Catherine Gilbert and William E. Dowling

Supported by the Directors' Fund

Global Health Travel Award Deadline: March 21, 2022 / Scholarship Deadline: January 17, 2022 / Abstract Deadline: January 17, 2022 / Discounted Registration Deadline: February 10, 2022

SUNDAY, APRIL 10

Arrival and Registration

MONDAY, APRIL 11

Welcome and Keynote Address

Anthony S. Fauci, NIAID, National Institutes of Health, USA
Remote Presentation: The Good, Bad and Ugly Aspects of COVID-19 Responses

Challenges in Balancing Outbreak Investigation and Basic Research

Maria D. Van Kerkhove, World Health Organization, Switzerland
Challenges in Responding to COVID-19

John N. Nkengasong, Africa Centres for Disease Control and Prevention, Ethiopia
Remote Presentation: Talk Title to be Announced

Speaker to be Announced

Short Talks Chosen from Abstracts

Early Warning and Reporting for Emerging Zoonotic Diseases

Lawrence C. Madoff, University of Massachusetts Medical School and ProMED, USA
WHO and ProMED: Have We got the Early Warning Systems Right?

Linfa Wang, Duke-NUS Medical School, Singapore
Are we ready for SARS-CoV-3?

Short Talks Chosen from Abstracts

Poster Session 1

TUESDAY, APRIL 12

Rapid Development and Deployment of Diagnostics in an Outbreak Setting

Daniel G. Bausch, FIND, Switzerland
The 100 Day Mission for Pandemic Diagnostics: The Marathon Before the Sprint

Christian T. Happi, Redeemer's University, Nigeria
Experience in Assay Development: From Lassa to COVID-19

Speaker to be Announced

Short Talks Chosen from Abstracts

Career Roundtable

Pathogen Biology and Model Systems

César Muñoz-Fontela, Bernhard-Nocht-Institute for Tropical Medicine, Germany
Filoviruses and Arenaviruses

Florian Krammer, Icahn School of Medicine at Mount Sinai, USA
Influenza

Thomas W. Geisbert, University of Texas Medical Branch, USA
NHP Models for Hemorrhagic Fever Viruses

Emmie de Wit, NIAID, National Institutes of Health, USA
Models for Coronaviruses and Henipaviruses

Short Talks Chosen from Abstracts

Poster Session 2

WEDNESDAY, APRIL 13

Countermeasures: Vaccines, Therapeutics and Rapid Manufacturing I

William E. Dowling, Coalition for Epidemic Preparedness Innovations, USA
COVAX and CEPI Disease "X" Platforms

Sarah Catherine Gilbert, University of Oxford, UK
ChadOx1 Vaccines against Different Viral Targets

Kizzmekia S. Corbett, Harvard T.H. Chan School of Public Health, USA
Coronavirus Vaccines - NIAID Pandemic Preparedness Program

Sue Ann Costa Clemens, Oxford University, UK
Testing a Pandemic Vaccine during a Pandemic

Short Talks Chosen from Abstracts

Countermeasures: Vaccines, Therapeutics and Rapid Manufacturing II

Catherine Green, University of Oxford, UK
Developing a Highly Transferrable Vaccine Manufacturing Process for Worldwide Use

Kathrin U. Jansen, Pfizer, USA
Vaccine Manufacturing for Public Health Emergencies

Erica Ollmann Saphire, La Jolla Institute for Immunology, USA
Hemorrhagic Fever Consortium and Coronavirus Immunotherapy Consortium

Dan Hartman, Bill and Melinda Gates Foundation, USA
Therapeutics Accelerator

Short Talks Chosen from Abstracts

Meeting Wrap-Up: Outcomes and Future Directions (Organizers)

THURSDAY, APRIL 14

Departure

* Session Chair † Invited but not yet accepted Program current as of ***January 14, 2022.*** Meal formats are based on meeting venue.
For the most up-to-date details, visit ***https://www.keystonesymposia.org.***

From: (b) (6)
To: (b) (6)
Cc: (b) (6) Eric Laing; (b) (6) ; Christopher Broder; (b) (6)
Subject: Re: Urgent: Time sensitive: abstract for Keystone Symposia. please respond by Monday 3pm
Date: Saturday, January 15, 2022 1:49:53 PM
Attachments: Keystone Nipah Abstract d1.docx
ATT00002.bin
Keystone symposia program.pdf
ATT00004.bin

Thanks (b) (6), I'm good with this as well, thanks for spearheading.

(b) (6)

On Jan 15, 2022, at 2:43 AM, (b) (6) wrote:

Dear colleagues,
I've just become aware of an upcoming Keystone Symposium on pandemic prevention being co-organized by CEPI.
www.keystonesymposia.org/conferences/conference-listing/meeting?eventid=6862

I think it would be a good opportunity to submit an abstract for a short talk to showcase our Nipah virus work. The deadline for submission is Monday night (Eastern Time)

With apologies for the short notice, I would be grateful if you would review the attached abstract and send me your consent to be a co-author, along with any comments, by Monday 3pm.

Cheers,
(b) (6)

(b) (6) [redacted]

[redacted]

[redacted]

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From:	(b) (6)
To:	(b) (6) ; Eric Laing; Chris Broder
Cc:	(b) (6)
Subject:	NSF PIPP final versions submitted, and single powerpoint slide
Date:	Friday, October 1, 2021 5:18:36 PM
Attachments:	NSF_PIPP_ResearchDescription_v14.1.pdf ATT00002.bin Project Management Plan_v3.pdf ATT00004.bin Project Summary_v3.pdf ATT00006.bin PIPP_VisionSlide_(b) (6)_1Oct2021.pptx ATT00008.bin
Importance:	High

Dear NSF PIPP Proposal Colleagues,

Our NSF PIPP proposal was successfully submitted via grants.gov about an hour ago! Please see my email below to NSF, as it was requested that all applicants submit single powerpoint slide of our project's vision via email right after we submit ((b) (6) caught this just a couple hours before it was due, and we whipped the slide together).

Also attached for your reference are the **final Project Description, Project Summary,** and **Project Management Plan** that we submitted.

Please note in my message below that there seems to be a problem with grants.gov relaying our proposal over to research.gov (formerly Fastlane to get to NSF). Seems there is a backlog in the system (probably too many submissions), so we're hoping for positive news from research.gov that it clears (as of now still pending).

Thank you all for your hard work and collaboration on this, finger's crossed the submission full clears and we win this! Huge thanks to the EHA team: (b) (6) for a huge team effort to get this done on our end.

Best regards,
(b) (6)

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From: (b) (6)
To: (b) (6)
Cc: (b) (6) ; Eric Laing; Chris Broder; (b) (6)
Subject: Re: [EXT] Penultimate draft of NSF PIPP Research Description to submit today (Oct 1))
Date: Friday, October 1, 2021 1:14:07 PM
Attachments: NSF_PIPP_ResearchDescription_v12.1_(b)_-2021-10-01 1310.docx

My edits to the main research description attached
--

(b) (6)

On Fri, Oct 1, 2021 at 12:01 PM (b) (6) wrote:

> Dear all,
>
> Project Summary draft attached Please let me know ASAP if you have any specific edits, and track changes
>
> Cheers,
> (b) (6)

> On Oct 1, 2021, at 10:30 AM, M (b) (6) wrote:
>
> Glad to see the proposal coming along I have a long day ahead with back to back meetings :-(
>
> Do you have the summary page written up yet? I would be happy to take a quick look at it
>
> Best,
> (b) (6)

> On Fri, Oct 1, 2021 at 9:42 AM (b) (6) wrote:
>
> > Thanks (b) (6) (cc'ing all for awareness) Will include your edits and also try and add a short paragraph on "Associated Risks and Mitigation Plans"

(b) (6)

(b) (6)

On Oct 1, 2021, at 9:11 AM, (b) (6) wrote:

Hi (b) (6),

Amazing work, this reads really well. Attached are just some suggestions to fix a couple minor typos.

Something else I just caught on the solicitation (just before Section III - Award Information)

Several bullet points are listed under "Investigations that are outside the scope of this PIPP announcement include:" and one of them is:

- Projects that do not discuss associated risks and mitigation plans.

I do not think we have touched upon this explicitly in the proposal. Probably too late now, but if somebody finds a way to incorporate a paragraph that should be super.

Best,
(b) (6)

From: (b) (6)
Sent: Friday, October 1, 2021 4:07 AM
To: (b) (6) Eric Laing (b) (6); Chris Broder (b) (6)
Cc: (b) (6)
Subject: [EXT] Penultimate draft of NSF PIPP Research Description to submit today (Oct 1))

Dear all,

Here is the cleaned up, penultimate version of our NSF PIPP Proposal Research Description (15 page main doc)

Please review this morning if possible and send back to me with tracked changes before 12:30pm today so we can incorporate edits and get this uploaded in the system before 5pm!

Attached also is the cleaned up Data Management Plan I still need to clean up 1 page Summary and Project Management (2 pages) later this morning, but for now have a eye twitch and need to get some zzzzzzs

Cheers,
(b) (6)

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<NSF_PIPP_ResearchDescription_v12_(b) (6) docx>

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From: (b) (6)
To: (b) (6)
Cc: (b) (6) ; Eric Laing; Chris Broder; (b) (6)
Subject: Re: [EXT] Penultimate draft of NSF PIPP Research Description to submit today (Oct 1))
Date: Friday, October 1, 2021 10:35:59 AM
Attachments: NSF_PIPP_ResearchDescription_v12_(b) (6).docx
ATT00002.bin

Dear ALL,

See page 15 on the attached. I accepted (b) (6)'s minor edits and revised the scaling operations section on p15 and added in a "Associated Risks and Mitigation Plans" section… but now we're a bit more over length, so need to cut back more. How does this new section look?? Missing any big risks?

Associated Risks and Mitigation Plans: We envision two primary risks associated with our proposed work 1) data access and privacy violations, and 2) biosafety concerns inherent with emerging zoonoses research. For data related risks, we have already established a strong collaborative relationship with FB's Data for Good team in developing this proposal and already have access to all the relevant datasets for our pilots. Data privacy concerns are taken seriously by our group, including deidentification of any personal identifying information following strict protocols on using human subject data from FB and our already approved IRBs as part of EHA's NIH-funded work. We will amend or apply for additional IRBs as needed. For biosafety related risks, there will be no active sample collection under our proposed NSF project. We have already secured appropriate approvals (IRB, IACUCs, sampling permits) that outline PPE use and biosafety monitoring plans for our NIH-funded work. Our lateral flow assay development will be undertaken in laboratories designate at the appropriate Biosafety Level following US regulations (USU and other partner labs to be confrimed upon award).

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From:	(b) (6)
To:	(b) (6) ; Eric Laing; Chris Broder
Cc:	(b) (6)
Subject:	Re: Penultimate draft of NSF PIPP Research Description to submit today (Oct 1))
Date:	Friday, October 1, 2021 4:09:32 AM
Attachments:	NSF_PIPP_ResearchDescription_v12.1.docx ATT00002.bin
Importance:	High

Sending again making sure file extension works…

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From: (b) (6)
To: (b) (6)
Broder, Christopher
Cc: (b) (6)
Subject: CEPI Biosketches
Date: Friday, September 17, 2021 4:15:10 PM
Attachments: (b) (6) NIH Biosketch Example.docx

Hello, all!

I am hoping that everyone can create or update a NIH-formatted biosketch and forward it to me for inclusion in our CEPI proposal.

A recent version of (b) (6) biosketch is attached as an example. Section A, the personal statement, should be adjusted to be relevant to the CEPI proposal and your Nipah virus work. We've also removed Section D (funding/financial information).

For those whose biosketches we already have, from previous recent proposals, I will follow up separately in response to this email and ask that you update your biosketch as necessary. ((b) (6))

Please let me know if you have any questions, and have a great weekend!

(b) (6)

ANNUAL REVIEWS

Annual Review of Virology

Vaccines to Emerging Viruses: Nipah and Hendra

Moushimi Amaya and Christopher C. Broder

Department of Microbiology and Immunology, Uniformed Services University of the Health Sciences, Bethesda, Maryland 20814, USA; email: christopher.broder@usuhs.edu

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Abstract

Hendra virus (HeV) and Nipah virus (NiV) are bat-borne zoonotic paramyxoviruses identified in the mid- to late 1990s in outbreaks of severe disease in livestock and people in Australia and Malaysia, respectively. HeV repeatedly re-emerges in Australia while NiV continues to cause outbreaks in South Asia (Bangladesh and India), and these viruses have remained transboundary threats. In people and several mammalian species, HeV and NiV infections present as a severe systemic and often fatal neurologic and/or respiratory disease. NiV stands out as a potential pandemic threat because of its associated high case-fatality rates and capacity for human-to-human transmission. The development of effective vaccines, suitable for people and livestock, against HeV and NiV has been a research focus. Here, we review the progress made in NiV and HeV vaccine development, with an emphasis on those approaches that have been tested in established animal challenge models of NiV and HeV infection and disease.

INTRODUCTION

Nipah virus (NiV) and Hendra virus (HeV) are bat-borne viral zoonoses that were discovered in the mid- to late 1990s in outbreaks of severe disease in livestock and people in Australia (HeV) and Malaysia [NiV-Malaysia (NiV-M)] (1). They are the prototype members of the genus *Henipavirus* in the family *Paramyxoviridae* (2). NiV outbreaks have also been recorded in Bangladesh and India by a closely related strain, NiV-Bangladesh (NiV-B) (3). Three other henipaviruses are also recognized: Cedar virus (CedV) as an isolate and Ghana virus (GhV) and Mojiang virus (MojV) known only from sequence data (4–7). Both NiV and HeV are highly pathogenic in a broad range of mammalian hosts that are capable of infecting and causing severe disease in humans, monkeys, pigs, horses, cats, dogs, ferrets, hamsters, and guinea pigs and that span six mammalian orders including bats, although bats do not exhibit disease when infected (8–21). In contrast, CedV is nonpathogenic in well-characterized models of HeV and NiV disease including ferrets and hamsters (4, 22). The pathogenic potential of GhV and MojV is unknown.

Several species of *Pteropus* fruit bats are the natural reservoir hosts of NiV, HeV, and CedV (4, 23–27). NiV- or HeV-mediated disease has not been reported in wild or experimentally infected bats (13, 28–30). NiV and HeV infections in people and many animals manifest as severe systemic and often fatal neurologic and/or respiratory diseases (31–33). Both NiV and HeV are regarded as transboundary biological threats to both human and animal health and are classified as biosafety level 4 (BSL-4) select agents (34, 35). NiV and henipaviral diseases are included in the World Health Organization (WHO) R&D Blueprint list of priority pathogens with epidemic potential that need research attention (36). This review summarizes the important characteristics of the NiV and HeV pathogens, the modes of virus transmission, and the immunization strategies being developed against them.

Emergence and Outbreaks of Hendra and Nipah Viruses

In 1994 in the Brisbane suburb of Hendra, Australia, an outbreak of severe respiratory disease resulted in the deaths of 14 horses and their trainer, along with the nonfatal infection of 7 other horses and 1 other person. This led to the discovery of a novel paramyxovirus initially termed equine morbillivirus, now known as HeV (37–39). The first known cases of HeV in horses and a human actually occurred a few months prior, where one person became ill after assisting in the necropsies of two horses later shown to have died from HeV (40, 41). This individual experienced a relapsed fatal encephalitis caused by HeV 13 months later (42). HeV has since re-emerged in Australia 62 times with a total of 104 horse deaths (fatal or euthanized), along with 4 human fatalities of 7 cases (43). Every recorded occurrence of HeV in Australia has involved horses, all resulting in a severe or fatal disease, and all cases of human infection were acquired from virus-shedding horses (31, 44).

In 1998, an outbreak of encephalitis among pig farmers in Peninsular Malaysia occurred and a virus was isolated from samples of cerebrospinal fluid (CSF) of two patients who had died; cells infected with this virus cross-reacted with antibodies against HeV (45). Genetic studies revealed a new paramyxovirus that was closely related to HeV, and it was named Nipah after the village in Malaysia where one of the patients had lived (45). There were 265 cases of human infection with 105 fatalities in Malaysia and 11 cases and 1 fatality among abattoir workers in Singapore (46, 47). This outbreak was controlled through the culling of more than 1 million pigs, resulting in significant economic impacts to the region (48, 49).

A genetically similar but distinct strain of NiV was identified as the causative agent of fatal encephalitis in people in Bangladesh (NiV-B) (3, 50). Since 2001, nearly annual occurrences of human NiV-B infections have occurred in Bangladesh, and there have been three outbreaks in

India (51–54). The recent 2018 NiV outbreak in Kerala, India, was significant, having occurred in a new geographic region far from locations in Bangladesh and India where all prior outbreaks had occurred and with a case fatality rate of 91% (51). In 2014, an outbreak of NiV-M encephalitis occurred in the Philippines with 9 fatalities of 11 human cases of acute encephalitis and influenza-like illness or meningitis in another 6 individuals (55). Altogether, there have been over 650 cases of human NiV infection (combined ~60% fatality rate) in South Asia and Southeast Asia in five countries (54, 56).

Transmission of Hendra and Nipah Viruses

The routes of transmission of virus infection to humans from animals are different for HeV and NiV, with horses the only spillover host of HeV in Australia, while for NiV it was pigs in Malaysia and horses in the Philippines (**Figure 1**). However, human NiV infections in Bangladesh, India, and the Philippines also include bat-to-human and human-to-human transmission (57–60). Transmission routes of HeV and NiV to animals are likely urine from infected bats contaminating pastures or pigsties and/or virus-contaminated fruit spat from bats that is ingested (61, 62) (**Figure 1**). Recoverable virus is shed in the urine of experimentally infected bats and can also be detected in throat and rectal swabs (13, 28–30). Pooled urine samples from flying foxes are also routinely used to detect and isolate henipaviruses (4, 13, 23, 27, 63–65).

It was previously suggested that infected horses could transmit HeV to people during the feeding of ill animals (38). Also, the majority of all HeV-infected horse cases have involved a single animal, suggesting that HeV is not readily transmitted between horses, and multiple horse outbreaks are likely via contamination of fomites (43, 66). The transmission risk of HeV from infected horses to humans appears to be virus-contaminated fluids or tissues during examination procedures and/or the necropsy of horses (31, 67) (**Figure 1**). Indeed, all cases of human HeV infection have been associated with postmortem examination of horses or close contact with ill horses (31, 38, 42, 68). In Malaysia, it was contact with infected pigs or fresh infected pig products that was required for transmission of virus to humans (45, 69, 70) (**Figure 1**). NiV shedding in respiratory fluids of infected pigs suggested that it probably spread among farmed animals by aerosol droplets or direct contact (16, 71, 72). In Bangladesh, the transmission of NiV from bats to people has been linked to the consumption of virus-contaminated fresh date palm sap, and bats will consume sap during its collection (57, 73, 74). Domestic animals have also been linked to NiV infection in people in Bangladesh from unwell animals (cows and goats) and pigs (50, 59). Human-to-human transmission of NiV has been well documented in Bangladesh and India (52, 58–60, 75) (**Figure 1**). A study of human NiV-B cases in Bangladesh spanning 14 years reported that of 248 cases studied, one-third were caused by human-to-human transmission (56). Human-to-human transmission of NiV-M was not apparent in Malaysia (76, 77), whereas in the Philippines' NiV-M outbreak, human cases were linked to horse slaughtering and horse meat consumption or exposure to other human patients, indicating both horse-to-human and human-to-human transmission (55) (**Figure 1**). The NiV-B outbreak in Kerala had a very high rate of human-to-human transmission (22 of 23 cases) at three different hospital locations (51).

Naturally acquired NiV infections were also recorded in cats, dogs, and horses in the initial Malaysian outbreak (**Figure 1**), and serological evidence of natural NiV infection in dogs was linked to outbreak farms (11, 61, 78). In the Philippines, both dogs and cats were linked to NiV-M infection, with cats dying after eating horse meat and dogs having NiV-neutralizing antibodies (55) (**Figure 1**). In Australia, a dog was found to be seropositive for HeV and later euthanized but showed no signs of disease, and a second HeV-positive dog was identified in 2013 following exposure to blood from an infected horse (79) (**Figure 1**). Dogs are susceptible to experimental HeV infection and shed virus but show little evidence of clinical illness (80).

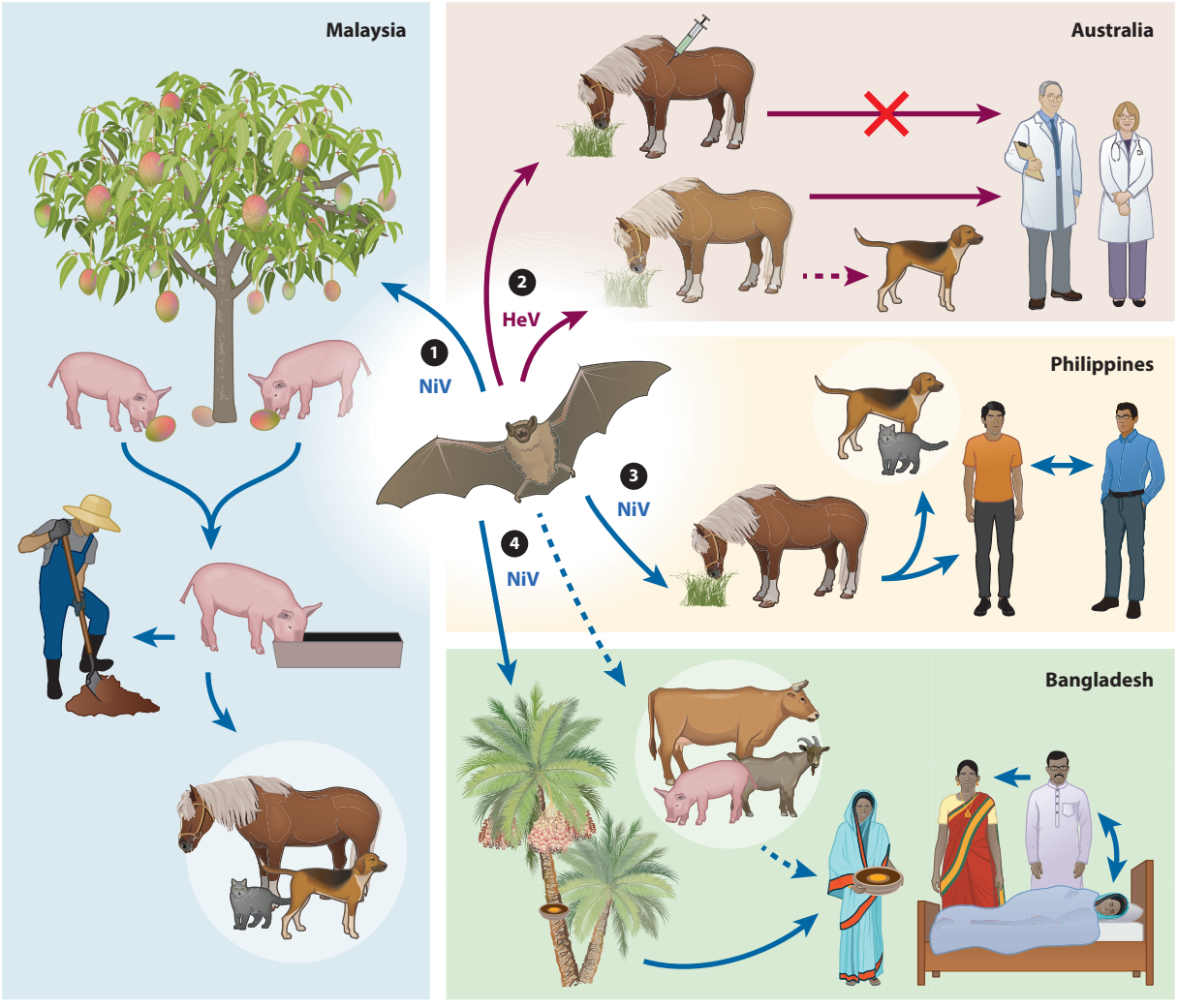

Figure 1

Nipah virus (NiV) and Hendra virus (HeV) modes of transmission in different countries. The transmission routes of NiV in Malaysia (*left*), Philippines (*middle right*), Bangladesh (*bottom right*), and HeV (*top right*) are depicted. Solid lines represent transmission that has been observed and documented, and dashed lines represent suspected transmission in natural conditions. Fruit bats are the natural reservoirs of NiV and HeV. (①) Pigs are infected by consuming partially eaten or contaminated fruit from infected bats (urine, saliva) and transmit NiV to other pigs, pig farmers, or other animals (dogs, cats, and horses) through close or direct contact. (②) Horses can be infected from grazing in contaminated pastures and transmit HeV to humans and on occasion domestic dogs through close contact. A One Health vaccine approach was developed for vaccination of horses in Australia with the dual purpose of saving horses from lethal HeV infection and preventing HeV transmission from horses to humans. (③) NiV is transmitted to humans through close contact with infected horses. NiV transmission to humans, cats, and dogs appears to have occurred following close contact with or consumption of infected horse meat. Human-to-human NiV transmission can occur through close contact. (④) Bat-to-human NiV transmission occurs through consumption of contaminated date palm sap. Human-to-human transmission can occur through close contact with infected patients. Humans may also become infected through contact with infected animals. Figure adapted with permission from Reference 171.

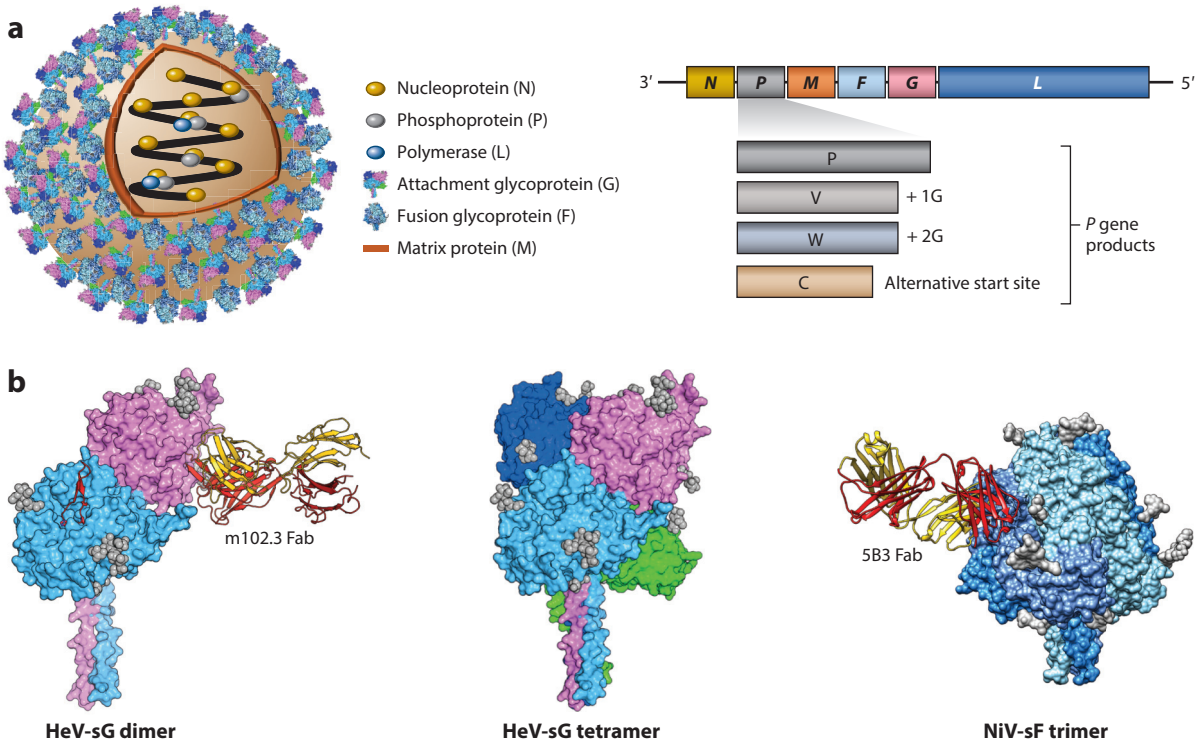

Figure 2

Henipavirus structure and genome organization and models of the G and F glycoprotein soluble ectodomains, Hendra virus (HeV-sG) and Nipah virus (NiV-sF), respectively, and their complexes with respective NiV and HeV cross-reactive neutralizing monoclonal antibodies m102.3 (anti-G) and 5B3 (anti-F). (*a*) Schematic representation of a henipavirus particle with the structural proteins depicted in different colors (*left*) and the henipavirus genome (*right*). HeV and NiV *P* genes encode 3 nonstructural proteins: The C protein is expressed from an alternative start site, and the V and W proteins are expressed following the addition of one or two G residues at the messenger RNA editing site, respectively (*right*). (*b*, *left*) HeV-sG shown as a dimer solvent-accessible surface view with one monomer (*cyan*) overlaid with the monoclonal antibody m102.3 CDR-H3 loop (*red*) at the receptor binding site, and the other monomer (*magenta*) in complex with m102.3 Fab, which has an identical heavy chain and a similar light chain, that was used in place of the m102.4 monoclonal antibody (mAb) in the structural solution of the complex (109). The HeV-sG consists of amino acids 76–604, and the structures of the two globular head domains of HeV-sG are derived from the crystal structure (103, 172). The stalk regions of each G monomer (residues 77–136) are modeled (173). The light chain of m102.3 Fab is colored in yellow, and the heavy chain is colored in red. (*b*, *middle*) The HeV-sG tetramer surface view is modeled with one dimer (*cyan* and *magenta*) in front and the other dimer (*blue* and *green*) in back. *N*-linked glycans are gray spheres. (*b*, *right*) Structural model of the NiV-sF trimer in complex with the 5B3 Fab derived from the cryo–electron microscopy structure (110). The NiV-sF consists of amino acid residues 1–494 with a FLAG tag (DYKDDDK) introduced between residues L104-V105 and a C-terminal GCN4 motif. Each monomer of NiV-sF is in a different shade of blue, 5B3 heavy chain is in red, and light chain is in gold. *N*-linked glycans are illustrated in gray.

Entry and Tropism of Nipah and Hendra Viruses

NiV and HeV are enveloped viruses containing an unsegmented, single-stranded, negative-sense RNA genome (2). **Figure 2*a*** is an illustration of the viral particle and the associated viral proteins. The genomes of HeV and NiV, and also CedV, GhV, and MojV, are considerably longer than the genomes of other paramyxoviruses, at greater than 18 kb. Henipavirus genomes encode 6 structural proteins: nucleoprotein (N), phosphoprotein (P), matrix protein (M), fusion glycoprotein (F), attachment glycoprotein (G), and the polymerase protein (L) (**Figure 2*a***). The N, P, and L proteins comprise the replication complex. The *P* gene undergoes RNA editing

to produce 2 additional nonstructural proteins, V and W, that are interferon (IFN) antagonists (81–84). The C protein is transcribed from a second open reading frame in the *P* gene (**Figure 2*a***). NiV has been central to understanding the V, W, P, and C protein roles in antagonizing the innate immune responses via a diverse set of mechanisms (85, 86). Recent in vivo studies with recombinant NiV variants have further defined the varying importance of these nonstructural proteins in pathogenesis, but only a lack of the V protein results in a nonlethal infection (87–89).

The henipavirus virion bears surface projections composed of the F and G glycoproteins that are anchored in the viral membrane and together mediate infection of host cells, and they are the major antigens of vaccine strategies (1) (**Figure 2*a***). The F glycoprotein facilitates membrane fusion between the virus and host cell. The G glycoprotein consists of a characteristic stalk with a globular head that engages entry receptors on host cells, leading to the fusion activation of F and virus infection. The native structure of G is a tetramer while F is a trimer, and together they are the key determinants of infection and tropism (90–92). Models of the soluble ectodomain of the HeV G (HeV-sG) as a dimer and tetramer and the soluble ectodomain of the NiV F (NiV-sF) as a trimer are shown in **Figure 2*b***. NiV and HeV utilize the host cell proteins ephrin-B2 and ephrin-B3 for entry (93–96). Ephrin-B2 and ephrin-B3 are members of a large family of ligands that bind to Eph receptors and are highly sequence conserved among mammals (97, 98). Ephrin-B2 expression is prominent in the vasculature of multiple organs, whereas ephrin-B3 is found predominantly in the nervous system (99–101). The ability of HeV and NiV to use these ephrins as receptors provided explanations of their broad host and tissue tropism (32, 33, 102). The NiV and HeV G head domain structures alone and in complex with ephrin-B2 and ephrin-B3 receptors have been determined (103–106). The structures of both the NiV and HeV F in their prefusion conformation have also been determined (107, 108). These studies have provided insights into understanding the virus entry receptors and host tropism features of the viruses on the molecular level and also facilitated further structural studies of henipavirus G and F glycoproteins in complexes with specific virus-neutralizing antibodies, providing valuable information that has aided vaccine design and choice (109, 110).

Nipah Virus and Hendra Virus Infection in Humans and Animals

Human NiV and HeV infections are generally accepted to occur via the oronasal route, and the incubation periods for both have been estimated to be 1 to 2 weeks (31, 51, 111). Acute infection in people is a systemic infection likely via hematogenous spread of the virus from the respiratory system (112). In general, HeV and NiV disease onset is characterized by fever, myalgia, shortness of breath, and cough (38, 111). Human HeV infections have resulted in both fatal respiratory or encephalitic disease and also recovery from infection (31, 38, 42, 68). The predominant clinical feature in the NiV-M outbreak in Malaysia was encephalitis, but respiratory symptoms were also common with fever, cough, and headache (47, 111, 112). The clinical presentation of NiV-B infections in Bangladesh also includes severe respiratory disease. In the 2018 NiV-B outbreak in Kerala, 83% of cases presented with acute respiratory distress syndrome (ARDS) (51, 113). Central findings of human NiV and HeV infection are a widespread endothelial cell tropism and systemic vasculitis, with prominent parenchymal cell infection in most major organs with the brain and lung significantly affected (45, 112, 114). Human NiV and HeV infections can also take a protracted course following apparent recovery, and some patients can experience late-onset encephalitis or relapsed encephalitis can occur in patients who previously recovered (42, 115). Relapsed encephalitis caused by NiV appears to result from a recrudescence of virus replication in the central nervous system (CNS), with cases presenting from a few months to as long as 11 years later (116–118). Recrudescence of virus has important implications for vaccine development.

The development of animal models of NiV and HeV infection and pathogenesis has been a major focus since the late 1990s and an essential component of vaccine development and testing. Also, the approval process of countermeasures for NiV and HeV would fall under the animal rule requirement set forth by the US Food and Drug Administration (FDA) in 2002 as an alternative licensing pathway for countermeasures against highly pathogenic agents when human efficacy studies are not feasible or ethical (119, 120). Several animal models of NiV and HeV infection have emerged that well reflect the pathogenesis seen in infected people, which includes a systemic vasculitis with both respiratory and neurological diseases. Detailed reviews of NiV and HeV infections of a variety of mammalian species have recently been published (33, 121–123). It is generally accepted that the pathogenic processes of NiV and HeV infection in the hamster, ferret, and African green monkey (AGM) best reflect the pathogenesis observed in humans, whereas the most appropriate models for livestock are the horse and pig themselves.

VACCINATION

The attachment and fusion glycoproteins of paramyxoviruses such as measles, mumps, and parainfluenza viruses are the viral antigens to which virtually all neutralizing antibodies are directed (124–126). Likewise, immunization strategies for NiV and HeV have largely targeted their G and F glycoproteins.

Passive Immunization Strategies

Early passive immunization studies in the hamster model demonstrated that polyclonal antiserums or mouse monoclonal antibodies (mAbs) to NiV F or G could provide complete protection against NiV-M or HeV when administered before and immediately after virus infection (10, 127, 128). These studies demonstrated a major role of a viral glycoprotein-specific antibody in protection.

Recombinant human antibody technology was used to generate a potent cross-neutralizing mAb against NiV and HeV (m102.4) (129, 130). The m102.4 mAb epitope maps to the ephrin receptor binding site of G and blocks virus infection (see the left side of **Figure 2*b***), and it can neutralize NiV-M, NiV-B, and HeV (8, 109). The m102.4 mAb provided complete protection from NiV-M-mediated disease in ferrets as a single 50 mg dose administered 10 h post-challenge (8). In the AGM model, m102.4 administered as two 20 mg/kg doses, intravenously, at 10 h and again on day 3, on days 1 and 3 (days 1/3), or on days 3/5, after HeV challenge [4×10^5 50% tissue culture infectious dose ($TCID_{50}$)] by intratracheal (i.t.) administration, protected 100% of treated subjects (131). All treated subjects seroconverted against HeV F glycoprotein with a rise in antibody titer over time, indicating all animals had become infected with HeV and recovered, whereas untreated control subjects succumbed to HeV disease and failed to mount a protective immune response. No clinical signs were evident at any time in the early treatment groups; although neurological symptoms were observed in subjects in the late treatment group (days 3/5), all later recovered from infection. There was no HeV antigen or virus-specific histopathology detected in the lung or brain at the conclusion of the study in any treated subject, and infectious virus could not be recovered from any tissue. A similar study evaluated m102.4 against NiV-M disease in the AGM model at several time points following virus challenge (5×10^5 PFU), including a late cohort where treatment was initiated at the onset of clinical illness (day 5) (132). All subjects became infected after challenge, and all subjects that received m102.4 survived infection and all controls succumbed to disease. Subjects in the late day 5/7 treatment group exhibited disease, but all recovered. A comparative study in AGMs using NiV-M and NiV-B [5×10^5 PFU divided by i.t. and intranasal (i.n.) administration] revealed that NiV-B caused a more aggressive disease, with a

shortened time to death and higher virus loads in tissues and fluids (133). When m102.4 was tested in this model, all subjects in the days 1/3 and days 3/5 post-infection treatment groups survived NiV-B challenge, but subjects in the days 5/7 treatment group succumbed, indicating a shorter therapeutic window in treating NiV-B infection (133). Another well-characterized, humanized mouse mAb, 5B3 (h5B3.1), that is cross-reactive to the F glycoprotein of NiV and HeV and binds a prefusion conformation epitope on F, preventing membrane fusion, was recently tested (110, 134) (**Figure 2*b***). The h5B3.1 mAb was given to ferrets in 20 mg/kg doses by intraperitoneal (i.p.) injection, at 1 to several days post-challenge, with either NiV or HeV ($\sim 5 \times 10^3$ PFU) delivered i.n. (135). All subjects that received h5B3.1 after infection were protected from disease and had increasing neutralizing antibody titers, whereas all controls died. No pathology was observed, and no infectious virus could be isolated at the study endpoint. Altogether, these studies demonstrate that passive immunization with mAbs can provide therapeutic benefit and allow the infected host an extended period to mount a protective immune response. The findings from these experiments were also important because they suggest that vaccine approaches designed to induce adequate neutralizing antibody responses to NiV and HeV should be effective.

The m102.4 mAb producing cell line was provided to the Queensland Government, Australia, to produce the mAb for compassionate use in future cases of high-risk human HeV infection. To date, 14 individuals exposed to either HeV in Australia ($n = 13$) or NiV in the United States ($n = 1$) have been given high-dose m102.4 therapy (15–20 mg/kg) by emergency use protocols, and all have remained well. In Australia, m102.4 was used in a randomized, controlled phase I study in healthy adults (136). The study included four single and one repeat dosing groups, and the m102.4 mAb was found to be safe and well tolerated, with a half-life ranging between ~16.5 and 27 days, and no observed immunogenicity was reported. Two doses of 20 mg/kg (days 1/3) were as well tolerated as a single dose. This study's findings will aid in the design of future dosing regimens of mAbs for evaluating their ability to prevent and/or treat HeV and NiV human infections.

Active Immunization Strategies

A variety of immunization strategies have been developed to prevent NiV and HeV infection including several live-recombinant virus vectors, protein subunit, and virus-like particle (VLP) approaches, and all target the virus attachment and entry steps of infection by employing the G and/or F glycoprotein antigens. Here we summarize these various vaccination countermeasure approaches to NiV and HeV infection (**Tables 1** and **2**).

Poxvirus vectored. Poxviruses have a long history as a platform for the expression of heterologous genes to study protein function and serve as vaccine candidates as a live-attenuated viral vaccine platform capable of inducing both cell-mediated and humoral immune responses (137). The F and G glycoproteins of NiV and HeV were functionally characterized using recombinant vaccinia viruses in the early 2000s (138, 139). The first NiV vaccine tested used a highly attenuated vaccinia virus strain (NYVAC) encoding either the F or G glycoproteins from NiV-M (127). Hamsters were vaccinated by subcutaneous (s.c.) injection in a prime-boost strategy with NYVAC-NiV-F or NYVAC-NiV-G, individually and in combination, and then 3 months later challenged i.p. with NiV-M. Vaccination yielded complete protection from NiV-M with no detection of viral RNA, and control subjects succumbed 7–10 days after challenge (127) (**Table 1**). Another poxvirus-based approach was examined as a vaccine for pigs using canarypox (ALVAC) vaccine vectors encoding either NiV-M F or G glycoprotein (140). A prime-boost strategy with ALVAC-NiV-F or ALVAC-NiV-G vectors was tested alone or in combination in pigs. The animals were then challenged 28 days later with NiV-M via i.n. administration. All vaccinated animals survived NiV-M challenge

Table 1 Virus vectored vaccine strategies for NiV and HeV

Approach	Name(s)	Animal model	Vaccination dose, route, schedule	Adjuvant	Challenge	Challenge dose, route, schedule	Survival	Correlate(s) of immunity/ protection	Reference
Poxvirus	NYVAC-NiV-F and/or -G	Hamster	2 doses at 1×10^7 PFU, s.c., 1 month apart	None	NiV-M	1×10^3 PFU, i.p., 3 months later	100%	NAb response, viral RNA	127
	ALVAC-NiV-F and/or -G	Pigs	2 doses at 1×10^8 PFU, i.m., 2 weeks apart	None	NiV-M	2.5×10^5 PFU, i.n., 28 days later	100%	NAb response, viral RNA, infectious virus, viral shedding, cytokine production	140
	ALVAC-HeV-F and/or -G	Hamster	2 doses at 7.4 or 5.4 log10 $CCID_{50}$, s.c., 3 weeks apart	None	HeV	1×10^3 LD_{50}, i.p., 21 days later	89% and 63%	NAb response, viral RNA, viral antigen, viral shedding	141
		Ponies	2 doses at 6 log10 $CCID_{50}$, i.m., 3 weeks apart	None	NT	NA	NA	High NAb titers	
	MVA-NiV-sG and/or MVA-NiV-G	IFNAR−/− mice	1 or 2 doses at 1×10^8 PFU, i.m., 3 weeks apart	None	NT	NA	NA	High serum IgG titers, NiV-G-specific CD8 and CD4 T cells	142

(*Continued*)

Table 1 (*Continued*)

Approach	Name(s)	Animal model	Vaccination dose, route, schedule	Adjuvant	Challenge	Challenge dose, route, schedule	Survival	Correlate(s) of immunity/ protection	Reference
VSV	VSV-NiV-F and/or -G	Mice	5×10^3 PFU, i.n. or i.m.	None	NT	NA	NA	High NAb titers	144
		Hamster	1×10^6 infectious particles, i.m.		NiV-M	1×10^5 $TCID_{50}$, i.p., 32 days later	100%	NAb response, viral RNA, viral antigen	145
	VSV-NiV-B F and/or G	Ferret	1×10^7 PFU, i.m.	None	NiV-M	5×10^3 PFU, i.n., 28 days later	100%	Serum IgG response, viral RNA, viral antigen	146
		AGM			NiV-B	5×10^5 PFU, i.t. and i.n., 28 days later		NAb response, viral RNA, viral antigen	147
	VSV-ZEBOV-GP-NiV F, G, or N	Hamster	1×10^5 PFU, i.p.	None	NiV-M	1×10^3 LD_{50}, i.p., 28 days later	100%	NAb response, viral RNA, infectious virus	148
		AGM	1×10^7 PFU, i.m.			1×10^5 $TCID_{50}$, i.t., 29 days later		NAb response, viral RNA, infectious virus, viral shedding	149
	VSV-HeV-G	Mice	1×10^5 PFU, i.m.	None	NT	NA	NA	Serum IgG, NAb response	150
AAV	AAV8 NiV.G	Mice	2×10^{10} genome particles, i.m. or 1×10^{10} genome particles, i.d.	None	NT	NA	NA	Serum IgG, NAb response	151
		Hamster	6×10^{11} genome particles, i.m.		NiV-M	1×10^4 PFU, i.p., 5 weeks later	100%	Serum IgG, NAb response, viral RNA, viral antigen	
					HeV		50%		

(*Continued*)

Table 1 (*Continued*)

Approach	Name(s)	Animal model	Vaccination dose, route, schedule	Adjuvant	Challenge	Challenge dose, route, schedule	Survival	Correlate(s) of immunity/ protection	Reference
Adenovirus	ChAdOx1 NiV-B G	Hamster	2 doses at 1×10^8 IU, i.m., 28 days apart	None	NiV-B	5.3×10^5 $TCID_{50}$, i.p., 28 days later	100%	NAb response, viral RNA, infectious virus, virus shedding	153
			1×10^8 IU, i.m.		NiV-M	6.8×10^4 $TCID_{50}$, i.p., 28 days later	100%		
					HeV	6×10^3 $TCID_{50}$, i.p., 28 days later	33%		
Measles virus	rMV-Ed-G or rMV-HL-G	Hamster	2 doses at 2×10^4 $TCID_{50}$, i.p., 3 weeks apart	None	NiV-M	1×10^3 $TCID_{50}$, i.p., 1 week later	100%	Serum IgG response	NA
	rMV-Ed-G	AGM	2 doses at 1×10^5 $TCID_{50}$, s.c., 4 weeks apart			1×10^5 $TCID_{50}$, i.p., 1 week later		Serum IgG response, viral RNA	154
Inactivated RABV	RABV-HeV-G	Mice	3 doses at 10 μg, i.m., 2 weeks apart	None	NT	NA	NA	High NAb titers, serum IgG response	150
	RABV-NiV-B G		2 doses at 10 μg, i.m., 4 weeks apart						155
RABV	RABV-NiV-F and/or -G	Mice	$1 \times 10^{6.5}$ FFU, oral	None	NT	NA	NA	Serum IgG, NAb response	156

All NiV glycoprotein vaccines employ the NiV-M strain unless otherwise indicated.

Abbreviations: AAV, adeno-associated virus; AGM, African green monkey; $CCID_{50}$, 50% cell culture infectious dose; ChAdOx1, chimpanzee adenovirus Oxford 1; F, fusion glycoprotein; FFU, focus forming units; G, attachment glycoprotein; HeV, Hendra virus; i.d., intradermal; i.m., intramuscular; i.n., intranasal; i.p., intraperitoneal; i.t., intratracheal; IFNAR, interferon receptor; IgG, immunoglobulin G; IU, infectious unit; LD_{50}, 50% lethal dose; MVA, modified vaccinia virus Ankara; NA, not applicable; NAb, neutralizing antibody; NiV, Nipah virus; NiV-B, Nipah virus Bangladesh; NiV-M, Nipah virus Malaysia; NT, not tested; PFU, plaque forming unit; RABV, rabies virus; rMV-Ed, recombinant measles virus Edmonston; rMV-HL, recombinant measles virus HL; s.c., subcutaneous; sF, F glycoprotein soluble ectodomain; sG, G glycoprotein soluble ectodomain; $TCID_{50}$, 50% tissue culture infectious dose; VSV, vesicular stomatitis virus; ZEBOV-GP, *Zaire ebolavirus* glycoprotein.

Table 2 VLP, subunit, and nucleic acid–based vaccine strategies for NiV and HeV

Approach	Name	Animal model	Immunization dose, route, schedule	Adjuvant	Challenge	Challenge dose, route, schedule	Survival	Immune correlate(s) of survival	Reference
VLPs	VLPs-NiV M/F/G	Mice	2 doses at 1.75, 3.5, 7, or 14 μg and 6 μg, s.c., 2 weeks apart	None	NT	NA	NA	High NAb titers	159
		Hamster	1 dose or 3 doses at 30 μg, i.m., 3 weeks apart	Alhydrogel/MPLA or Alhydrogel/CpG	NiV-M	1.6×10^4 PFU (3-dose trial) or 3.3×10^4 PFU (1-dose trial), i.p., 28 days later	100%	NAb response, viral RNA	160
Subunit vaccines	NiV-sG	Cat	3 doses at 100 μg, s.c., 2 weeks apart	CSIRO triple adjuvant	NiV-M	5×10^2 $TCID_{50}$, s.c., 2 months later	100%	NAb response, viral antigen, viral genome	14
	HeV-sG	Cat	3 doses at 100 μg, s.c., 2 weeks apart	CSIRO triple adjuvant	NiV-M	5×10^2 $TCID_{50}$, s.c., 2 months later	100%	NAb response, viral antigen, viral genome	14
			2 doses at 50, 25, or 5 μg, i.m., 3 weeks apart	CpG/Alhydrogel		5×10^4 $TCID_{50}$, o.n., 2 weeks later	100%	Serum IgG, NAb response, viral RNA, viral shedding, infectious virus	162
		Ferret	2 doses at 100, 20, or 4 μg, i.m., 20 days apart	CpG	HeV	5×10^3 $TCID_{50}$, o.n., 3 weeks later	100%	NAb response, viral RNA, infectious virus	163
				CpG/Alhydrogel	NiV-B	5×10^4 $TCID_{50}$, 20 days later or 12 months later	100%	viral RNA, viral antigen, infectious virus	164

(*Continued*)

Table 2 (*Continued*)

Approach	Name	Animal model	Immunization dose, route, schedule	Adjuvant	Challenge	Challenge dose, route, schedule	Survival	Immune correlate(s) of survival	Reference
		AGM	2 doses at 100, 50, or 10 μg, i.m., 3 weeks apart	CpG/Alhydrogel	NiV-M	1×10^5 $TCID_{50}$, i.t., 3 weeks later	100%	Serum IgG, NAb response, viral RNA, viral antigen, infectious virus	165
			2 doses at 100 μg, i.m., 3 weeks apart	Alhydrogel or CpG/Alhydrogel	HeV	5×10^5 PFU, i.t., 21 days later	100%	NAb response, viral RNA	166
		Horse	2 doses at 100 or 50 μg, i.m., 3 weeks apart	Zoetis	HeV	2×10^6 $TCID_{50}$, o.n., 28 or 194 days later	100%	NAb response, viral RNA, viral antigen, infectious virus	167
		Pig	2 doses of 2 mL preformulation, i.m., 3 weeks apart	Zoetis	HeV	5×10^5 PFU, i.n., 35 days later	Partial	NAb response, viral RNA, infectious virus, viral shedding	168
					NiV-M		0%		
Nucleic acid–based vaccine	HeV-sG mRNA LNP	Hamster	10 or 30 μg, i.m.	None	NiV-M	1×10^5 $TCID_{50}$, i.p., 30 days later	30% or 70%	Serum IgG, NAb response	169

All NiV glycoprotein vaccines employ the NiV-M strain.

Abbreviations: AGM, African green monkey; CSIRO, Commonwealth Scientific and Industrial Research Organisation; F, fusion glycoprotein; G, attachment glycoprotein; HeV, Hendra virus; i.m., intramuscular; i.n., intranasal; i.p., intraperitoneal; i.t., intratracheal; IgG, immunoglobulin G; LNP, lipid nanoparticle; M, matrix protein; MPLA, monophosphoryl lipid A; mRNA, messenger RNA; NA, not applicable; NAb, neutralizing antibody; NiV, Nipah virus; NiV-B, Nipah virus Bangladesh; NiV-M, Nipah virus Malaysia; NT, not tested; o.n., oronasal; PFU, plaque forming unit; s.c., subcutaneous; sG, G glycoprotein soluble ectodomain; $TCID_{50}$, 50% tissue culture infectious dose; VLP, virus-like particle.

as determined by the lack of NiV RNA and infectious virus from nasal washes, pharyngeal swabs, and a variety of sampled organs (140).

ALVAC-vectored vaccines encoding HeV F or G glycoprotein for potential use in horses were also examined (141). ALVAC-HeV-F or ALVAC-HeV-G vectors were combined and first used to vaccinate hamsters at a high or low dose of each vector, by s.c. injection, and then challenged with HeV by i.p. administration. Vaccination did not result in complete protection, with 8 out of 9 subjects in the high-dose group and 5 out of 8 subjects in the low-dose group surviving challenge. No signs of disease were noted, and viral antigen or viral RNA could not be detected in survivors. Nine ponies vaccinated using the same prime-boost regimen were able to develop high cross-neutralizing antibody titers to HeV and NiV-M at day 28 after vaccination. Although ponies were not challenged, most animals yielded titers of at least 1:32 and were considered likely protective (141).

More recently, a modified vaccinia virus Ankara (MVA) vector encoding NiV-M G glycoprotein and a soluble version of G (NiV-sG) were examined in interferon receptor α and β (IFNAR−/−) knockout mice (142) (**Table 1**). IFNAR−/− mice were immunized once with MVA-NiV-G or MVA-NiV-sG or prime-boosted. IFNAR−/− mice developed high serum immunoglobulin G (IgG) titers to NiV-G and also generated NiV-G-specific CD8 and CD4 T cells following vaccination. MVA-NiV-sG vaccination induced rapid and significantly higher amounts of NiV-G epitope-specific CD8 T cells compared with the MVA-NiV-G candidate vaccine, suggesting superior immunogenicity. Together, these immunization studies with poxvirus vectors highlight that both T cell and B cell responses play a role in an adaptive immune response to NiV and HeV. However, detailed studies on the adaptive immune responses in animal experiments with henipaviruses have been limited. Future studies evaluating the role of NiV-specific T cells will be important because two human survivors of NiV-B infection in the 2018 outbreak in Kerala showed marked elevation of activated $CD8^+$ T cells, which coincided with virus clearance (143).

Vesicular stomatitis virus vectored. Recombinant vesicular stomatitis virus (rVSV) vectors as a vaccine platform suitable for single immunization strategies to potentially meet emergency use requirements have been tested by several groups (**Table 1**). A method of using two defective VSVΔG vectors each expressing only the NiV G or F glycoprotein was devised using VSV G glycoprotein complementation that can generate replication-defective VSV vectors that could elicit NiV-neutralizing antibodies (144). Using this technique, researchers tested rVSV vaccines expressing either NiV-M F or G glycoproteins (VSV-ΔG-NiVG, VSV-ΔG-NiVF) in hamsters by intramuscular (i.m.) vaccination (145). Hamsters were then challenged 32 days later with NiV-M by i.p. administration. All vaccinated animals survived lethal infection with no clinical signs of disease. No viral RNA or viral antigen could be detected in the sampled tissues when compared with controls, and there was a lack of an anamnestic immune response in vaccinated subjects following challenge, suggesting the induction of sterilizing immunity.

Another study used rVSV-ΔG vectors expressing NiV-B F or G glycoprotein and also tested them as single-injection vaccinations in NiV-M-challenged ferrets (146). Ferrets were vaccinated i.m. with rVSV-NiV-B F or rVSV-NiV-B G complemented with VSV G or a mix of both vectors, rVSV-NiV-B F/G, that was generated as a complementing pair in the absence of VSV G and then challenged at 28 days with NiV-M by i.n. administration. All vaccinated ferrets were completely protected against NiV-M challenge. Although viral RNA was detected in blood at day 6 post-challenge in 2 of 5 animals in each group, those levels were 100 times lower than in the unvaccinated controls, and by day 21 no viral RNA was detected (146). In a second study, rVSV-NiV-B F and rVSV-NiV-B G were assessed separately and in combination in AGMs (147). Cohorts were

vaccinated with the rVSVs by i.m. injection and challenged 28 days later with NiV-B divided between the i.t. and i.n. routes (147). Complete protection was recorded from NiV-B disease with no gross pathology and no detectable NiV antigen in lung or spleen tissues. Viral RNA was detected in nasal and oral swabs of the vaccinated groups, but no viral RNA could be detected in blood samples.

Replication-competent rVSV-NiV-M F or G vectors, generated by the retention of the envelope glycoprotein from *Zaire ebolavirus* (ZEBOV-GP), which allowed virus stocks to be propagated (rVSV-ZEBOV-GP-NiVF, rVSV-ZEBOV-GP-NiVG, and rVSV-ZEBOV-GP-NiVN), have also been tested (148). These rVSVs were used to immunize hamsters by i.p. administration and were challenged 28 days later with NiV-M. All subjects vaccinated with either the NiV F or G glycoprotein encoding rVSV vectors were completely protected with no clinical disease or pathology, whereas those vaccinated with the NiV N protein were only partially protected (2 of 6 animals) with no clinical signs of disease and the other subjects succumbed to infection. The protective efficacy of the rVSV-ZEBOV-GP-NiVG was also tested in AGMs, where vaccinated subjects were challenged with NiV-M by i.t. administration 29 days later (149). All vaccinated subjects were protected from lethal challenge and showed no signs of clinical disease, no viral RNA was detected in the blood or oral and nasal swabs, and no infectious virus could be recovered. Another study using a rVSV vector expressing a codon-optimized HeV *G* gene together with an inactivated counterpart was evaluated in mice for humoral immune responses only as a comparator to a recombinant rabies virus vaccine encoding HeV G as a HeV vaccine candidate (150). Here, the live rVSV vectors induced greater levels of HeV G-specific antibodies and higher levels of HeV-neutralizing antibodies than did the recombinant rabies virus vectors (see the section titled Rabies Virus Vectored).

Adeno-associated virus and adenovirus vectored. Adeno-associated virus (AAV) vectors as a vaccine platform against infectious diseases, particularly viral pathogens, have been explored. AAV is a small, single-stranded DNA virus in the family *Parvoviridae*. Immunization of hamsters with an AAV vector expressing NiV-M G glycoprotein (AAV8 NiV.G) by i.m. injection demonstrated complete protection from a challenge of NiV-M by i.p. administration, and no signs of clinical disease were recorded (151) (**Table 1**). Neutralizing antibodies to NiV were induced, no viral RNA or viral antigen was detected in any of the sampled tissues, and there was only a moderate anamnestic response observed in a single subject, suggestive of potential sterilizing immunity. However, in a cross-protection study, AAV8 NiV.G protected only 50% of hamsters challenged with HeV.

Chimpanzee adenoviral (ChAd) vectors circumvent issues of the preexisting immunity observed with human adenovirus vectors (152). Adenoviruses are double-stranded DNA viruses in the family *Adenoviridae*. An engineered replication-deficient ChAd vector, Oxford 1 (ChAdOx1), was tested as a NiV/HeV vaccine (153). Here, ChAdOx1 encoding NiV-B (ChAdOx1 NiV-B) G glycoprotein was used to vaccinate hamsters by i.m. injection, either as a single dose or as a prime-boost protocol. Hamsters were challenged by i.p. administration with NiV-B 42 days following the booster or the single vaccination. Neutralizing antibodies were detectable, and all vaccinated hamsters were protected against lethal disease with no lung pathology, suggesting that a single dose of ChAdOx1 NiV-B was sufficient to completely protect against NiV-B. No viral RNA in the lung tissue and no viral shedding in oropharyngeal swabs could be detected, and no infectious virus could be isolated. A second cohort using a single dose of ChAdOx1 NiV-B to vaccinate hamsters was trialed, and these animals were challenged 28 days later with NiV-M or HeV. All vaccinated animals were protected from lethal NiV-M challenge, but 4 out of 6 hamsters succumbed to HeV disease between days 5 and 7 post-challenge. Neither virus shedding in oropharyngeal swabs nor

infectious virus was detected in the lung or brain tissues of NiV-M-challenged vaccinated hamsters. In contrast, infectious virus was detected in the lung tissues of 75% of the HeV-challenged vaccinated animals. The lower cross-protection observation using NiV G vaccination followed by HeV challenge was not unexpected, as it was previously shown that when the G glycoprotein (as a recombinant soluble subunit immunogen) of either HeV or NiV was used to vaccinate cats, both could completely protect against lethal NiV-M challenge, and that the HeV-sG elicited greater heterologous neutralizing antibody responses in comparison to NiV-sG (14).

Measles virus vectored. Recombinant measles virus vectors based on the HL (rMV-HL) and Edmonston (rMV-Ed) measles virus strains have also been explored in which they encoded the NiV-M G glycoprotein (rMV-HL-G and rMV-Ed-G) (154) (**Table 1**). Hamsters were immunized twice by i.p. administration of rMV-HL-G or rMV-Ed-G. All vaccinated animals produced NiV G-specific antibody titers after the booster immunization. Animals were challenged 1 week after the second immunization with NiV-M by i.p. administration. All immunized hamsters exhibited no clinical symptoms and survived challenge. The study was extended to non-human primates (NHPs), where 2 AGMs were immunized twice by s.c. injection with rMV-Ed-G. Subjects were challenged 2 weeks after the second immunization with NiV-M by i.p. administration. Here, immunization completely protected the AGMs with no observed clinical disease and no detectable pathological changes, and no viral RNA could be detected in sampled tissues. Although this was a small study, the safety profile and success of the live-attenuated measles virus vaccine suggests that a recombinant platform encoding the NiV G glycoprotein as a NiV vaccine candidate is promising and should induce a balanced and long-lasting immune response against NiV.

Rabies virus vectored. A rabies virus (RABV) SAD B19 vaccine strain, BNSP333, expressing HeV or NiV G glycoproteins has been evaluated (150, 155). Recombinant BNSP333 encoding either the wild-type or a codon-optimized HeV *G* gene, together with their inactivated counterparts, was used in mice (150) (**Table 1**). Mice were immunized by i.m. injection with a single dose of the RABV-based vectors or with 3 doses of their inactivated versions. The inactivated RABV-based vectors induced higher and more rapid HeV G-specific antibody responses and higher neutralizing antibody titers than their live counterparts. The inactivated RABV-coHeV-G induced cross-neutralizing antibodies against NiV. A similar study used the BNSP333 vector expressing NiV-B G glycoprotein (RABV-NiV-BG) (**Table 1**) and elicited NiV G-specific neutralizing antibodies (155).

Recently, the recombinant RABV Evelyn-Rokitnicki-Abelseth (ERA) strain ($rERAG_{333E}$) expressing either NiV-M F or G glycoproteins was evaluated in mice and pigs (156) (**Table 1**). This vector, $rERAG_{333E}$, serves as an oral vaccine in dogs. Here, mice were orally immunized with RABV-NiV-F or RABV-NiV-G either individually or in combination. Pigs were also immunized in a similar manner but with 2 doses of each vector either alone or in combination. RABV-NiV-F and/or RABV-NiV-G immunization induced NiV F- and G-specific IgG antibody responses and neutralizing antibodies in both mice and pigs with the combination vaccine inducing higher titers. Although not suitable for human use, the live-attenuated $rERAG_{333E}$ vector is of interest as a potential veterinary vaccine for NiV because it is already approved for use in some animals and could be adapted for emergency use to protect against NiV infection in livestock, particularly swine.

Many of these virus-vectored vaccines for NiV are promising candidates because of their established safety profiles and ease of genetic modification. Several of these virus-vectored vaccines also require no adjuvants, and some are clearly efficacious as a single immunization strategy, suitable features for emergency use circumstances. In addition, several of these platforms are able to induce both cell-mediated and humoral immune responses, which may also be desirable but as yet

are not fully explored in the development of vaccines for NiV and HeV. Although animals immunized with viral vectors encoding the NiV G glycoprotein and challenged with the homologous virus were completely protected, cross-protection studies with some of these vaccines against a HeV challenge were less effective. For example, only 50% of AAV8 NiV.G-immunized hamsters or 33% of ChAdOx1 NiV-B G glycoprotein–immunized hamsters were protected from a lethal HeV challenge (151, 153). In addition, the ALVAC-HeV-F and ALVAC-HeV-G vaccination studies showed that these vectors did not provide 100% protection in hamsters challenged with HeV, perhaps due to either a suboptimal immunization dose or the immunization route (141).

Virus-like particles. VLPs have been explored as a vaccine platform because of the resemblance of their surface structure, dimensions, and compositions to authentic virus yet are of high safety because of the lack of viral genetic material. Earlier studies revealed that the M protein of NiV was capable of orchestrating the formation and budding of NiV VLPs when expressed in cells that appeared structurally similar to authentic NiV virions, and these VLPs could also incorporate other viral proteins such as the F and G glycoproteins (157, 158). VLPs composed of NiV M, F, and G were used to vaccinate mice s.c. at weeks 0, 2, and 4 and demonstrated they could induce high neutralizing antibody titers by day 35 (159) (**Table 2**). NiV VLPs were later used in NiV-M challenge studies either alone or in combination with adjuvant, monophosphoryl lipid A (MPLA) and Alhydrogel^{TM} (15 μg/50 μg) or CpG and Alhydrogel (40 μg/50 μg) (160). Hamsters were vaccinated i.m. either as a single dose or as a 3-dose regimen and then challenged via i.p. administration of NiV-M at 28 days or 58 days, respectively. In all cohorts, 100% of the vaccinated animals survived with no clinical signs of disease and no detection of viral RNA in any of the sampled tissues, regardless of the presence of adjuvant. VLPs are thus an alternative means, with inherent safety, of producing an inactivated whole virus vaccine from an otherwise highly pathogenic virus.

Subunit vaccine. The HeV-sG subunit vaccine has been extensively evaluated in several studies. Here, a brief summary of earlier reports is made, but the focus is on studies in NHPs and livestock. Recombinant HeV-sG and NiV-sG can elicit a potent neutralizing antibody response and were first tested as vaccine immunogens in the feline model (14, 161) (**Table 2**). Both HeV-sG and NiV-sG vaccination of cats completely protected against lethal NiV-M challenge, and HeV-sG elicited greater heterologous neutralizing titers than did NiV-sG, demonstrating that a single subunit vaccine may be effective against both NiV and HeV (14). Other studies using lower doses of HeV-sG (**Table 2**) demonstrated that a pre-challenge neutralizing titer of 1:32 could protect against NiV-M (162). Additional studies in ferrets showed that low doses of HeV-sG could protect against HeV and NiV-B (163, 164) (**Table 2**). Also, a longevity study showed that vaccinated ferrets challenged with NiV-B at 14 months post-immunization, with pre-challenge neutralizing titers of 1:16 to 1:128, were also protected (164).

The HeV-sG vaccine has been extensively evaluated in AGMs (**Table 2**). In a cross-protection study, 100 μg, 50 μg, or 10 μg doses of HeV-sG in combination with Alhydrogel and CpG were administered i.m. as a prime-boost, on days 0 and 21. Pre-challenge 50% neutralization titers ranged from 1:28 to 1:379. All subjects were challenged with NiV-M by i.t. administration on day 42. All vaccinated subjects were completely protected, displaying no clinical signs of disease, and no viral RNA could be detected in blood and tissues and no infectious virus was isolated (165). Similarly, HeV-sG vaccination HeV challenge in AGMs has also been performed. Using a prime-boost regimen, AGMs were vaccinated twice, 3 weeks apart, by i.m. injection with 100 μg HeV-sG with Alhydrogel or HeV-sG with Alhydrogel and CpG, and then challenged 3 weeks later with HeV by i.t. administration (166). All vaccinated animals were completely protected from clinical

disease, and no HeV RNA or viral antigen could be detected in swabs, blood, or tissues, and notably HeV-sG formulated in only Alhydrogel protected (166).

The efficacy and inherent safety of the HeV-sG subunit led to its development as an equine vaccine to prevent HeV infection of horses and also reduce the risk of HeV transmission to people, as a One Health concept (**Figure 1**). HeV-sG, formulated in an approved equine adjuvant (Zoetis, Inc.), was evaluated in two efficacy studies; the first tested 50 μg and 100 μg doses of the same HeV-sG used in prior animal studies to vaccinate horses, and the second used 100 μg doses of HeV-sG produced in Chinese hamster ovary cells (Zoetis, Inc.). Two vaccinations were given by i.m. administration 3 weeks apart. All horses in these efficacy studies were challenged by oronasal inoculation with HeV (**Table 2**). Seven horses were challenged at 28 days and 3 horses were challenged at 194 days after the second immunization. All vaccinated horses remained clinically healthy following challenge; pre-challenge neutralization titers ranged from 1:128 to more than 4,096 in horses challenged 21 days after vaccination and only from 1:16 to 1:32 in horses challenged at 6 months. There was no gross or histologic evidence of infection in any of the vaccinated horses at study completion, and all tissues examined were negative for viral antigen, with no viral genome detected in any tissue. In 9 of 10 vaccinated horses, HeV nucleic acid was not detected in daily nasal, oral, or rectal swab samples or from blood, urine, or fecal samples collected before euthanasia, no recoverable virus was present, and no rise in antibody titer was detected in any vaccinated horse following challenge (167). The HeV-sG horse vaccine (Equivac® HeV) was launched by Zoetis, Inc., in November 2012 on a minor use permit by the regulatory authority, the Australian Pesticides and Veterinary Medicines Authority (APVMA), and is the first commercially developed and deployed vaccine against a BSL-4 agent. All vaccinated horses are microchipped, and a database is maintained. Equivac HeV received full registration by the APVMA in 2015. To date, more than 765,000 doses of Equivac HeV vaccine have been administered to more than 179,000 unique horses, and laboratory-confirmed HeV infections in horses have since occurred only in unvaccinated animals.

Studies showed HeV-sG as a NiV vaccine in the pig model (which is a non-lethal challenge model) was much less effective in comparison to results observed in the cat, ferret, NHP, and horse, and HeV-sG was only partially protective against HeV challenge and unprotective against NiV-M in the pig (168). These experiments also indicated that both humoral and cellular immune responses were required for protection of swine against NiV and HeV. Here, pigs were immunized with HeV-sG in a proprietary adjuvant (Zoetis, Inc.), and subjects were challenged with HeV or NiV via i.n. administration (**Table 2**). HeV-sG-vaccinated pigs developed neutralizing titers ranging from 1:160 to 1:320 to HeV, but only partial protection was achieved with reduced viral RNA in tissues and no recoverable virus, and there was no reduction of viral shedding in nasal washes. These HeV-sG-vaccinated pigs did not develop neutralizing antibodies to NiV-M that were considered protective (low), nor did they have measurable activation of cellular immune memory. Only a comparative group of pigs that were first orally infected (vaccinated) with NiV (and recovered) were subsequently protected against an i.n. rechallenge with NiV. This group of pigs developed protective antibody levels and cell-mediated immune memory responses (168).

Single-dose lipid nanoparticle mRNA, HeV-sG vaccine. More recently, messenger RNA (mRNA)-based vaccines have emerged as an attractive vaccine strategy because of safety, efficacy, and rapid implementation features. In a recent study, the efficacy of an mRNA vaccine approach was assessed in a NiV-M animal challenge model (169). mRNA transcripts encoding HeV-sG were complexed with lipid nanoparticles (LNPs) to generate HeV-sG mRNA LNP. Two groups of 10 hamsters were vaccinated with a single dose of HeV-sG mRNA LNP at either 10 μg or 30 μg by i.m. injection. Subjects were challenged with NiV-M by i.p. administration 30 days

post-vaccination (**Table 2**). The HeV-sG mRNA LNP was only partially protective, with 3 hamsters from the low-dose group and 7 hamsters from the high-dose group surviving challenge. Of the surviving animals, signs of clinical disease were observed in 2 low-dose group and 6 high-dose group hamsters; however, disease symptoms were gone by study termination. NiV *N* gene RNA levels in the blood and a variety of tissues in surviving hamsters were lower compared with nonsurvivors, but NiV RNA copy levels were not different compared with controls. No anti-NiV IgG or virus-neutralizing activity was detected in vaccinated animals prior to challenge; however, all post-challenge survivors were positive for anti-NiV IgG antibodies, and all survivors (in both groups) had similar neutralizing titers ranging from 1:160 to 1:640. Euthanized animals had little to undetectable neutralizing activity, highlighting the correlation of this immune response to protection. Although promising, the partial efficacy of HeV-sG mRNA LNP observed in this study suggests that further optimization of vaccination route, addition of an adjuvant, and/or a prime-boost regimen is needed.

SUMMARY AND FUTURE PERSPECTIVES

The frequency of henipavirus outbreaks and human infections is a significant global health concern. A promising passive immunization strategy has been developed using a human mAb, m102.4, shown effective in the NHP challenge model, which has also been administered numerous times to people by compassionate use protocol and has successfully completed a phase I safety trial in Australia. In addition, the Equivac HeV vaccine is available, targeting the protection of horses and also people by breaking the chain of HeV transmission to people, and is an example of a One Health approach to counter an infectious disease threat. Over the past 15 years, nearly a dozen NiV and HeV vaccine approaches have been trialed in animal challenge models, and many show promise as effective human-use vaccines. Recently, the formation of the Coalition for Epidemic Preparedness Innovations (CEPI), a global partnership between public and private organizations, was undertaken with the goals of developing vaccines against emerging infectious diseases and offering equitable access to those vaccines (170). Indeed, without the support of CEPI, the prospects of having a NiV or HeV vaccine suitable for use in people, at a deployable stage in the event of a significant outbreak, would have remained academic. Research teams can now capitalize on the large body of basic and preclinical vaccine development data on a half-dozen important emerging viral threats including NiV and, with the support of CEPI, can develop vaccine candidates for clinical use and future licensure. Several of the NiV human vaccine candidates described in this review are now supported by CEPI.

DISCLOSURE STATEMENT

C.C.B. is a US federal employee and co-inventor on US and foreign patents pertaining to soluble forms of HeV and NiV G and F glycoproteins and monoclonal antibodies against HeV and NiV whose assignee is the United States as represented by the Henry M. Jackson Foundation for the Advancement of Military Medicine (Bethesda, Maryland). The soluble forms of the HeV and NiV G glycoproteins are licensed to Zoetis, Inc., and Aurobindo Pharma USA Inc. M.A. declares no competing interests.

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Errata

An online log of corrections to *Annual Review of Virology* articles may be found at http://www.annualreviews.org/errata/virology

From: Broder, Christopher (b) (6)
To: (b) (6)
Cc: (b) (6) ; Eric Laing; (b) (6)
Subject: Re: CEPI RfP on Nipah epidemiology
Date: Wednesday, August 18, 2021 12:06:28 PM
Attachments: AA-Amaya-Published-annurev-virology-021920-113833.pdf

---

hi (b) (6)

Sure, that's fine.

Sorry but what I meant before on the last question, was "we" (b) (6), myself and (b) (6) **do not** know who could address all 3 workflows easily .

I guess my HeV point is:

If the goal of the RFP is aimed at gathering genetic data in support of vaccine development, why
is the RFP focused only on Nipah virus isolates, all of which are highly similar (92-99.9% identical), when we know now
that the Hendra G glycoprotein (only 78% identical to Nipah G) can completely protect against Nipah Malaysia and Nipah Bangladesh
in multiple animal models?

Nipah from Thailand being essentially identical to Bangladesh and all Bangladesh isolates essentially identical amongst themselves (99,9%),
and Nipah Malaysia to Bangladesh >92% identical among all proteins with G being 95.5% identical. Any vaccine using G as the immunogen
of choice delivered in a way to induce a broad polyclonal response will be effective against all the isolates of Nipah virus we know of.

Importantly, Nipah G does not provide complete protection against a Hendra virus challenge; this has been done with ChAdOx1 and AAV.
We settled on HeV-sG as the vaccine of choice in 2004, because the first test in the cat model showed it to be superior in eliciting higher cross-reactive
neut titers to Hendra and Nipah, as compared to Nipah sG

What if a new Nipah virus emerges in SEA or Oceana that is more related to Hendra?
Those Nipah G vaccines based on the Bangladesh G will likely not be as effective, whereas the Hendra G vaccine will be.

(b) (6)

(b) (6) will inquire with his partners in a soon to be funded DTRA project for virus surveillance in the Philippines. But we do not think there are any human
samples available. (b) (6) has active surveillance projects in India and Bangladesh and Malaysia now,
but most of these activities target wildlife/livestock but some human sampling, and are serology based.

best wishes
(b) (6)

On Tue, Aug 17, 2021 at 3:32 PM (b) (6) wrote:

> Hello (b) (6),
>
> Thank you for your email – always good to hear from you!
>
> This RfP was prepared and launched by CEPI's Epidemiology group, which is primarily responsible for its contents. I disseminated the RfP to our Task Force members as a courtesy, and I will be supporting the Epi team tangentially by reviewing applications and by ensuring that we engage external stakeholders in a coordinated fashion.
>
> On that note, you raise questions and comments along the lines of others who have replied. Would it be OK with you if your questions highlighted in yellow are logged to be followed up in CEPI's public response to questions raised? The questions would be anonymized. If you have additional questions to these, please let us know. The HeV point is an important one and I would suggest framing into a question for public follow up.
>
> Best wishes,
>
> (b) (6)
>
> ---
>
> **From:** Broder, Christopher (b) (6)
> **Sent:** Tuesday, August 17, 2021 8:43 PM
> **To:** (b) (6)
> **Subject:** CEPI RfP on Nipah epidemiology
>
> CAUTION: This message was sent by an address external to CEPI. Please do not click on links or open attachments unless you recognize the source of this email and know the content is safe. Contact support@intility.no or it@cepi net if uncertain.
>
> hi (b) (6)

--

(b) (6)
[redacted]

viruses

MDPI

Review

Overview of Bat and Wildlife Coronavirus Surveillance in Africa: A Framework for Global Investigations

Marike Geldenhuys [1], Marinda Mortlock [1], Jonathan H. Epstein [1,2], Janusz T. Pawęska [1,3], Jacqueline Weyer [1,3,4] and Wanda Markotter [1,*]

[1] Centre for Viral Zoonoses, Department of Medical Virology, Faculty of Health Sciences, University of Pretoria, Pretoria 0001, South Africa; marike.geldenhuys@up.ac.za (M.G.); marinda.mortlock@up.ac.za (M.M.); epstein@ecohealthalliance.org (J.H.E.); januszp@nicd.ac.za (J.T.P.); jacquelinew@nicd.ac.za (J.W.)
[2] EcoHealth Alliance, New York, NY 10001, USA
[3] Centre for Emerging Zoonotic and Parasitic Diseases, National Institute for Communicable Diseases, Johannesburg 2131, South Africa
[4] Department of Microbiology and Infectious Diseases, School of Pathology, University of Witwatersrand, Johannesburg 2131, South Africa
* Correspondence: wanda.markotter@up.ac.za

Abstract: The ongoing coronavirus disease 2019 (COVID-19) pandemic has had devastating health and socio-economic impacts. Human activities, especially at the wildlife interphase, are at the core of forces driving the emergence of new viral agents. Global surveillance activities have identified bats as the natural hosts of diverse coronaviruses, with other domestic and wildlife animal species possibly acting as intermediate or spillover hosts. The African continent is confronted by several factors that challenge prevention and response to novel disease emergences, such as high species diversity, inadequate health systems, and drastic social and ecosystem changes. We reviewed published animal coronavirus surveillance studies conducted in Africa, specifically summarizing surveillance approaches, species numbers tested, and findings. Far more surveillance has been initiated among bat populations than other wildlife and domestic animals, with nearly 26,000 bat individuals tested. Though coronaviruses have been identified from approximately 7% of the total bats tested, surveillance among other animals identified coronaviruses in less than 1%. In addition to a large undescribed diversity, sequences related to four of the seven human coronaviruses have been reported from African bats. The review highlights research gaps and the disparity in surveillance efforts between different animal groups (particularly potential spillover hosts) and concludes with proposed strategies for improved future biosurveillance.

Keywords: coronaviruses; surveillance; biosurveillance; Africa; bat; emerging; African bat coronaviruses; wildlife; domestic animals; COVID-19; HCoV-229E; HCoV-NL63; MERS-CoV; SARS-CoV; SARS-CoV 2; surveillance strategies

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1. Introduction

In the past two decades, four novel coronaviruses of public and veterinary health importance have emerged. These include the three agents originating from China; severe acute respiratory syndrome coronavirus (SARS-CoV) in 2002 [1,2], swine acute diarrhea syndrome coronavirus (SADS-CoV) among localized pig farms in 2017 with re-emergence in 2019 [3,4], and SARS-CoV 2 towards the end of 2019 [1,4–6]. The fourth emergent coronavirus, Middle East respiratory syndrome coronavirus (MERS-CoV), emerged in the Arabian Peninsula in 2012 [7,8]. These events show that coronaviruses have the potential to spillover from natural hosts into different species and cause severe diseases with devastating consequences. Dromedary camels are considered the reservoirs of MERS-CoV,

though the original source and transmission routes from animals are still uncertain for SARS-CoV and SARS-CoV 2 [9–13], with related viruses identified in bats. Different amplification hosts are considered to be involved in all three human coronavirus (HCoV) outbreaks.

The link between bats and emerging coronaviruses was first considered in 2005 following the identification of coronaviruses related to SARS-CoV in specific Asian rhinolophid bat species [14–16]. Since then, a high diversity of coronavirus nucleic acids has been detected in bats, several of which are related to coronaviruses infecting human and domestic animals, with hundreds of unclassified sequences pending characterization. The expanding knowledge of coronavirus diversity has additionally allowed for novel insights into their evolutionary history, including linking bats as the ancestors of specific mammalian coronavirus lineages [17,18]. More specifically, bat coronaviruses with genetic similarity to known coronavirus species, such as HCoV229E and HCoVNL63, are suggested to have acted as ancestors of these human viruses from previous spillover events [19].

Biosurveillance of wildlife hosts, including bats, are one of the first steps towards understanding how viruses emerge [20,21] and include identifying viral diversity, host species, and distribution ranges. However, several factors have been implicated in spillover events, including genetic, ecologic, epidemiological, and anthropological elements [22]. Unless the underlying factors are also identified and mitigated, coronaviruses are likely to continue to emerge in the future.

The high biodiversity on the African continent supports viral species richness, which has been correlated with disease hotspot mapping and novel viral diseases that have emerged or re-emerged in Africa to date [22]. Many communities in Africa live in close contact with wildlife, domesticated animals, and livestock. Some surveillance for bat coronaviruses has been performed in Africa. A recent review by Markotter et al. [23] provides a comprehensive summary of potentially zoonotic coronaviruses reported from Africa (relatives of HCoV229E, HCoVNL63, MERS-CoV, and SARS-CoV), focusing on the distribution of the host bat species, and concluding that inferences on zoonotic potential based on the genetic relatedness is limiting. This review focuses in greater detail on the total coronavirus diversity identified among African animal species. We review published literature concerning bat species targeted, sample sizes, viral genetic diversity, and evolutionary links to specific host species. The review was also expanded to include the currently available surveillance data among non-bat wildlife and domesticated livestock as hosts of coronavirus diversity. We highlight surveillance approaches from previous studies, important findings, and gaps in current surveillance and propose a surveillance framework to guide the design of future biosurveillance studies.

2. The Importance of Viral Taxonomy

The hierarchical levels of the coronavirus taxonomy are well described [24]. There are currently four genera in the *Orthocoronavirinae* subfamily: the *Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus*, and *Deltacoronavirus*. The *Alphacoronavirus* and *Betacoronavirus* genera predominantly infect mammals and are further divided into subgenera (Figure 1A,B). Human coronaviruses group within either the *Duvinacovirus, Setracovirus, Sarbecovirus, Merbecovirus*, or *Embecovirus* subgenera (Figure 1A,B). Coronavirus genomes consist of several non-structural genes in open reading frame (ORF) 1 (encoding the replicase polyprotein pp1ab), followed by four structural genes and several accessory genes depending on the species (Figure 1C). Current classification criteria for coronaviruses (ICTV code 2019.021S) rely on comparative amino acid sequence analysis of five domains within the replicase polyprotein pp1ab: 3CLpro, NiRAN, RdRp, ZBD, and HEL1 [6,25]. Computational approaches are used to estimate genetic divergence, and thresholds are utilized as demarcation criteria at various taxonomic levels (Figure 1C,D) [24]. Moreover, only complete genomes are considered for formal taxonomic placement.

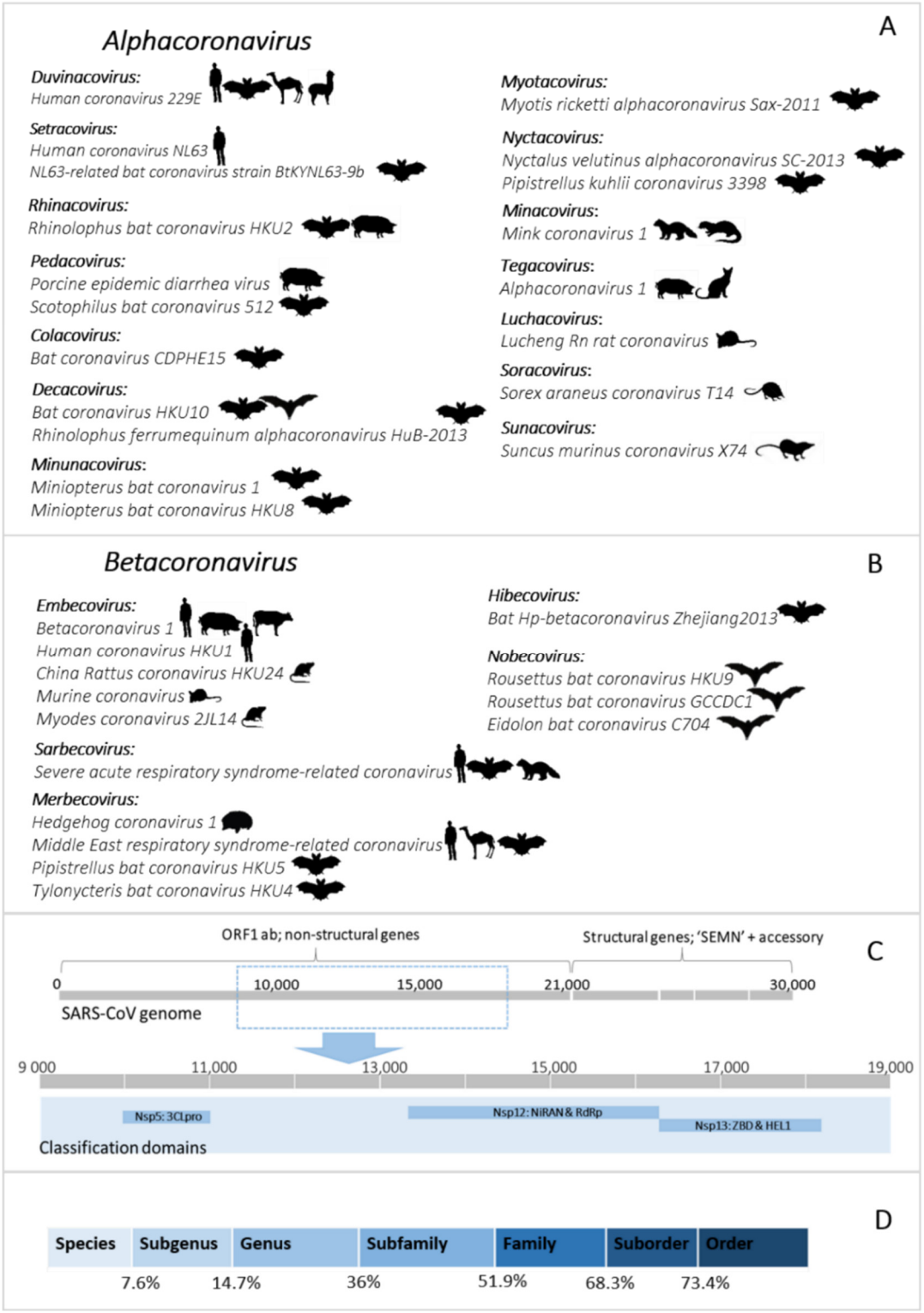


Figure 1. (**A**,**B**) Current coronavirus subgenera (bold) and species of the *Alphacoronavirus* and *Betacoronavirus* genera. The images indicate host species associated with the virus species. Figure constructed with the species listed on the 2019 Release of the ICTV Virus Taxonomy 9th Report MSL#35: (Available at https://talk.ictvonline.org/ictv-reports/ictv_9th_report/positive-sense-rna-viruses-2011/w/posrna_viruses/222/coronaviridae accessed on 12 December 2020). (**C**) Representation of the coronavirus genome (based on the reference genome NC_004718.3 SARS coronavirus Tor2) depicting the locations of important domains for classification of species (NSP5 (3CLpro), NSP12 (NiRAN and RdRp), and NSP13 (ZBD and HEL1)). (**D**) Thresholds of the taxonomic demarcation criteria [24]. Novel viruses are part of a taxonomic level if the divergence within the five concatenated replicase domains is less than the indicated amino acid percentage.

Since the initial identification of bat coronaviruses in 2005, a total of 16 formally recognized coronavirus species have been described from bats. Biosurveillance research mainly report on partial sequences of the coronavirus genome and can only be described to a limited extent by their phylogenetic grouping or similarity percentages. Sequences are considered 'related' to genetically similar sequences in a phylogenetic cluster, pending the viral diversity included in the inference. This 'related' terminology has become widely misrepresented. It is frequently used to indicate the relatedness of sequences to the closest human coronavirus (HCoV) in a phylogeny, even if these sequences may be significantly distant. For example, SARS-CoV belongs to the *Sarbecovirus* subgenus; and the *Hibecovirus* subgenus forms a sister-clade to the sarbecoviruses (Figure S2). Sequences with low similarity to sarbecoviruses, and which should be part of the hibecoviruses, have (even recently) been deemed as 'SARS-related'. Erroneous conclusions may be readily avoided by including all representative diversity of the current taxonomy in phylogenies. In this review, we will employ the convention of limiting the use of 'related' only to describe bat coronaviruses deemed sufficiently similar to known species according to demarcation criteria (e.g., MERS-related, SARS-related, 229E-related, and NL63-related). All others will be described in relation to phylogenetic clusters, using sequence similarities where possible, or indicating possible grouping within a subgenus (Figure 1A).

3. Biosurveillance Studies Based on Nucleic Acid Detection in Africa

Table 1 stipulates the selection criteria utilized to identify and classify publications included in the review. Several surveillance studies focused on bat species were identified [19,26–47], though few studies were found in regards to surveillance among other wild animals or livestock [40,48–51] (Table 1). This may be due to the 'reactive' nature of surveillance among livestock, domestic animals, and non-bat wildlife in response to outbreak events among farmed animals or human populations; such events have not been regularly reported in Africa. Global examples include studies involving farmed civets following the first SARS-CoV outbreak, surveillance in camel herds after identifying MERS-CoV and detecting SARS-CoV 2 among mink farms in Europe [9,52,53]. Coincidentally, the use of passive unbiased metagenomic next-generation sequencing among illegally smuggled pangolins identified sarbecoviruses with overall genome similarity of 85.5% to 92.4% to SARS-CoV 2 in Asia [10,54,55].

Table 1. Selection and classification criteria of studies included in the review.

Search criteria:	Google scholar searches with keywords: "bat, bats, fruit bats, insectivorous bats, animal, mammal, livestock, domestic, domesticated, wildlife, coronavirus, coronaviruses, detections, Africa, Sub-Saharan, Southern Africa, Eastern Africa, nucleic acid, molecular detection, serology, serological, surveillance, survey" were used to search for peer-reviewed publications documenting surveys for coronaviruses in mammals from Africa (mainland Africa as well as islands associated with Africa such as Madagascar, Reunion Island, Seychelles).
Selection criteria:	For a suitably thorough synopsis of the findings, publications were limited to research available until the end of December 2020 and excluded dissertations, theses, or non-peer-reviewed publications. Sequences included in phylogenetic analyses in this review also excluded sequences from dissertations, theses, or unpublished sequences on GenBank that are not linked to available publications. However, PREDICT surveillance data ('PREDICT 1 and 2 surveillance and test data') linked to a 2017 publication was accessed online from Healthmap.org [56] and included both surveillance among bats and other wildlife and livestock.
Criteria for 'primary surveillance reports':	Reports containing a description of the collection and testing of samples from animals for coronavirus surveillance. For bat surveillance, we focused on surveillance strategies using nucleic acid detection methodologies such as family-wide consensus PCR analysis or unbiased high throughput metagenomic sequencing. This includes re-testing samples from an earlier report with a different assay and reporting additional coronaviruses detected. Primary surveillance reports may contain varying levels of characterization for detected viruses. We expanded this criterion for livestock and non-bat wildlife to include both nucleic acid and serological surveillance.
Criteria for 'secondary characterization reports':	Refers specifically to studies based on a primary surveillance report that does not describe new sample collection but a detailed characterization of viral sequences identified in a previous publication or more in-depth analysis of data obtained from primary surveillance reports.

3.1. Surveillance in African Bats

Several surveillance studies focused on bats have been performed in Africa since the first reports in 2009 [26,37]. We identified 23 primary surveillance reports and four subsequent secondary characterization reports [57–60] (Table 2 and Figure 2) that included sampling in 24/54 African countries (www.un.org, accessed on 6 September 2020). Several reports originate from Kenya, Ghana, Gabon, and South Africa (Table 2, Figure 2), with limited surveillance in Morocco and Tunisia [33]. Most studies focused on one or more sites within a single country (Table S1), though few studies include once-off sampling from multiple African countries [30,33,38,45]. Anthony et al. [30] describe the PREDICT surveillance performed over a 5-year timespan in more than 20 countries, seven of which took place in Africa (with Rwanda surveillance further detailed in Nziza et al. [36]). Furthermore, nine reports identified coronaviruses while conducting broader virological surveillance [29,31,32,34–36,39,45], whereas others were coronavirus specific. Supplementary Tables S1–S4 summarize the different reports in terms of approach, species and sample numbers, nucleic-acid detection strategy, and overall findings, including when the information was omitted or not sufficiently described.

Table 2. Bat coronavirus surveillance performed in Africa, per country.

Country (3 Letter Country Code)	References [Primary Surveillance]/(Characterization Report) *
Cameroon	[30,34]
Central African Republic (CAF)	[45]
Democratic Republic of the Congo (DRC)	[30]
Egypt (EGY)	[27]
Gabon (GAB)	[30,40,45]
Ghana (GHA)	[37,44,46]
Guinea (GIN)	[39]
Kenya (KEN)	[19,26,29,(57)]
Madagascar (MDG)	[38,47]
Mauritius (MUS)	[38]
Mayotte (MYT)	[38]
Morocco (MAR)	[38]
Mozambique (MOZ)	[38]
Nigeria (NGA)	[28,41]
Republic of the Congo (COG)	[30,45]
Reunion Island (REU)	[38]
Rwanda (RWA)	[30,35,36,(60)]
Senegal (SEN)	[45]
Seychelles (SYC)	[38]
South Africa (RSA)	[32,42,43,(58)]
Tanzania (TZA)	[30,(60)]
Tunisia (TUN)	[33]
Uganda (UGA)	[30,(59,60)]
Zimbabwe (ZWE)	[31]

* References in square brackets indicate primary surveillance reports; Round brackets refer to 'secondary characterization reports'.

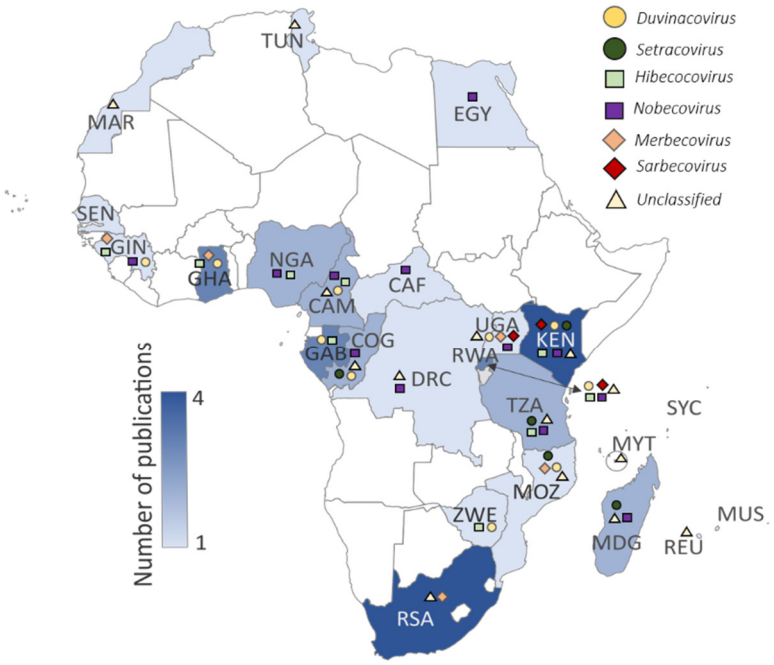


Figure 2. Published bat coronavirus surveillance studies per country (shading denoting the number of publications). Symbols in the key above the map represent different coronaviruses detected in the respective countries: Duvinacovirus as a yellow circle (HCoV229E-related viruses), Setracoronavirus as a dark green circle (HCoVNL63-related viruses), Sarbecoviruses as a red diamond (HCoV-SARS-related viruses), Merbecoviruses as an orange diamond (HCoV-MERS-related viruses), Nobecoviruses as a purple square, Hibecoviruses as a green square, and unclassified viruses as a black triangle. Further details on coronaviruses identified can be reviewed in Table S4. Three-letter ISO country code abbreviations are shown on the map.

3.1.1. Sampling Approaches and Methodologies of Bat Coronavirus Surveillance

Overall, the primary aim of most of the reports was to detect the presence of coronavirus RNA in bat species, with limited subsequent genetic characterization. Bat species and sample numbers were opportunistically sampled at roosts in mainly cross-sectional once-off sampling focused on a targeted population, region, or species. The frequency of sampling was generally poorly described (Table S1). Exceptions include reports from Madagascar, Nigeria, and Zimbabwe, where multiple sampling events (2 or more) were performed at the same roosts [28,31,47]. Figure 3 provides a graphical summary of the approaches employed by surveillance efforts for bat coronaviruses (Tables S1 and S2).

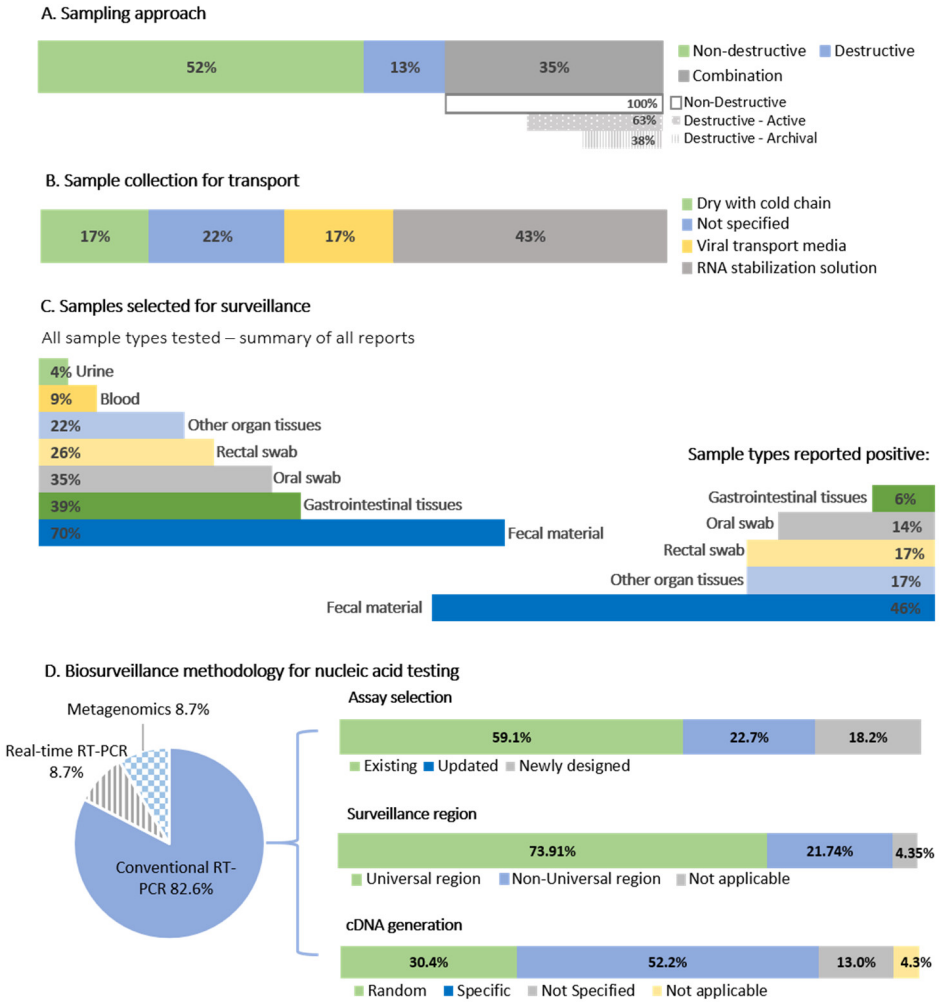


Figure 3. A summary of coronavirus sampling approaches and methodology. (**A**) The sampling approaches of the 23 primary surveillance reports. Combination studies are split into those employing new or archival destructive sampling. (**B**) Sample preservation methods. (**C**) Sample types selected for surveillance and samples testing positive. (**D**) Biosurveillance methodology for nucleic acid testing, percentage of studies using conventional, real-time, or metagenomic approaches. The conventional assays were further split into existing assays from the literature, updated exiting assays, or whether new assays were developed. The percentages of studies targeting the 'universal surveillance region' (see text for an explanation) contrast to those using different genome regions, and whether specific or random primers were chosen for cDNA preparation.

It is well established that coronaviruses display a gastrointestinal tropism in bats [61], and fecal material or other gastrointestinal sample types such as rectal swabs (non-destructive) or intestinal tissue (destructive) is the preferred sample types for surveillance (Figure 3). Sample collection was mostly non-destructive (52% of studies), including fecal material collected beneath roosting bats in caves and trees [28,29,31,33,37] or fecal material and rectal swabs from individual bats [19,26,27,30,32,34,35,37,42–44,46,47]. For this review, we are assuming fecal swabs are the same as rectal swabs. Only 13% of studies solely implemented destructive sampling (collection of organ tissues), and 35% of studies (Figure 3) combined both methodologies to collect sample material for multi-pathogen surveillance [27,30,35,41,43,45] or were tested due to availability within archival tissue banks [32,39,42]. Along with gastrointestinal samples, oral (or throat) swabs were also collected [19,26,27,30,47], but infrequently contained coronavirus RNA [19,27,30,36,39]. Due to limited reporting information provided per study, coronavirus detection among oral swabs can only be roughly estimated. Of all reports investigated, only 35% tested oral swabs (Figure 3). From these reports, 62.5% identified coronavirus RNA, representing positive

oral swabs from only 14% of studies overall (Table S1). Coronaviruses were also opportunistically detected within lung and liver tissues [27,38,45], though it is unclear what other positive individuals' organs were also tested.

The basic methodology implemented in all but two studies [32,34] involved RNA extraction of samples followed by nucleic acid detection targeting a conserved region of the genome. A region of the RNA dependent RNA polymerase (RdRp) gene within the open reading frame (ORF) 1b of the coronavirus genome (Figure 4) is mostly targeted and corresponds to approximate nucleotide position 15,200–15,600 in the coronavirus genome (using reference NC_004718.3 SARS coronavirus Tor2) (Figure 4, Table S2). Targeting of this "universal coronavirus surveillance region" enables comparison between studies, though 74% of the African bat surveillance studies utilized assays based on the region (22% either used a non-universal region or combination of both; Table S2). The addition of a nested step is generally essential for the detection of low concentration viral RNA. A small number of studies in Africa quantified viral concentrations of positive samples, obtaining as little as 50–450 RNA copies/mg fecal material for some low concentration samples, or between 323 to 1.5 × 10^8 RNA copies/g of fecal material [37,44].

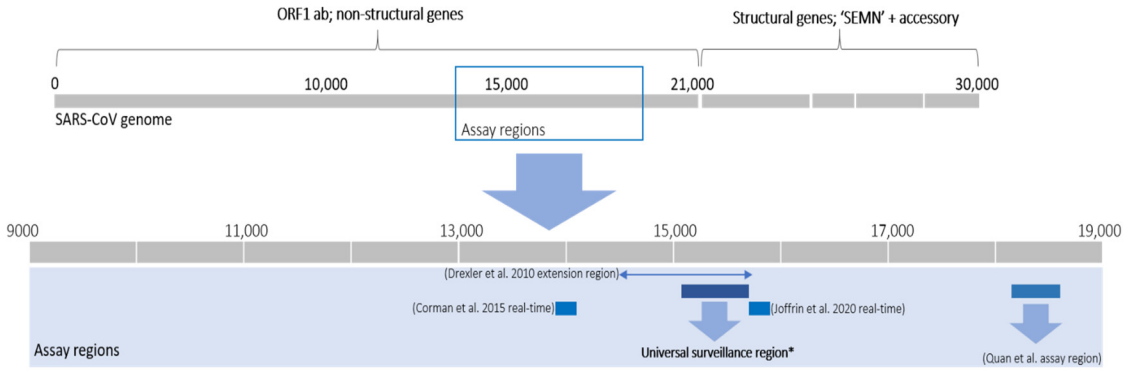


Figure 4. Representation of the coronavirus genome (based on the reference genome NC_004718.3 SARS coronavirus Tor2) depicting the assay regions. The assays corresponding to this universal region included in Tong et al. [26], de Souza Luna [62], Geldenhuys et al. [42] and Geldenhuys et al. [32] (based on primers from Woo et al. [63]), Razanajatovo et al. [47] (based on Poon et al. [14]), Shehata et al. [27], Waruhiu et al. [29] (based on Watanabe et al. [64]), Chu et al. [65], Gouilh et al., [33]. The RdRp grouping units (RGU) amplification region by Drexler et al. [66] is indicated with the line and arrows.

The majority of surveillance studies (52.2%) implemented a one-step kit approach (i.e., utilizes RNA templates in a single reaction with target-specific primers for cDNA followed directly by PCR amplification), with seven (30.4%) implementing an unbiased methodology for the preparation of cDNA with random hexamers before PCR amplification [31–33,35]. An unbiased approach is more beneficial where only limited sample material is available and multi-pathogen surveillance is done. Suitable assays were either selected from the literature (with the assay from de Souza Luna et al. [62] most frequently employed), constitute newly developed assays (included if no reference was provided for assay modifications), or were updated/modified from the literature (Table S2 and Figure 3). Assays selected from the literature were constructed using the available sequence information known at that point in time. The expanding genetic diversity of coronaviruses is high, and even though these assays target a conserved region, existing primers may be less sensitive toward the detection of more diverse viruses. For example, primers developed before the 2012 emergence of MERS-CoV might not be sufficiently sensitive to detect diverse coronaviruses from the Merbecovirus subgenus. Developing new assays or updating available primers have the added advantage of ensuring that some of the expanding sequence diversity of emerging human coronaviruses and newly detected animal coronaviruses can be incorporated; reducing the probability of highly diverse clades going undetected.

Exceptions to this 'universal CoV surveillance' region are represented mainly by the nested RT-PCR assay developed by Quan et al. [41], targeting a region downstream of the universal CoV surveillance region, corresponding to the approximate nucleotide position 18,300–18,700 (Figure 4). Sequences amplified with the assay from Quan et al. [41] cannot be directly incorporated in phylogenies using the short universal CoV surveillance region and may only be compared to viruses for which this corresponding genome region is available or with full genomes. The PREDICT surveillance described in Anthony et al. [30] and Nziza et al. [36] utilized two surveillance assays to test samples; that of Watanabe et al. [64] based on the universal region and Quan et al. [41]. In total, the Watanabe assay detected 950 coronavirus sequences compared to the 654 sequences from the Quan assay, with only a 27% overlap [30].

Overall, it is not possible to directly compare methodologies to conclude best practices for coronavirus surveillance. However, non-destructive sampling methodologies (swab collection or fecal material from underneath roosting bats) associated with a gastrointestinal origin allow for successful coronavirus identification with minimal injury to the hosts or ecosystem. Proper preservation of sample material is good practice (cold chain or using preservation media), and unbiased cDNA preparation approaches allow for the conservation of reagents and sample material. The use of appropriate assays and overlapping target regions are essential to enable comparisons between studies.

3.1.2. Summary of Sample Sizes and Bat Species Tested

The surveillance data from the 23 publications were compared to the 2019 African Chiropteran Report (comprehensive report of the current taxonomy with data based on museum records from bats collected across the continent) to determine an estimate of total bats sampled per species (Table S4; [67]). There are 13 extant bat families in Africa, with an estimated 324 species [67]. Eleven families have been included in coronavirus surveillance reports (Table 3). Several publications provided the total bats sampled within a study though may not have specified per species or country, and thus 1966 sampled bats could not be included [29,41]. The sample numbers (per species per country) were not specifically indicated in Anthony et al. [30], but total PREDICT surveillance data for the seven African-surveyed countries was accessed online from Healthmap.org and included in the analyses. We acknowledge that the data likely exceeds the sample size for the countries used for the analysis in the 2017 publication; however, we felt that including the data in our assessment greatly contributes to the total bats sampled in Africa per species—by over 10,000 individuals. Moreover, this data was also used in Tables 2 and S1–S3. Of the approximate 127 total bat species included in studies, bat coronaviruses were identified in 59. Nearly 26,000 bat individuals are estimated to have been tested for coronaviruses in African surveillance studies using one or more assays. However, this number comprises mainly pteropid and hipposiderid bats (41.8% and 33%, respectively) and varies per family. The table below highlights the need for additional surveillance in several families, such as the Vespertilionidae. These are abundant bats, and increasing the sample size tested of species in this family may provide a greater understanding of the host ecology of coronavirus species such as MERS-related viruses.

Table 3. Coronavirus detections according bat host taxonomy.

Bat Families Tested	Number of Species	Species Tested	Bat Species Positive	Number of Individuals Tested Per Family *	Positive Individuals #
Pteropodidae	44	22	14	10,851	881 (8.1%)
Hipposideridae	21	10	8	8563	257 (3%)
Molossidae	44	16	8	2144	286 (13.3%)
Miniopteridae	22	12	5	1464	120 (8.2%)
Vespertilionidae	114	37	9	918	41 (4.5%)
Rhinolophidae	38	14	9	728	68 (9.3%)
Emballonuridae	11	4	0	678	0
Nycteridae	15	6	3	299	51 (17.1%)
Rhinonycteridae	6	3	2	250	74 (29.6%)
Megadermatidae	2	2	1	25	3 (12%)
Rhinopomatidae	3	1	0	1	0
Myzopodidae	2	0	0	0	-
Cistugonidae	2	0	0	0	-
Totals	324	127	59	25,921	1779 (6.9%)

* Counts for number of individuals tested reflect individuals from publications reporting total individuals tested per species per country, or total positive individuals in reports where total sampled are not provided. These counts exclude 1966 bats tested in [29,41] from which species totals were not provided, and studies testing colony-level fecal samples [28,31].
# Approximate number of positives from Table S5.

Coronavirus RNA has been detected in nine of the eleven families sampled, excluding the Emballonuridae and Rhinopomatidae. The Rhinopomatidae represents only one tested individual; approximately 678 bats from four species in the Emballonuridae family have been investigated (*Coleura afra, Taphozous perforates*, *Taphozous mauritianus,* and *Taphozous hildegardeae*). This includes surveillance from eight countries with sample sizes varying from 1 to 172 (Tables S4 and S5). Comparatively, coronaviruses have been identified from families like the Megadermatidae, Rhinonycteridae, or Nycteridae, from which far fewer individuals were analyzed (25–299). The lack of viral detection from the Emballonuridae family could be due to insufficient sample sizes, extremely low prevalence, time of sampling, highly diverse viruses missed by consensus primers, or the absence of coronaviruses. The remaining unsampled Myzopodidae and Cistugonidae families are small (two species each), with limited distributions in Madagascar and Southern Africa, respectively.

Primary surveillance reports investigating one or two species/genera typically focus on abundant hosts that may form large populations with frequent opportunities for contact with human communities [28,31,32,34]. Studies sampling many diverse genera/species (83% of primary surveillance reports) mostly sample species opportunistically present at one or more surveillance sites (Table S3). To estimate sample sizes per species, we looked at the total and average number of individuals per species tested in these reports and specifically noted sample sizes of less than ten individuals (Table S3). For some species, below ten individuals were tested, whereas several hundred [19,27,30,36,45,47] or even thousands of individuals from other species were sampled [44,46]. It was more common for less than 100 individuals to be sampled per species, though a few reports averaged 100–150 per species [19,27,30,36,45,47]. The percentage of species within a report for which less than ten individuals were sampled ranged between 18.5 to 100% of species (Table S3). This constituted more than 50% of species sampled from 11 of the reports and likely represented opportunistically caught individuals. This could not be determined for a further four reports, as sufficient detail was not specified, or samples collected represent colony or population-level sample collection.

A guideline for optimal sample sizes per species was proposed by the meta-analysis of coronavirus surveillance in 20 countries by Anthony et al. [30], with the optimal sampling number being approximately 397 individuals. This was calculated to detect the av-

erage number of unique coronavirus groups relating to probable viral species (2.67) estimated to be present in each bat species. Their findings identified that sampling less than 154 individuals per species constituted poor returns on investment and sampling effort [30]. The percentage of species per report from which coronavirus nucleic acids were detected varied between 8.3% to 66.7% (excluding when only one species was sampled). Overall, the percentage positivity of coronaviruses per total samples ranged from below 1% to 25.7% (excluding pools) (Table S3). As expected, increasing either sample sizes or number of species tested show correlation with increased positivity percentages (Pearson's product correlation $t = 8.9289$, df = 21, $p < 0.001$ and $t = 5.4952$, df = 20, $p < 0.001$, respectively). The differences in positivity can be attributed to many factors, including the nucleic acid detection assay, the methodology for sample collection (preservation of nucleic acids), time of sampling coinciding with coronavirus excretion, species sampled, and sufficient sample numbers per species. Tables S4 and S5 highlight species commonly detected to host coronaviruses; a detailed description of 'high-risk' viruses identified from host species is described below.

3.1.3. Importance of Accurate Bat Species Identification

Correct identification of bat species is essential to conclude potential virus-host associations and estimation of host-viral distribution ranges. This is especially important for complex bat species with similar morphological markers, such as members of the Hipposideridae, Rhinolopidae, and Vespertilionidae. Since the start of coronavirus nucleic acid surveillance among bat species in Africa in 2009, several bat species have undergone species reassignments and name changes. We could not update all new species names for this review and used the taxonomy described in the 2019 African Chiropteran report [67]. However, recent changes of note are among the Hipposideridae, Rhinolophidae, Miniopteridae, and Vespertilionidae families, with additions of new genera (*Afronycteris*, *Pseudoromicia, Vansonia* (elevated to genus)) and the reassignment of species to existing and new genera [68–71]. Some of these include *Hipposideros* species reassignments to the genus *Macronycteris* and the resolution of some *Neoromicia* species with reassignments to *Laephotis, Afronycteris,* and *Pseudoromicia* genera [68,69]. Currently recognized species may be accessed at www.batnames.org (accessed 18 November 2020) [72], and new species need to be correctly correlated to geographical distributions.

We investigated the methodologies for host identification implemented by the primary surveillance reports (Table S3). No identification methodologies for bat species were stipulated in seven (30%) of the bat coronavirus surveillance studies; five (22%) report the use of keys to determine morphological identities by either field teams, veterinarians, or experienced chiroptologists; and two (9%) report the use of molecular means of species confirmation. Only nine reports (39%) describe both morphological and molecular methods to identify and confirm host species (Table S3). Molecular methods include either mitochondrial cytochrome B gene or cytochrome C oxidase subunit I sequencing [73,74]. Not only is this good practice in ensuring accurate determination of host species identity, but if deposited on public reference databases, it ensures that the records of these sequences for sampled species are expanded. However, depositing sequences of individuals lacking accurate morphological identification and failure to update taxonomic changes generally leads to confusion and incorrect host reporting. Thus, reference material on these databases must be associated with correctly identified individuals where morphological identification was conducted by highly trained individuals or experienced bat taxonomists.

3.1.4. Characterization of Bat Coronavirus Genomes and Virus Isolation Attempts

Bat coronavirus surveillance in Africa primarily focused on amplifying and sequencing short amplicon sequences and subsequent diversity determination. The majority of African bat coronaviruses are therefore unclassified and are only represented by a short-sequenced region. Further characterization of the detected coronaviruses is essential for

improved phylogenetic placement and comparisons of various genes/proteins for phenotypic analyses. Studies aiming to further characterize identified coronaviruses employed diverse methodologies (Table S2). Sequence-specific primers have been successful in extending the sequenced regions of the ORF1ab [28,47] or recovering complete coding regions of structural genes like the nucleoprotein gene [27,37]. Sequencing these regions generally involved primer-walking strategies with conventional Sanger sequencing or even high throughput sequencing platforms to overcome the length limit of conventional sequencing. The informal RdRp gene grouping units (referred to as RGU; Figure 4) developed by Drexler et al. [66] amplifies an 816 nucleotide amplicon of the RdRp gene. The pairwise distances of the translated 816 nucleotide fragments (272 amino acids) have been used to delimit different groups as a surrogate system for taxonomic placement of detected bat coronaviruses that lack complete genomes. Grouping units of alphacoronaviruses differ by 4.8% and betacoronaviruses by 5.1% [61]. These grouping units have been used as an extension assay by 22% of African bat coronavirus studies [32,35,43,44,46]. It is worth noting that these units are an unofficial estimate of possible species groupings and may be subject to revision as new diversity is detected (as evident by previous decreasing betacoronavirus thresholds from 6.3% to 5.1%) [61].

The number of bat coronaviruses that can correctly be assigned to a viral species is limited to those with available complete genomes. From African studies, there are over 1840 partial coronavirus gene sequences available among public domains (such as NCBI's GenBank), though only 13 complete genomes and 12 near-complete genomes [19,32,34,41,46,57–59]. The MERS-related *Pipistrellus* bat coronavirus from Uganda was recovered with unbiased sequence-independent high throughput sequencing on the MiSeq platform [59] and a near-complete genome of Zaria bat coronavirus from Nigeria using 454 pyrosequencing [41]. Sanger sequencing with classic primer-walking spanning the entire genome with 70 overlapping hemi-nested PCR assays was implemented to recover a MERS-related *Neoromicia* bat coronavirus from South Africa [58], with a second variant from the same host sequenced using 11 overlapping hemi-nested PCR assays on the MiSeq platform [32]. For more novel viruses, amplification of more conserved coronavirus genome segments with nested consensus degenerate primers are frequently required before being able to sequence more diverse regions with long-range PCRs [19,46,57].

The limited number of complete African bat coronavirus genomes are reflective of the challenges involved. These include the limited scope of certain studies, low viral RNA concentrations, unavailability of sufficient material, lacking related reference genomes for primer design, availability of high throughput sequencing platforms, expertise, and cost [32,37,46]. To overcome some of these constraints, such as limited availability of material, virus culturing can be attempted. However, coronaviruses are notoriously difficult to isolate in vitro, with various methodologies utilized (reviewed in Geldenhuys et al. [75]). Only bat coronaviruses closely related to SARS-CoV have thus far been successfully isolated in Vero cells because the bat viruses could use the same receptors as SARS-CoV [76,77]. This challenge and limited sample material available after nucleic acid extraction and high-biocontainment requirements are likely contributing factors to why none of the 23 primary surveillance publications or secondary characterization reports attempted cultivation of coronaviruses in cell culture (nor described attempts).

It is important to note the formats of naming conventions among bat coronavirus studies, with only some providing sufficient information on the origins of sequences (Table S2). The Coronavirus Study Group of the ICTV recommends adopting a standardized format for nomenclature that has been used for Influenza viruses and avian coronaviruses [6]. Namely, the reference to a host organism from which the viral nucleic acid was derived, the place of detection, a unique strain identifier as well as mention of the time of sampling (e.g., virus/host/location/isolate/date or as an example BtCoV/Neoromicia/RSA/UP5038/2015). This format also allows rapid identification of inter-genus viral sharing in phylogenetic trees and highlights similar clades of viruses occurring in related

species independent of geography. More importantly, this naming convention makes no inference of belonging to a particular species, as species assignments may only be performed once the requirements have been met (i.e., sequencing the genome according to species demarcations).

3.1.5. Coronavirus RNA Identified in African Bats

Global coronavirus surveillance in bats has established several generalizations, with which African studies are in agreement. Namely, bat coronaviruses generally display host specificity, which is usually evident at the genus-level [19,61,78–80]. As a result, certain viral species or even subgenera may be predominantly associated with specific host genera (e.g., rhinolophid bats and *Sarbecovirus*). This association has been observed to be independent of the geographical isolation of the bat hosts [38,81,82]. The evolution of coronaviruses has been suggested to involve a combination of two mechanisms, co-evolution between viral and host taxa and frequent cross-species transmission events [78]. Co-evolution is evident by genus-specificity and the large diversity of bat coronaviruses globally sampled, though many taxa host more than one species/group of coronaviruses [37,78]. Meta-analyses of publicly available bat coronavirus sequences confirmed long-term evolution among bats and determined that frequent cross-species transmissions occur, particularly among sympatric species, though often result in transient spillover among distantly related host taxa [19,30,78]. Such transmissions potentially create viral adaptation opportunities to new hosts and increase overall genetic diversity [83]. Uniquely for Africa, the genetic information of bat coronaviruses sharing similarity to human coronaviruses have been identified in four of the five subgenera associated with human coronaviruses—*Duvinacovirus, Setracovirus, Merbecovirus,* and *Sarbecovirus* (Figure 1A,B). Such findings suggest opportunities for transmission from bats to other animals or directly to humans may have occurred in the past. Though these viruses are still circulating among these hosts, discerning current risks of spillover is limited by available evidence.

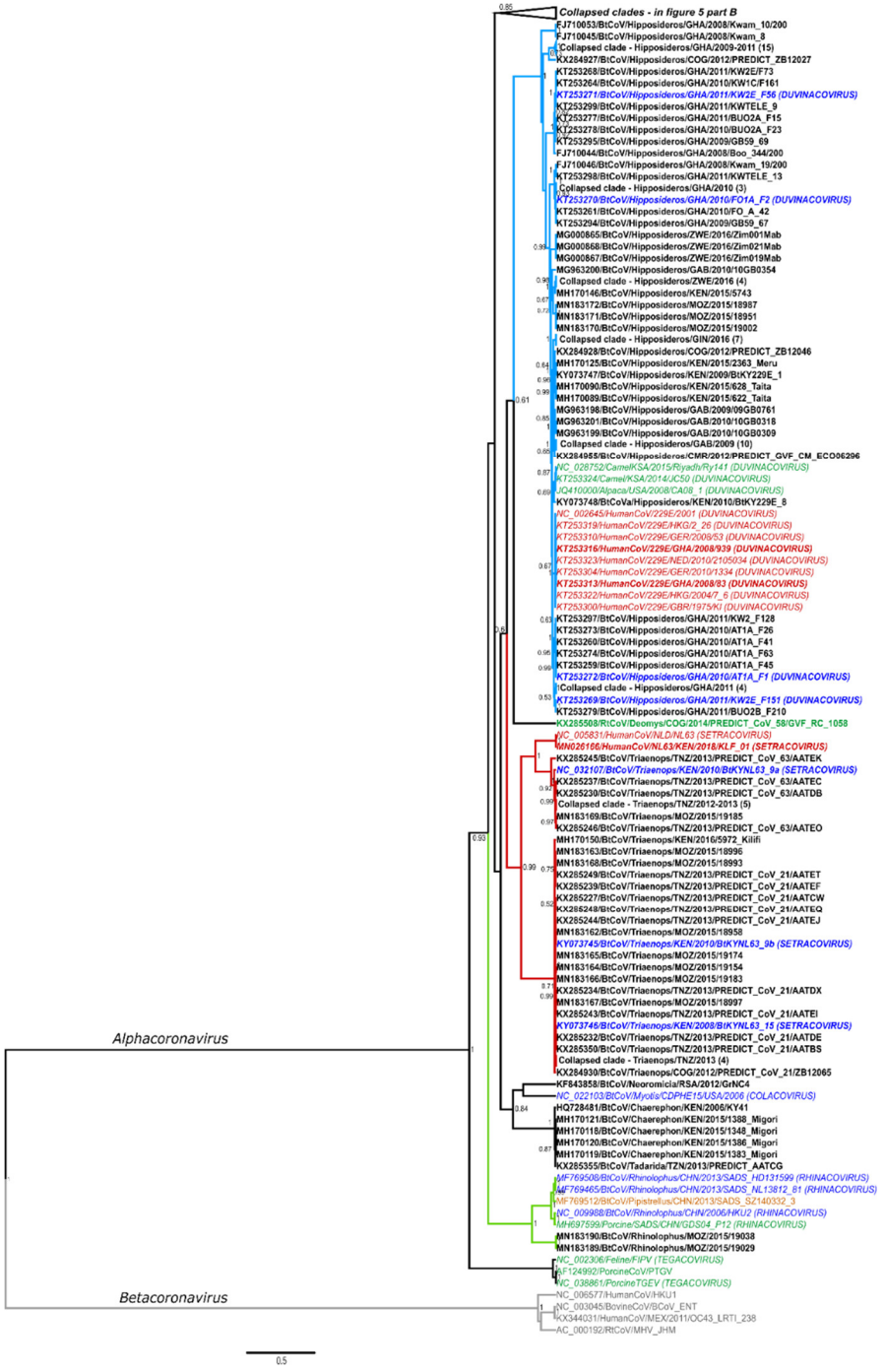
Together with highly variable mutation rates [84,85], coronaviruses are also known for recombination events, where homologous recombination between similar coronaviruses is the most likely. However, recombination between different co-infecting coronaviruses from different subgenera/genera has also been documented [86–88]. Opportunities also increase when bats are co-infected by more than one species of coronavirus. Moreover, heterologous recombination between viral families has also led to the assimilation of novel genes in certain coronaviruses [86,87]. Recombination hotspots within the spike gene have been identified for diverse coronaviruses originating from humans, domestic animals, and bats [89]. Some of the new resultant variants may have improved fitness advantages within their native or new hosts, and new recombinants may be more suited to the usage of new receptor molecules.

Phylogenies were constructed with the sequences from the 23 primary surveillance reports and secondary characterization research studies, representing the sequence diversity of African bat coronaviruses compared to formally classified species and relevant reference sequences (see Appendix A and complete phylogenies in Figures S1 and S2). The following sections summarize the information available regarding detected bat coronaviruses associated with known human coronaviruses and highlight the importance of recombination in the emergence of novel viruses. We also discuss the large diversity of unclassified and unstudied viruses in some highly abundant host species and consider possible interaction opportunities between humans and bat hosts.

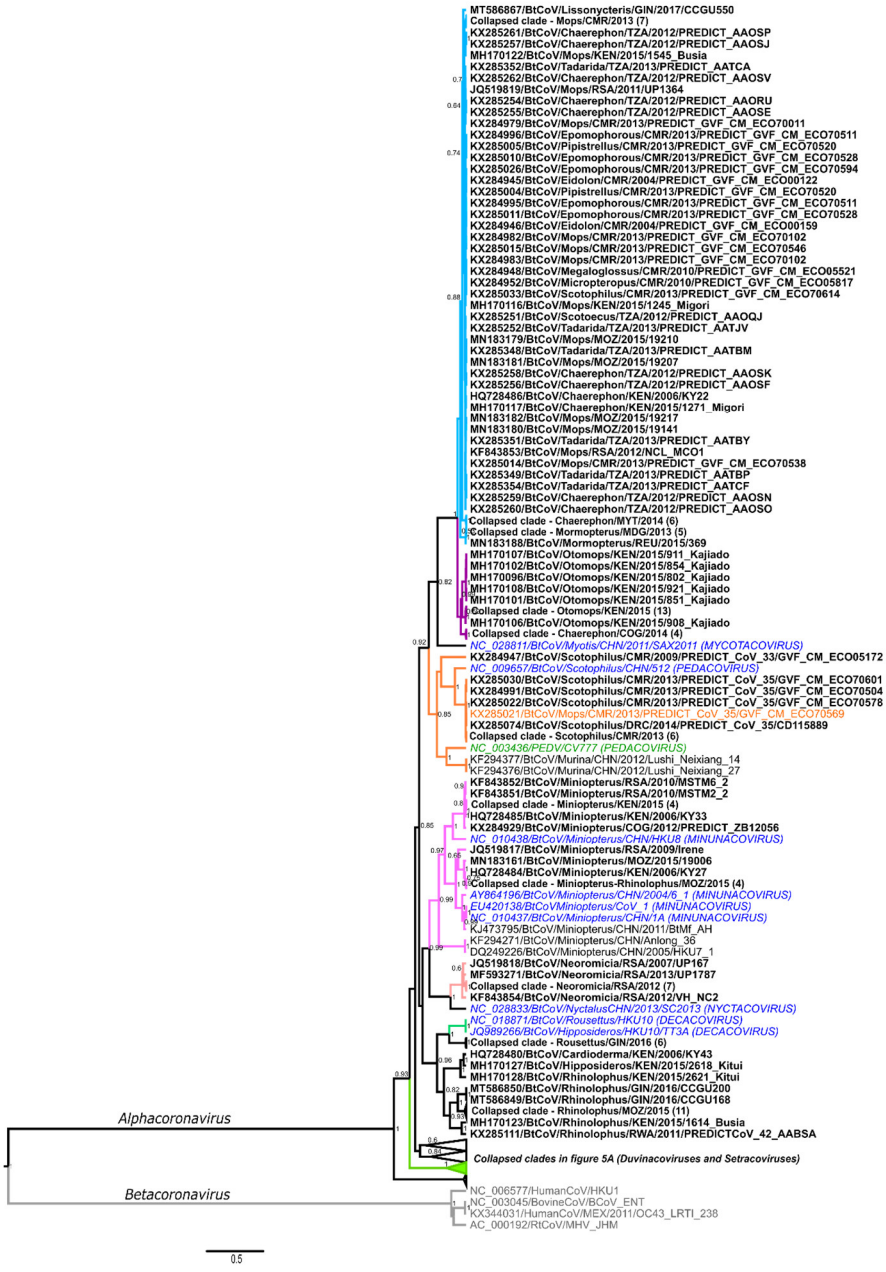
Alphacoronaviruses—*Duvinacovirus*, *Setracovirus*, and Unclassified Virus Relatives of Human Alphacoronaviruses

Several African bat coronaviruses share genetic similarity with the two human alphacoronaviruses, HCoV229E (*Duvinacovirus*) and HCoVNL63 (*Setracovirus*). As seen in Figure 5A, hipposiderid bats (genus *Hipposideros*) are associated with coronavirus se-

quences similar to HCoV229E and have been reported across a wide geographical distribution (Ghana, Kenya, Cameroon, Zimbabwe, Republic of the Congo, Rwanda, Uganda, Gabon, Mozambique, and Guinea) [19,29–31,37–39,45,46]. Due to taxonomic revisions and reassignments [69], the *Macronycteris* genus (Hipposideridae) may also be associated with duvinacoviruses (Table S5).



(**A**)



(**B**)

Figure 5. (**A**,**B**): Alphacoronavirus Bayesian phylogeny of the RdRp partial region (corresponding to approximately 15,200–15,400 nt of the coronavirus genome). Clades collapsed in A are shown in B (and vice versa). To include the maximum number of sequences, sequence lengths were trimmed to a generally useable length of 260 nucleotides. Sequences resulting in shorter lengths were omitted. Sequences in italics indicate formally recognized species (subgenera indicated in capital letters at the end of sequence names); sequences in bold originate in Africa; red highlights human viruses; green indicate non-bat animal hosts; blue/italics indicate formally recognized bat species; orange indicate viral detections from hosts not typically associated with a particular group of coronaviruses. All sequence names were edited to conform to the correct convention, with the modification of the unique sequence identifier listed last due to convenience. Only posterior probabilities of greater than 0.5 are indicated. No unpublished sequences are shown.

Full genomes of four *Hipposideros* alphacoronaviruses from Ghana were compared to current and historical isolates of HCoV229E and an alpaca coronavirus (similar to HCoV229E) from the USA [46]. Sufficient similarity was found between genomes to consider them members of the same *Human coronavirus 229E* species within the *Duvinacovirus* subgenus. The analysis suggested multiple recombination events have occurred among

genomes, including gene losses (e.g., ORF8 within human viruses) and deletions within the spike gene [46]. Several of the bat viruses with similarity to HCoV229E for which no complete genomes are available indicate that there are sequence divergences of approximately 13.5% among RdRp partial gene segments, suggesting circulation of highly diverse HCoV229E-related viruses. The scenario would suggest that HCoV229E may have originated from the large diversity of *Hipposideros* HCoV229E-related bat coronaviruses in the past 200 years (based on the current sequence diversity), with camelids (alpacas, camels, etc.) as possible intermediate hosts [46].

Similarly, several African bat sequences cluster around HCoVNL63 (Figure 5A) and originate from the genus *Triaenops* (Rhinonycteridae family). *Triaenops afer* is the only mainland Africa species currently recognized within the genus after it was split from *T. persicus*, which only occurs in the Middle East [67,90] (with *Triaenops menamena* from Madagascar). Partial and complete genomes were first reported in Kenya [19] with additional partial genomes from the Republic of the Congo, Tanzania, Mozambique, and Madagascar [30,38] (Table S4). Three full genomes were recovered from Kenyan *T. afer* bats and compared to HCoVNL63 [19]. Much like 229E-related bat viruses and HCoV229E, comparisons of the bat viruses to HCoVNL63 identified additional ORFs (ORFx) in bat viral genomes that were absent in HCoVNL63 [19]. The new species, *NL63-related bat coronavirus strain BtKYNL63-9b* (*Setracovirus*), comprised of *Triaenops* coronavirus strains, has been recognized. *Triaenops* virus 9a shares the closest similarity to HCoVNL63 with 78% overall nucleotide identity. The spike was the most divergent gene, with gene phylogenies showing the spike gene of HCoVNL63 grouping with *Hipposideros* 229E-related bat viruses detected in the same study [19]. Recombination analysis of HCoVNL63 indicates multiple breakpoints within the spike gene and suggests a history of recombination between the *Triaenops* NL63-related viruses and *Hipposideros* 229E-related viruses giving rise to the lineage of HCoVNL63 before its introduction into human populations [19]. As with HCoV229E, an intermediate host (and not bats directly) may likely have been involved in introducing progenitor HCoVNL63 viruses into the human populations. Such intermediate hosts are often domesticated livestock animals (such as camelids in the case of HCoV229E) as they have more frequent contact with people, underscoring the need for expansive surveillance within domestic animals to complement surveillance in wildlife.

Bats from the *Hipposideros, Myonycteris* and *Triaenops* genera are all small insectivorous bats and have many overlapping ecological features in terms of habitat. *Hipposideros* and *Myonycteris* primarily roost in caves, though certain species have been known to roost in rock crevices, under bridges, and in tunnels [67]. *Triaenops* have been found roosting in small trees and certain shrubs and mines and caves [91]. Moreover, bats from all three genera are sensitive to human activities that lead to habitat loss and roost disturbance [67]. The surveillance findings show that these viruses continue to circulate in these hosts, with the potential to recombine and create new variants. Establishing whether these viruses pose possible zoonotic risks is limited due to lacking evidence. In vitro studies can assist with determining permissivity or pathogenicity in different cell lines, and protein modeling can suggest the likelihood of receptor binding of bat viruses in spillover hosts. There is also a lack of nucleic or serological investigations into potential spillover animal species that overlap with the bat hosts' geographical distributions and ecological niches.

Alphacoronaviruses—Molossids and a Large Diversity of Uncharacterized Bat Coronaviruses

The diversity of bat alphacoronaviruses from Africa is high. Much of the reported sequences share genetic similarity to members of described subgenera, such as *Rhinacovirus*, *Pedacovirus*, and *Minunacovirus* (Figure 5B). Many of the other sequences represent undescribed diversity and may possibly belong to new subgenera. A large number of unclassified alphacoronaviruses have been identified from molossid bats (Figure 5B). Generally, these sequences form three clades, with sequences similar to a species of

Colacovirus detected in *Chaerephon* and *Tadarida*; a sister clade of the *Mycotacovirus* subgenus that split into an *Otomops*-specific species clade from Kenya; a predominantly *Mops*/*Chaerephon* group of alphacoronaviruses from several countries (Cameroon, Kenya, Tanzania, South Africa and the Republic of the Congo). The latter group also contains a large volume of sequences from various pteropid species (as well as a few vesper species) from Cameroon [30], making it a mixed family clade or a group of viruses frequently prone to host switching. Sequence information on these viruses largely constitutes short sequences from surveillance assays as well as a few partial genomes (HQ728486/BtCoV/*Chaerephon*/KEN/2006/KY22 and HQ728481/BtCoV/*Chaerephon*/KEN/2006/KY41) [57]. These coronaviruses were detected from molossid species such as *Chaerephon pumilus, Mops condylurus, Otomops martiensseni,* and *Tadarida aegyptiaca,* with only 16 of the 44 species from the Molossidae family having been included in surveillance studies. Of note are recent taxonomy changes among this family [72]. Moreover, as indicated in Table S4, large numbers of molossid bats tested are only specified to genus level, with nearly 171 *Chaerephon* spp., 30 *Mops* spp., and 64 *Tadarida* spp. reported. This again reiterates the need to identify hosts down to species level. These species are highly abundant with widespread distributions throughout Africa and are often encountered in urban settings. They are frequently found to be roosting in large populations (several hundred) in the rafters or roofs of buildings such as houses or public institutions like schools, universities, and libraries [67]. As a result, opportunities for contact arise between bat excreta and people (and domestic animals). Though there is no current zoonotic association with these coronaviruses, their abundance among a commonly encountered bat species, with possibly frequent exposure opportunities warrant investigation. Significant characterization of these viral groups is required to better understand this diversity and investigate the zoonotic potential of these alphacoronaviruses.

Betacoronaviruses—Merbecoviruses and Vespertilionid Bats

MERS-CoV emerged on the Arabian Peninsula in 2012 and is now considered endemic to the region due to the presence of the primary reservoir, the dromedary camel [7,92,93]. According to reports from Africa, Europe, Asia, and even South America, viruses sharing similarities to MERS-CoV (*Merbecovirus*) are associated with more than one bat host genus or family [32,43,44,59,83,94,95]. The MERS-related coronaviruses genomes currently sharing the highest similarity to human and camel MERS-CoV were detected in Africa from *Neoromicia capensis* (South Africa) and *Pipistrellus hesperidus* (Uganda) [32,58,59]. Both *Neoromicia* and *Pipistrellus* are small insectivorous bats belonging to the Vespertilionidae family, with several species reassignments occurring in 2020 [68]. Due to taxonomic rearrangements, the genera *Laephotis, Afronycteris,* and *Pseudoromicia*, necessitate inclusion into future MERS-related coronavirus surveillance due to possible intra-host sharing of coronaviruses. Sampling efforts into the previously recognized *Neoromicia* species include approximately 238 individuals and only 100 individuals among *Pipistrellus* species (Table S4), warranting intensified surveillance. According to published reports, very few individuals have been found to harbor MERS-related viruses from these bats sampled.

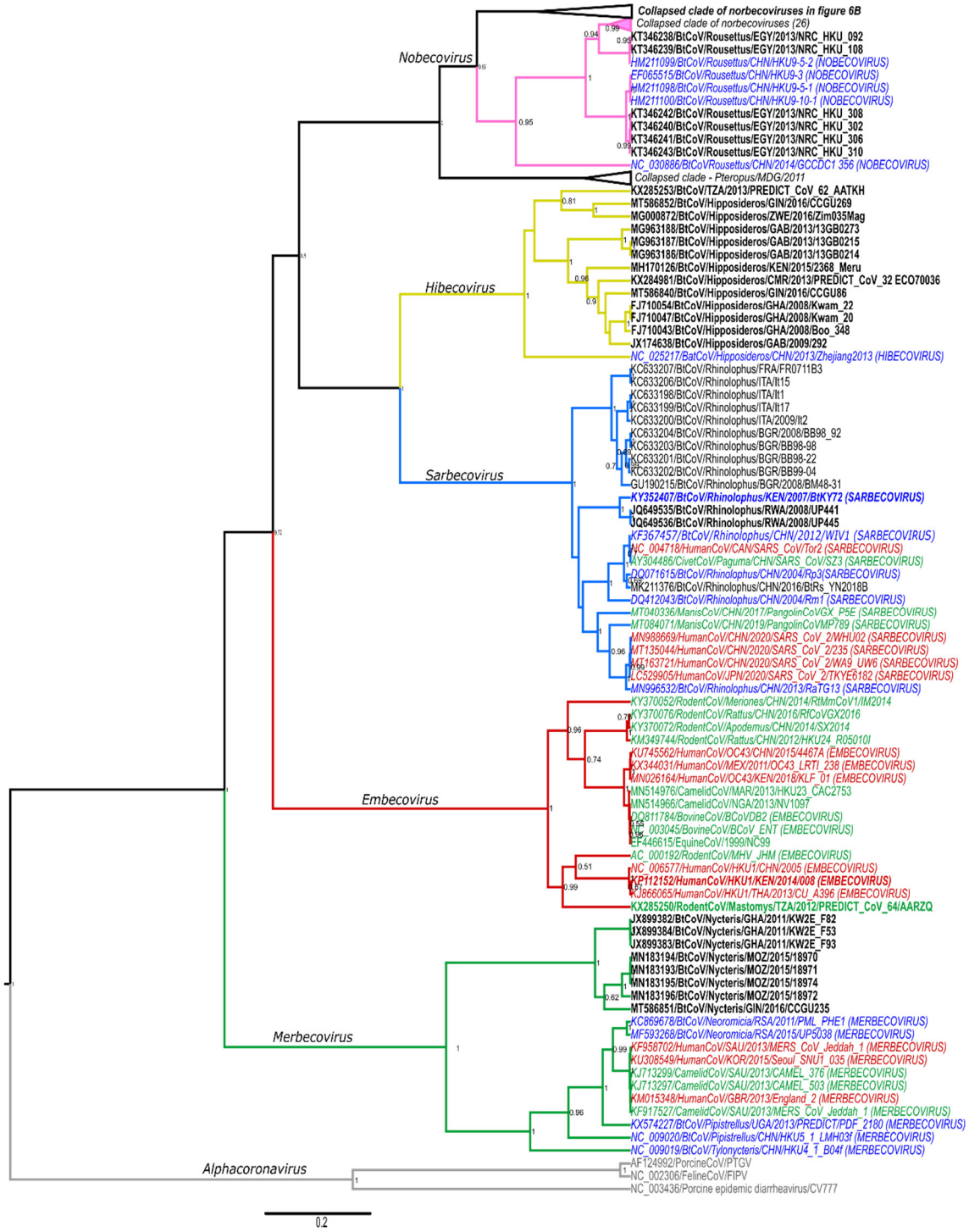
The three available viral full genomes recovered from *Neoromicia (Laephotis)* and *Pipistrellus* were used to classify the viruses as belonging to the same viral species as human and camel MERS-CoV. Within the bat-borne MERS-related viral genomes, the spike genes shared the lowest similarity to human and camel MERS-CoV spike genes (approximately 63–64% nucleotide identity) [32,58,59]. The latter viruses utilize the DPP4 (Dipeptidely peptidase 4) as an entry receptor. Using homology models based on the crystalized structure of the spike protein of the *Pipistrellus* MERS-related virus, Anthony et al. [59] determined that the bat virus spike was unlikely to utilize DPP4 due to insufficient similarities among the required residues to facilitate binding of the spike to the receptor. This was practically demonstrated when recombinant MERS-CoV particles containing the spike from the *Pipistrellus* MERS-related virus were unable to enter Vero cells (unlike wild-type

MERS-CoV) [59]. Moreover, recombination analysis also identified potential breakpoints within the spike gene for *Neoromicia (Laephotis)* MERS-related virus PML/PHE1 and *Pipistrellus* MERS-related virus PREDICT/PDF-2180 [58,59]. The data thus suggests that the identified bat-borne MERS-related viruses have not served as direct progenitors of MERS-CoV detectable in camels and humans, though whether recombination occurred in a bat host or an intermediate host is uncertain.

Depending on the species, both *N. capensis (reassigned as Laephotis capensis)* and *P. hesperidus* have widespread distributions in various parts of Africa [67,68]. *N. capensis* (*L. capensis*) is an abundant and adaptable species distributed from sub-Saharan Africa to South Africa. They typically roost under bark or rock crevices that limit roost sizes to a few individuals [96]. However, these bats have adapted to occupy increasingly available urban roost sites such as cracks in walls and the roofs of houses, which allow populations over 50 individuals to congregate [96,97]. As a result, *N. capensis (L. capensis)* is a common species in urban areas that beneficially aid in decreasing insect populations attracted by city lights. Conversely, *P. hesperidus* is not very abundant and sparsely populated within its distribution from sub-Saharan Africa (Ethiopia down) to South Africa [98].

Betacoronaviruses—Sarbecoviruses with African Rhinolophids

Bat coronavirus sequences sharing similarity to human sarbecoviruses (SARS-CoV and SARS-CoV 2) have been identified throughout the geographic distribution of rhinolophid bats in Asia, Europe, and Africa. The highest genetic similarities between human and bat sarbecoviruses (Rp3, HKU3, WIV1, WIV16, ZXC21, ZC45, RaTG13, RmYN02) originate in Asia [5,76,77,82,99]. Bat species from the *Rhinolophus* genus are considered the main hosts for the genetic diversity of bat sarbecoviruses [16,66,88]. Some species occurring in Europe have also been reported from Northern Africa, such as *Rh. ferrumequinum* and *Rh. euryale*; and are known hosts of sarbecoviruses [66], but very few sequences with similarity to members of the *Sarbecovirus* subgenus have been identified in Africa (Figure 6A). Reports include partial RdRp sequences from two species (*Rh. hildebrandtii* and *Rh. clivosus*) from Kenya, Rwanda, and Uganda (non-universal surveillance region) with similarity to SARS-CoV [19,35,36,100]. Further sequencing of the complete genome of BtCoVKY72 detected from a *Rhinolophus* sp. from Kenya identified the virus as a member of the *Severe acute respiratory syndrome-related coronavirus* species within the *Sarbecovirus* subgenus [100].


Collapsed clade of norbecoviruses in figure 6B
Collapsed clade of norbecoviruses (26)
KT346238/BtCoV/Rousettus/EGY/2013/NRC_HKU_092
KT346239/BtCoV/Rousettus/EGY/2013/NRC_HKU_108
HM211099/BtCoV/Rousettus/CHN/HKU9-5-2 (NOBECOVIRUS)
EF065515/BtCoV/Rousettus/CHN/HKU9-3 (NOBECOVIRUS)
HM211098/BtCoV/Rousettus/CHN/HKU9-5-1 (NOBECOVIRUS)
HM211100/BtCoV/Rousettus/CHN/HKU9-10-1 (NOBECOVIRUS)
KT346242/BtCoV/Rousettus/EGY/2013/NRC_HKU_308
KT346240/BtCoV/Rousettus/EGY/2013/NRC_HKU_302
KT346241/BtCoV/Rousettus/EGY/2013/NRC_HKU_306
KT346243/BtCoV/Rousettus/EGY/2013/NRC_HKU_310
NC_030886/BtCoVRousettus/CHN/2014/GCCDC1 356 (NOBECOVIRUS)
Collapsed clade - Pteropus/MDG/2011
KX285253/BtCoV/TZA/2013/PREDICT_CoV_62_AATKH
MT586852/BtCoV/Hipposideros/GIN/2016/CCGU269
MG000872/BtCoV/Hipposideros/ZWE/2016/Zim035Mag
MG963188/BtCoV/Hipposideros/GAB/2013/13GB0273
MG963187/BtCoV/Hipposideros/GAB/2013/13GB0215
MG963186/BtCoV/Hipposideros/GAB/2013/13GB0214
MH170126/BtCoV/Hipposideros/KEN/2015/2368_Meru
KX284981/BtCoV/Hipposideros/CMR/2013/PREDICT_CoV_32 ECO70036
MT586840/BtCoV/Hipposideros/GIN/2016/CCGU86
FJ710054/BtCoV/Hipposideros/GHA/2008/Kwam_22
FJ710047/BtCoV/Hipposideros/GHA/2008/Kwam_20
FJ710043/BtCoV/Hipposideros/GHA/2008/Boo_348
JX174638/BtCoV/Hipposideros/GAB/2009/292
NC_025217/BatCoV/Hipposideros/CHN/2013/Zhejiang2013 (HIBECOVIRUS)
KC633207/BtCoV/Rhinolophus/FRA/FR0711B3
KC633206/BtCoV/Rhinolophus/ITA/It15
KC633198/BtCoV/Rhinolophus/ITA/It1
KC633199/BtCoV/Rhinolophus/ITA/It17
KC633200/BtCoV/Rhinolophus/ITA/2009/It2
KC633204/BtCoV/Rhinolophus/BGR/2008/BB98_92
KC633203/BtCoV/Rhinolophus/BGR/BB98-98
KC633201/BtCoV/Rhinolophus/BGR/BB98-22
KC633202/BtCoV/Rhinolophus/BGR/BB99-04
GU190215/BtCoV/Rhinolophus/BGR/2008/BM48-31
KY352407/BtCoV/Rhinolophus/KEN/2007/BtKY72 (SARBECOVIRUS)
JQ649535/BtCoV/Rhinolophus/RWA/2008/UP441
JQ649536/BtCoV/Rhinolophus/RWA/2008/UP445
KF367457/BtCoV/Rhinolophus/CHN/2012/WIV1 (SARBECOVIRUS)
NC_004718/HumanCoV/CAN/SARS_CoV/Tor2 (SARBECOVIRUS)
MY304486/CivetCoV/Paguma/CHN/SARS_CoV/SZ3 (SARBECOVIRUS)
DQ071615/BtCoV/Rhinolophus/CHN/2004/Rp3(SARBECOVIRUS)
MK211376/BtCoV/Rhinolophus/CHN/2016/BtRs_YN2018B
DQ412043/BtCoV/Rhinolophus/CHN/2004/Rm1 (SARBECOVIRUS)
MT040336/ManisCoV/CHN/2017/PangolinCoVGX_P5E (SARBECOVIRUS)
MT084071/ManisCoV/CHN/2019/PangolinCoVMP789 (SARBECOVIRUS)
MN988669/HumanCoV/CHN/2020/SARS_CoV_2/WHU02 (SARBECOVIRUS)
MT135044/HumanCoV/CHN/2020/SARS_CoV_2/235 (SARBECOVIRUS)
MT163721/HumanCoV/CHN/2020/SARS_CoV_2/WA9_UW6 (SARBECOVIRUS)
LC529905/HumanCoV/JPN/2020/SARS_CoV_2/TKYE6182 (SARBECOVIRUS)
MN996532/BtCoV/Rhinolophus/CHN/2013/RaTG13 (SARBECOVIRUS)
KY370052/RodentCoV/Meriones/CHN/2014/RtMmCoV1/IM2014
KY370076/RodentCoV/Rattus/CHN/2016/RfCoVGX2016
KY370072/RodentCoV/Apodemus/CHN/2014/SX2014
KM349744/RodentCoV/Rattus/CHN/2012/HKU24_R05010I
KU745562/HumanCoV/OC43/CHN/2015/4467A (EMBECOVIRUS)
KX344031/HumanCoV/MEX/2011/OC43_LRTI_238 (EMBECOVIRUS)
MN026164/HumanCoV/OC43/KEN/2018/KLF_01 (EMBECOVIRUS)
MN514976/CamelidCoV/MAR/2013/HKU23_CAC2753
MN514966/CamelidCoV/NGA/2013/NV1097
DQ811784/BovineCoV/BCoVDB2 (EMBECOVIRUS)
NC_003045/BovineCoV/BCoV_ENT (EMBECOVIRUS)
EF446615/EquineCoV/1999/NC99
AC_000192/RodentCoV/MHV_JHM (EMBECOVIRUS)
NC_006577/HumanCoV/HKU1/CHN/2005 (EMBECOVIRUS)
KP112152/HumanCoV/HKU1/KEN/2014/008 (EMBECOVIRUS)
KJ866065/HumanCoV/HKU1/THA/2013/CU_A396 (EMBECOVIRUS)
KX285250/RodentCoV/Mastomys/TZA/2012/PREDICT_CoV_64/AARZQ
JX899382/BtCoV/Nycteris/GHA/2011/KW2E_F82
JX899384/BtCoV/Nycteris/GHA/2011/KW2E_F53
JX899383/BtCoV/Nycteris/GHA/2011/KW2E_F93
MN183194/BtCoV/Nycteris/MOZ/2015/18970
MN183193/BtCoV/Nycteris/MOZ/2015/18971
MN183195/BtCoV/Nycteris/MOZ/2015/18974
MN183196/BtCoV/Nycteris/MOZ/2015/18972
MT586851/BtCoV/Nycteris/GIN/2016/CCGU235
KC869678/BtCoV/Neoromicia/RSA/2011/PML_PHE1 (MERBECOVIRUS)
MF593268/BtCoV/Neoromicia/RSA/2015/UP5038 (MERBECOVIRUS)
KF958702/HumanCoV/SAU/2013/MERS_CoV_Jeddah_1 (MERBECOVIRUS)
KU308549/HumanCoV/KOR/2015/Seoul_SNU1_035 (MERBECOVIRUS)
KJ713299/CamelidCoV/SAU/2013/CAMEL_376 (MERBECOVIRUS)
KJ713297/CamelidCoV/SAU/2013/CAMEL_503 (MERBECOVIRUS)
KM015348/HumanCoV/GBR/2013/England_2 (MERBECOVIRUS)
KF917527/CamelidCoV/SAU/2013/MERS_CoV_Jeddah_1 (MERBECOVIRUS)
KX574227/BtCoV/Pipistrellus/UGA/2013/PREDICT/PDF_2180 (MERBECOVIRUS)
NC_009020/BtCoV/Pipistrellus/CHN/HKU5_1_LMH03f (MERBECOVIRUS)
NC_009019/BtCoV/Tylonycteris/CHN/HKU4_1_B04f (MERBECOVIRUS)
AF124992/PorcineCoV/PTGV
NC_002306/FelineCoV/FIPV
NC_003436/Porcine epidemic diarrheavirus/CV777
Nobecovirus
Hibecovirus
Sarbecovirus
Embecovirus
Merbecovirus
Alphacoronavirus
0.2

(**A**)

(**B**)

Figure 6. (**A**,**B**): Bayesian *Betacoronavirus* phylogeny of a 294-nucleotide sequence region of the RdRp gene. Shorter sequences were omitted. Clades collapsed in A are shown in B (and vice versa), and the collapsed clade of *Eidolon* nobecoviruses may be viewed in Figure S2). Sequences in italics indicate formally recognized species (subgenera are indicated in capital letters at the end of sequence names); sequences in bold originate in Africa; red highlights human viruses; green indicate non-bat animal hosts; blue/italics indicate formally recognized bat species; orange indicate viral detections from hosts not typically associated with a particular group of coronaviruses. All sequence names were edited to conform to the correct convention, with the modification of the unique sequence identifier listed last due to convenience. Only posterior probabilities of greater than 0.5 are indicated. No unpublished sequences were included.

This limited detection of sequences similar to sarbecoviruses may be due to lacking surveillance of individuals within the *Rhinolophus* host genus. There are 38 extant *Rhinolophus* species in Africa, with approximately 728 individuals from 14 species included in published surveillance efforts from 11 countries (Table S4). However, very small sample sizes averaging between 1–62 individuals have been tested per species. To our knowledge, no bat coronaviruses sharing high similarity to the SARS-CoV 2 clade sarbecoviruses have been identified from African bats. In addition to betacoronaviruses, unclassified alphacoronaviruses have also been identified from four *Rhinolophus* species, suggesting large diversities of coronaviruses to be present in these bats [19,29,33].

Rhinolophids are taxonomically challenging to identify with frequent revisions to species due to highly convergent morphology [67]. Certain species are widespread and have distributions spanning into other continents, such as *Rh. clivosus* from Africa and into South West Asia [101]. These bats generally roost in caves, unused mines, and buildings [67] and are threatened by disturbances to roosts such as mining and the use of pesticides and insecticides [102], though provide valuable ecosystem services by decreasing the populations of crop-damaging insects [102].

Sequences with similarity to sarbecoviruses have also been reported from non-rhinolophid genera, including *Chaerephon* spp. in Kenya and hipposiderids in Rwanda, Cameroon, and the Republic of the Congo [26,30,36]. The latter hosts' detections were few and may represent transient spillover between hosts (*Rhinolophus* and *Hipposideros*), possibly co-roosting. In addition, some other studies have reported the detection of viruses with homology to SARS-CoV in hipposiderid bats, though these viruses were part of a more distant sister clade than rhinolophid SARS-related viruses. Moreover, this sister-clade was later formally classified as the *Hibecovirus* subgenus. Due to the thorough surveillance of hipposiderid bats, these viruses have been reported from various countries, including Ghana, Gabon, Nigeria, Kenya, Rwanda, Zimbabwe, Guinea, and Rwanda (Tables S4 and S5).

Betacoronaviruses—Nobecoviruses and Fruit Bats

Members of the *Nobecovirus* subgenus are not currently associated with any known zoonotic diseases, though much like the aforementioned molossid alphacoronaviruses warrant further investigation due to their widespread occurrence in several abundant fruit bat species [79]. Nearly two-thirds of all the unclassified sequences in Figure S2 likely represent members of this subgenus. Described species in this genus include two Asian bat viruses, *Rousettus bat coronavirus HKU9* and *Rousettus bat coronavirus GCCDC1* detected in species such as *Rousettus leschenaultia* [80,103], as well as *Eidolon bat coronavirus C704* in Cameroon [34]. The African detections sharing similarities to members of the *Nobecovirus* subgenus are indicated in Table S5. These detections have been widespread and predominantly reported from fruit bat genera such as *Rousettus, Eidolon*, *Micropteropus, Epomophorus, Pteropus, Epomops, Myonycteris* (formerly *Lissonycteris*), and *Megaloglossus* [19,26–30,34,36,39,47]. Additionally, similar sequences have been reported from several insectivorous bat species, though whether these represent active maintenance of the virus in these hosts or transient spillover is unclear. Recombination events have been detected between species of the *Nobecovirus* subgenus identified in *R. leschenaulti* in Asia and rotaviruses (*Reoviridae;* double-stranded RNA viruses) co-infecting the same species, leading to the acquisition of the P10 orthoreovirus fusogenic gene [86].

E. helvum migrates over large distances throughout much of sub-Saharan Africa (Senegal to Ethiopia and down to southern Africa) and are tree-roosting fruit bats that form aggregates of thousands to millions of individuals. Large urban colonies have been recorded in trees of various cities (e.g., Accra in Ghana) [67]. Excreta from these urban colonies would provide ample opportunities for human contact with contaminated fecal and urine. *E. helvum* is also heavily harvested for bushmeat, with estimates of 128,000 bats being sold per year in markets in Ghana alone [67,104]. *R. aegyptiacus* also has a broad

distribution throughout sub-Saharan and parts of Northern Africa, as well as South East Asia and the Western Palaearctic region [67]. This species is a cave-dwelling fruit bat that forms large colonies in the thousands (e.g., 5000 to 50,000), and may co-roosts with multiple insectivorous bat species. Opportunities for contact and possible viral sharing may thus arise between different bat genera, though possible exposure events to humans are more infrequent and generally arise due to human activities. These bats are often threatened by farmers who view fruit-eating bats as destructive to their crops as well as due to mining and other cave disturbances [67,105].

3.1.6. Investigating Factors Affecting the Maintenance of Bat Coronaviruses

Understanding how bat coronaviruses are maintained in their host populations allows determination of infection duration and times that may be at 'higher risk' for coronavirus spillover opportunities. 'High risk' periods coincide with increased excretion of viruses from bats in a colony and may be associated with reproductive or seasonal factors affecting the viral infection dynamics of the colony. For example, an increase of mating activity and accompanying hormonal changes may affect the susceptibility of hosts to infection, or the increase in immunologically naive juveniles at the start of a birthing pulse creates a large population of bats susceptible to infection [106–108]. Understanding these dynamics allows the formulation of management plans to mitigate risks and facilitate engagement with communities at risk of frequent contact with particular bat populations. Behavioral changes may assist in reducing the associated risks of exposure and possible spillover interactions [109].

Limited African studies (only 5) expanded data analyses to include correlations between bat biology, ecology, and viral status of hosts [30,36,38,40,44]. Those investigating increased infection among age classes agree that subadults are more likely to host coronaviruses than adults [30,36,44], consistent with reports from other continents [109]. A higher frequency of infection was also identified among lactating females [44], though also males [30]. Most disagreements center around seasonality, with either no correlation identified [36] or a higher chance of detecting coronaviruses in the dry seasons [30]. Longitudinal surveillance projects would be able to assist with such interpretations in the future.

Bats occupy a wide range of niches, including diverse roost preference (e.g., cave-dwelling or tree-roosting), eating habits (frugivores, nectivores, insectivores, etc.), population sizes (less than 10 to thousands), and level of social interaction between the same and different species (gregarious or non-gregarious). It may also be possible that factors affecting the maintenance of coronavirus infection among bat species may not be universal to all bat species. Thus, combining coronavirus data from different species may result in biased conclusions. For example, it has been suggested that bat coronaviruses may amplify within maternity colonies [108], though the reproductive seasons of diverse bat species do not all overlap, and certain species are capable of reproducing more than once a year, depending on the geographic regions. For example, *Rousettus aegyptiacus* displays two birthing pulses among populations along the North of Africa [110], while populations in Southern Africa have only one [111]. Thus, if coronavirus maintenance is linked to its host species' reproductive biology, viral shedding may be predictable for certain species in particular climate zones.

A recent study predicted high-risk periods for different host species utilizing available surveillance data from three countries (Rwanda, Uganda, and Tanzania) and Bayesian modeling [30,60]. Though several assumptions were made regarding the duration of lactation and weaning, they determined that juveniles recently weaned were 3.34 times more likely to shedding coronavirus RNA than juveniles that were not recently weaned. Even adults were nearly four times more likely to be shedding coronaviruses when juveniles were being weaned [60], possibly due to increased coronavirus excretion levels within the colony. As described in Wacharapluesadee et al., [109], increased coronavirus shedding

among juvenile bats may be due to vertical transmission from mother to pup, which coincides with studies describing viral shedding from lactating females with increased frequency compared to non-lactating females [112]. The higher frequencies observed in recently weaned juveniles may be due to the loss of maternally received antibody protection following weaning [60,108]. These conclusions require confirmation with longitudinal surveillance among investigated bat species as well as serological studies determining changing antibody levels between lactating mothers, weaning and non-weaning juveniles, as well as other adults in the colony.

3.2. Surveillance in Other Wildlife and Domestic Animals (Livestock)

Coronavirus nucleic acid surveillance among non-bat wildlife, livestock, or other domestic animals in Africa is very limited, both in the frequency of research, sample sizes of animals tested, locations targeted, and are frequently investigated for only specific coronaviruses. Nucleic acid testing in animal populations where the prevalence of infection may be very low would yield limited data, provided that sampling was performed at a time when animals are infected or actively excreting viruses [113]. We only identified four reports in which other animals were tested for coronavirus nucleic acids, including anthroponoses of HCoVOC43 between humans and chimpanzees in Côte d'Ivoire [48], MERS-CoV specific surveillance among 4248 livestock animals from Ghana (cattle, sheep, donkeys, goats, and pigs) [50], general surveillance among 731 wildlife animals (rodents, non-human primates, and ad hoc samples of other wildlife) in Gabon [40], as well as just over 27,000 animals (birds, domestic animals, carnivores, pangolins, swine, rodents, and non-human primates) as part of the PREDICT surveillance initiative (accessed via Healthmap.org) (Table 4). Though this seems like a significant number of individuals tested, the total species diversity among all 16 countries sampled is much larger than the fraction represented by this surveillance. Moreover, not all hosts listed were surveyed in all countries (Table 4), with mostly opportunistic sampling from accessible individuals. However, even though the total positives detected in relation to the total number sampled is <1%, it still shows the presence of coronaviral RNA from among non-human primates (14 chimpanzees), ungulates (1 bush duiker), carnivores (1 African palm civet) and rodent species (13 individuals) from opportunistic surveillance [48,56].

Two of these sequences, publicly available and corresponding to the universal surveillance region (excluding the anthroponoses of HCoVOC43 from the chimpanzees), were included in the phylogenies in Figures 5A and 6A (KX285508 and KX285250). Most of the detected African rodent coronavirus partial sequences are phylogenetically placed in the *Embecovirus* subgenus, with human coronaviruses OC43 and HKU1 and other rodent coronaviruses from Asia [18,30]. Divergent rodent alphacoronavirus virus RNA was also identified (KX285508), as well as highly divergent shrew coronaviruses [30]. The sequence information confirms surveillance data from Asia and Europe, namely that rodents and shrews likely harbour additional undiscovered diversity of coronaviruses. Improved systematic and longitudinal surveillance of wildlife and domestic populations will provide more data on the presence of coronaviruses among these animal groups. The research is too limited to make any conclusions regarding the absence of viral sharing between animal groups. Additionally, serological surveillance would complement nucleic acid surveillance by providing data on hosts not actively infected with coronaviruses.

Not included in Table 4 is the expansive surveillance of dromedary camel populations for MERS-CoV. MERS-CoV is not only endemic to the dromedary camel populations of the Middle East but also populations in Northern Africa (Burkina Faso, Ethiopia, Kenya, Mali, Morocco, Nigeria, Somalia, Sudan, Tunisia) [93,114]. Seroprevalence of adult dromedaries is high (80–100%) and may result in respiratory disease with viral shedding via nasal discharge [93,115]. Despite this widespread occurrence, MERS infections among people from camels have only been reported from the Arabian Peninsula [114,115]. Viruses from African dromedaries form a separate basal lineage to the two clades of MERS-CoV identified from infected people and camels on the Arabian Peninsula [114,116],

though still share antigenic similarities through cross-neutralization [114]. Furthermore, within this African clade, genomes from the West and North African dromedary populations (Nigeria, Burkina Faso, Morocco) display deletions in specific accessory genes [114,117,118]. It has been suggested that these accessory genes are not required for the adaptation of the virus to dromedary camels and may have been necessary for a more historical host [114]. Whether bat-borne MERS-related viruses established in dromedary camel populations can only be addressed with better surveillance of African bat and dromedary populations, especially where bat and camelid distributions overlap [32].

Table 4. Summary of animals (non-bat) tested for coronavirus nucleic acids.

Animals Groups	Birds [1] and Poultry/ Other Fowl	Carivores [2]	Cattle/ Buffalo [3]	Dogs [4]	Goats/ Sheep [4]	Non-Human Primates	Pango-lins [5]	Rodents/ Shrews	Swine [4]	Ungu-lates [7]	Other [6]	Grand Total
Cameroon	-	**67**	-	-	-	3475	79	**4653**	-	144	16	8434
DR Congo	7	6	10	-	16	1574	3	1848	1	15	2	3482
Ethiopia	-	-	-	-	-	454	-	-	-	-	-	454
Gabon	1	11	-	-	-	82	18	1141	-	548	37	1838
Ghana	-	-	1230	-	2194	496	-	532	716	108	-	5276
Guinea	-	-	-	6	321	-	-	904	8	-	-	1239
Ivory Coast	12	-	-	-	-	**59**	-	293	-	-	-	364
Kenya	-	-	-	-	-	334	-	369	-	514	-	1217
Liberia	-	-	-	-	-	-	-	205	-	-	-	205
Republic of Congo	-	2	-	-	-	352	-	**461**	-	14	-	829
Rwanda	-	-	-	-	-	762	-	**708**	-	-	-	1470
Senegal	-	-	-	-	-	253	-	**263**	-	-	-	516
Sierra Leone	-	5	-	318	938	15	-	369	1012	-	-	2657
South Sudan	-	-	-	-	-	-	-	46	-	-	-	46
Tanzania	-	8	53	120	105	444	-	**1513**	95	**39**	1	2378
Uganda	-	-	-	-	13	1238	-	**762**	1	83	-	2097
Grand Total	20	99	1293	444	3587	9538	100	14,067	1833	1465	56	32,502
Coronavirus nucleic acid	-	1	-	-		14	-	13	-	1	-	29

[1] Unspecified; [2] carnivores (genets, mongoose, and civets; domestic cats); [3] domestic and African buffalo; [4] domestic; [5] tree and long-tailed pangolins; [6] ungulates (including camels, duikers, and antelope among others); [7] 'other' (reptiles, snakes, tortoise, hyraxes, and elephants). For species information review [56]. Numbers shaded in bold indicate positive detections from an animal group and country. No recorded surveillance is indicated with a '-'.

4. Coronavirus Serosurveillance

Coronavirus serology is complex and faces several challenges—even among human coronaviruses [113]. Serological targets include the immunogenic nucleoprotein that is abundant during infections and the spike protein that allows for the detection of more specific antibody responses and neutralizing antibodies [119]. Targeting a more conserved protein (such as the nucleoprotein) may yield high seropositivity levels due to potential cross-reactivity of conserved epitopes among related coronaviruses, without being able to discern between different viral species (or genera). Depending on the assay target, cross-reactivity could complicate human coronavirus assays due to conserved motifs between seasonal human coronaviruses, SARS-CoV, and MERS-CoV [113], as well as between SARS-CoV and SARS-CoV 2 [120]. Serosurveillance among animal populations is similarly hampered with cross-reactivity as they may be exposed to unidentified coronaviruses. Due to the challenges of cultivating certain animal coronaviruses, virus neutralization tests to exclude cross-reactions are not readily feasible. A lack of specific animal coronavirus assays often leads to the use of human coronavirus assays (generally based on the spike protein). However, interpreting the results should be made with caution as cross-reactivity to unknown epitopes and modifications to validated assays may allow for false assumptions [113]. There is a great need to develop suitable assays for serological surveillance of diverse coronaviruses in wildlife and domestic animals. The lack of well-

characterized reference sera to determine cut-off thresholds and limited species-specific biologics also challenges new assay development.

Bat coronavirus serology is demanding for all the aforementioned reasons and is further complicated by the large diversity of bat coronaviruses. Of note is that not all bat coronaviruses utilize the same receptor molecules. Angiotensin-converting enzyme two or ACE2 is the known receptor for SARS-CoV, SARS-CoV 2, and only the most closely related bat sarbecoviruses. The receptor-binding regions and important motifs even differ greatly between SARS-CoV and SARS-CoV 2 (see Andersen et al. [13]). The spike receptors for the larger majority of bat sarbecoviruses lack the required binding sites and are largely incompatible with human ACE2. The spike proteins of BtCoVKY72 only share 68–72% amino acid similarity to the spike proteins of SARS-CoV and SARS-CoV 2 and their most closely related bat viruses (unpublished data). Though, protein similarity alone cannot be used to determine if cross-reaction will occur due to the glycosylation and conformational folding of spike proteins [113].

In comparison to the number of studies investigating bat coronavirus nucleic acid surveillance, minimal serosurveillance studies have been performed on the continent. These include mainly Muller et al. [121], wherein a SARS-CoV ELISA kit with minor modifications was used to tests bat sera from the Democratic Republic of the Congo (DRC) and South Africa, as well as a MERS-CoV pseudo-particle neutralization assay to test *Rousettus* sera in Egypt and Lebanon by Shehata et al. [27]. Though no MERS-antibodies were detected in *Rousettus aegyptiacus*, antibodies reactive to SARS-CoV antigens were identified in 6.7% of bats tested (7 of 26 species) from the DRC and South Africa. These species include pteropid bats (*Rousettus*, *Myonycteris,* and *Hypsignathus*) as well as other insectivorous bat genera like *Mops, Miniopterus,* and *Rhinolophus*; many of these genera have since been identified to host either alpha- or betacoronaviruses. The results were confirmed with western blots, though no neutralizing antibodies were identified [121], cross-reactivity between potentially related bat coronaviruses. Increased bat coronavirus serological surveillance would provide better overall estimates of population exposure levels [119,122] and reduce false-negative assumptions from non-actively shedding hosts.

Wildlife, livestock or domestic animal serological surveillance in Africa is more frequent than serological surveillance among bats. A broad search of the literature found mainly studies focused on MERS-CoV serology and dromedary camel populations among various countries (reviewed in Dighe et al., [93]). Among domestic animals, several studies investigated livestock in Ghana [49–51]. Bovine coronavirus was determined to possibly be widespread among ruminants such as cattle and capable of spilling over into sheep and goats [51]. Cattle, sheep, goats, donkeys, and swineherds have been found lacking any serological response toward merbecoviruses like MERS-CoV or the similar *Nycteris* bat betacoronaviruses [50], or indeed HCoVNL63 and related bat viruses [49]. The authors highlight the need for such surveillance to be conducted in countries such as Kenya, where similar viruses to HCoV229E or HCoVNL63 were identified in bats.

Limited serosurveillance has been performed in wildlife. Though no feline coronavirus serological responses were identified among 13 lions from Botswana sampled between 2012 and 2014 [123], feline coronaviruses (particularly the highly pathogenic feline infectious peritonitis virus) have historically been shown to be actively circulating among captive cheetahs in the USA and free-living cheetah populations from Eastern and Southern Africa [124,125]. This lack of thorough surveillance in animals that may act as intermediate hosts and detecting spillover infections creates a gap in data not only for Africa but globally. Moreover, to our knowledge, no studies have investigated human populations in Africa for serological responses to bat coronavirus spillover [126].

5. Factors Associated with the Potential Emergence of Coronaviruses

Opportunities for potential pathogen exposure between humans and animals, including wildlife, are increasing. In Africa, the main factors include deforestation, agricultural intensification, and the collection, hunting, and butchering of bushmeat [22,127,128].

Interactions that are more specific to bats include ecotourism, mining, guano collection for fertilizer [128], or bat species that roost in man-made structures, such as houses, warehouses, schools, etc. Coronavirus nucleic acids are still detectable in guano fertilizer several days after collection, even if kept at room temperature (though viral isolation was not attempted) [129]. Although some factors may create opportunities for spillover, the exact routes of transmission are not yet clear. [130]. Research investigating potential interfaces in Africa is limited.

The bushmeat trade represents one of the most prominent points of contact between humans and bats on various continents [131], though it may practically represent a low risk of transmission for coronaviruses. Bushmeat serves as an important source of protein and household income in many African, Asian, and South American countries [132,133]. Large bats from the *Eidolon* or *Hypsignathus* genera are predominantly hunted, though smaller bats (*Hipposideros, Rhinolophus,* and *Myotis,* among others) are not excluded [133]. For sub-Saharan Africa alone, 52 African bat species (Table S4) are reportedly hunted in countries across their distribution [133]. *Alpha-* and *Betacoronavirus* sequences have been reported from at least 12 and 14 of these bat species, respectively (Table S4). Notably, viral sequences putatively grouping within the *Duvinacovirus*, *Sarbecovirus,* and *Hibecovirus* subgenera have been detected in one of the hunted bat species, namely *Hipposideros ruber* [30,36,37]. A large number of species deemed as bushmeat have, however, not been included in any coronavirus surveillance studies, and thus, their propensity as viral hosts and associated risk to humans remains to be determined.

Live animal markets have been labelled as an ideal interface for human exposure and disease emergence and have been scrutinized due to the ongoing global COVID19 pandemic [134]. As in specific regions of Asia where such markets are commonplace, live or cooked bats are sold in selected African countries [104,135,136]. These bats may also be used in traditional medicine. Additionally, festivals in Africa focused on bats, such as those in Buoyem (Ghana) and Idanre (Nigeria), may provide opportunities for viral spillover [137,138]. The emergence of SARS-CoV and the SARS-CoV 2 pandemic has led to the banning of wet markets from selling live animals in China [132]. Both bans were eventually lifted and remains a point of debate [139,140].

Human-bat interactions are motivated by social, economic and cultural drivers, which form an integral part of infectious disease research. Different cultures have multifaceted perspectives concerning bats, which may be shaped by the local beliefs, use in traditional medicine, knowledge of bat biology, disease risk, or change during periods of food shortages [141–144]. Though limited information is available in Africa, several recent studies have considered the risk perceptions of human populations to bats and their associations with zoonotic diseases [142–147]. Overall, the results suggest that communities have limited knowledge of bats and do not generally perceive bats as a threat [142,143,145]. These perceptions may likely have changed following the COVID-19 pandemic.

With the known diversity of coronaviruses in bat species from Africa and the association of a number of these bats in human activities, exposure to these viruses is inevitable. There have to date not been any reports of novel coronavirus-associated diseases speculated to be of bat origin on the African continent, contrary to the link between bats and sarbecoviruses from Asia [5,16]. There is a clear overlap between practices in Asian and African countries with regards to animal trade. An intricate relationship between the factors associated with disease spillover from bats to humans is likely involved. Identifying the synergistic effects of these factors is simply the first step in understanding their roles in disease emergence.

6. The Future of Coronavirus Surveillance

The majority of African coronavirus surveillance has been focused on nucleic acid detection, estimating the genetic diversity of coronaviruses from bats and largely excluding other wildlife. Very limited epidemiological information is available to understand

and support current assumptions regarding coronavirus maintenance among bat populations (effects of reproductive biology and ecologic impacts). Surveillance among other wildlife species and domesticated animals is so limited that no further conclusions can be reached on their risks. It is clear that bats host the genetic diversity of coronaviruses [17,18,78], but surveillance should be expanded to other species that share the same ecosystem as potential reservoir species and spillover hosts.

Longitudinal surveillance is essential towards understanding how bat coronaviruses are maintained within a species, as well as the occurrence and duration of shedding [109]. Identification of high-risk shedding periods can direct additional surveillance in other species and allow the formulation of preventative mitigation measures by decreasing possible interactions between human, livestock, and bat population. Determining possible increased shedding times can also allow better planning for surveillance studies to avoid sample collection of cross-sectional studies during the lowest shedding periods. This can be readily accomplished with non-destructive sample collection (colony-level fecal, swabs, or fecal collection). In addition, more basic research is also required for neglected species (Table S4), different animal groups (particularly rodents and livestock) [18] to expand surveillance regions and increase sample sizes.

The reliability of nucleic acid surveillance approaches would be much improved with standardized usage of updated, validated assays such as the recently published assay by Holbrook et al. [148], which updated the widely used Watanabe assay. There has also been an increasing shift away from only publishing short sequences to additional characterization of longer extended sequences or genes. This is both beneficial to the quality of research as well as disadvantageous to having basic surveillance data available. Better characterization of African bat coronaviruses will enable classification of more bat coronaviruses and identifying detectable recombination events. However, this requirement also hampers the frequency of newly published surveillance studies due to escalating costs and sequencing challenges leading to gaps of understanding and unreported diversity among different animal populations. A lack of such standardized approaches also results in technically challenging troubleshooting to be performed in resource-limited laboratories.

Moreover, the cost of fieldwork and sample collection in often remote regions of African countries, as well as the follow-up sample analyses, can be very high, with very little remaining for additional sequencing. Researchers should also be encouraged to publish data on the absence of coronavirus detections to assess species or regions of lower risk. Though not ideal, unpublished nucleic acid surveillance data can also be submitted to NCBI with all relevant collection data. As of August 2020, the user-friendly Database of Bat-associated Viruses (DBatVir) repository contained over 4600 bat coronavirus entries globally [149]. This repository is updated bimonthly, and accessing such a centralized source for bat coronavirus surveillance data (both published and unpublished) will allow for a more comprehensive comparison of detected viruses, assessment of surveillance coverage, and highlight areas where research is required.

We propose that surveillance studies publishing short sequences be bolstered by shifting from detecting viral presence alone to investigating questions concerning the epidemiology and maintenance of coronaviruses in selected populations of different species (Table 5). Bat surveillance in a specific region can be initiated, though it is important that surveillance of other species sharing the same ecological niche be done either concurrently or followed as soon as possible, including potential spillover hosts. Sampling of other animal groups and assessing anthropological and human behavioral risks should be included in the planning and implementation phase. Communities must be at the center of studies to understand societal and cultural issues. Initial surveillance at preselected sites may only provide an overall estimation of animal host species present (bat and non-bat), host movement patterns, and viral excretion, allowing informative planning decisions to be made for proper longitudinal surveillance appropriate sites. Surveillance using short

nucleic acid sequences from an updated assay is thus used to identify diversity and monitor changing excretion fluctuations of viruses in populations over time—either seasonally or based on a predetermined time frame (e.g., monthly). This would allow surveillance of both the presence and diversity of coronaviruses among bats and other sampled wildlife/domestic animals and investigate factors involved in viral maintenance with the collection of ecological data. Additionally, such data can be used for a basic assessment of risk regarding potential opportunities for spillover.

Further research is required to characterize detected coronaviruses, including recovery of complete genomes, incorporation of serological studies among bat populations and spillover hosts, or determination of host ranges and zoonotic potential with pathogenesis studies. Issues of cost or technical challenge may be overcome by collaborating with international institutions. In-country expertise and capacity building are essential to build sustainable surveillance programs and require an interdisciplinary approach.

Table 5. Framework for activity planning when implementing coronavirus surveillance in bat populations, other wildlife species, domesticated animals, and impacted human settlements.

Planning phase	Basic surveillance	Longitudinal surveillance	Extended surveillance

Consideration	Activity
Formulate a strong research question around the aim of the research to be conducted.	Scope of the surveillance—only coronaviruses or broader surveillance. What will the primary focus of the project be? Assessment of risk for settlements near known colonies? Review the literature and determine important species to target.
Assemble an interdisciplinary team	Collaborate with experts in virology, taxonomists, field biologists, veterinarians, ecologists, specific community leaders, social sciences, and policy-makers. A large interdisciplinary team is essential for accurate long-term surveillance.
Identify high-risk species or animal populations based on a predetermined research question	As a starting point, collaborations can assist in identifying accessible locations of interests, such as specific roosts (day or maternity roosts, etc.) for bat host species considered higher risk (from literature). The roosts can be assessed for population presence over time to enable longitudinal surveillance planning. The region must be assessed for nearby human settlements and the occurrence of animals (farmed, free-roaming, or other wildlife).
Perform initial surveillance targeting either large roosts or multiple smaller roosts	Assess viral presence and diversity with once-off or seasonal surveillance (statistically significant). Population-level sampling of excreted samples such as fecal collection (beneath roosting bats) is simple and non-invasive. Proper species identification should be conducted with both barcodes and morphological identification.
Nucleic acid testing with a suitable assay	Review the literature and use a recently updated assay to ensure detection of all available diversity. Test the assay sensitivity for comparisons. Based on the scope of the project and resource

	conservation—consider a specific or randomly primed approach.
Plan longitudinal surveillance (duration, types of samples collected, measurement, and ecological data collection). Plan to survey animal species in the region preferably concurrently or sequentially following bat surveillance.	Based on initial findings, plan for longitudinal surveillance according to specified intervals (based on bat presence at roosts or species movements): seasonal or periodic (monthly). Sampling must occur across different reproductive stages. Surveillance can be done at the population-level (overall) and individual-level (to determine demographics of infection prevalence).
Serological surveillance	Review options for serological assays (commercial or developed assays). Collaboration with experts may be critical. Serological testing (bats, non-bat animals, and humans) is important to understand coronavirus antibody responses, duration of protection, and exposure—optimize suitable assays.
Viral characterization	Recover complete genomes of selected viruses for classification and functional studies. Assessing possible zoonotic potential with pathogenesis studies and protein modeling. Collaborate with specialists that can assist and help develop local capacity.
Investigate human-animal interactions	Perform observational and behavioural studies to assess human-wildlife-livestock interactions.

7. Conclusions

Surveillance of coronaviruses in wildlife and potential spillover hosts is complicated with logistical, technical, and practical challenges. Proper biosurveillance requires detailed planning ahead of time with well-formulated research questions [150] and essential resources such as highly skilled staff, funding, and operating within ethical and regulatory requirements. Availability of research tools such as appropriate diagnostic assays, standardized protocols, and correct species (specifically related to wildlife) identification is paramount. Studies based on nucleic acid detection have been more commonly used, given the lack of suitable or validated serological assays. The development of such assays is further complicated with issues concerning coronavirus culture in vitro and stringent biosafety Level III conditions. The latter limits research to only a few groups when additional characterization, pathogenicity investigations, and determination of the zoonotic potential of newly discovered bat, rodent, and wildlife coronaviruses is needed. The development of recombinant proteins for serological assays and reverse-genetics systems for coronavirus rescue, though technically complex, are some of the only available options at present.

Much of the coronavirus biosurveillance studies reported, particularly in wildlife, has been reactive to outbreaks/newly emerging viruses and very opportunistic. The current coronavirus research identified many coronavirus host species among bats and rodents and provided novel insights into the possible evolutionary origins of some human coronaviruses [25,35,54]. Moreover, specific groups of coronaviruses have been identified for further research due to lack of characterization and high coronavirus diversity among abundant host populations with opportunities for human contact. The studies mainly provided "snap-shots" of diverse coronaviruses among different species, time points, and geographical locations. Such approaches do not allow long-term monitoring of these viruses in host species toward understanding the factors involved in viral maintenance, nor

does it provide cues for interpreting increased risk of spillover. Systematic longitudinal investigations of both natural and potential spillover hosts are needed. Additional layers of investigation must include studying human behavior and anthropological influences and the roles of virus/host interactions, pathogenicity, and the natural ecology of the virus. Investigations of coronavirus diversity among other wildlife (particularly rodents) and livestock are at infancy, with much still unknown. As a result, the future of coronavirus research in African has many topics to cover and will expand continent-wide, requiring an interdisciplinary collaborative approach and significant resource investment.

Supplementary Materials: The following are available online at www.mdpi.com/1999-4915/13/5/936/s1, Figure S1: complete Bayesian BEAST phylogeny of African alphacoronaviruses, Figure S2: complete Bayesian BEAST phylogeny of African betacoronaviruses, Table S1: overview of bat surveillance nucleic acid detection studies, Table S2: molecular methodologies employed by the bat surveillance nucleic acid detection studies, Table S3: summary of bat host species tested for coronavirus RNA and positive species reported, Table S4: coronavirus surveillance performed per bat host species, Table S5: bat species from which coronaviruses have been reported (positive species).

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Appendix A

Details of Phylogenetics

Short sequence lengths may hamper the resolution of a phylogeny, resulting in poor support for certain clades. The phylogenies in Figures S1 and S2 (and Figures 5 and 6) were constructed to include as many African bat coronavirus sequences as possible while still allowing for sequence lengths that would yield well-supported clades. Therefore, sequences that would have resulted in alignments of less than 200 nucleotides were omitted with final lengths between 260 and 294 nucleotides, respectively. For simplicity, all sequence names were converted to the standardized convention with the modification of listing unique sequence identifiers last. Sequences were obtained from Genbank (NCBI) by searching the accession numbers listed in the publications identified as described in Table 1, or manually searching for the publication title. The accession numbers of all sequences included are provided in the phylogenetic trees. Sequence alignments and editing were performed with ClustalW in Bioedit [151]. Maximum clade credibility trees were constructed using suggested models selected from jModelTest2.org [152]. Phylogenetic analyses were performed with Bayesian phylogenetics using BEAST v. 1.10 using the general time-reversible model (GTR) plus invariant sites and gamma distribution substitution

model [153]. The CIPRES Science Gateway was used to run computationally expensive analyses such as alignments, jmodeltest, and BEAST [154]. The Bayesian MCMC chains of the alphacoronavirus phylogeny was set to 20,000,000 states, sampling every 2000 steps, and the betacoronavirus phylogeny was set at 25,000,000 states (sampling every 2500 steps). Final trees were calculated from the 9000 generated trees after discarding the first 10% as burn-in. Trees were viewed and edited in Figtree v1.4.2.

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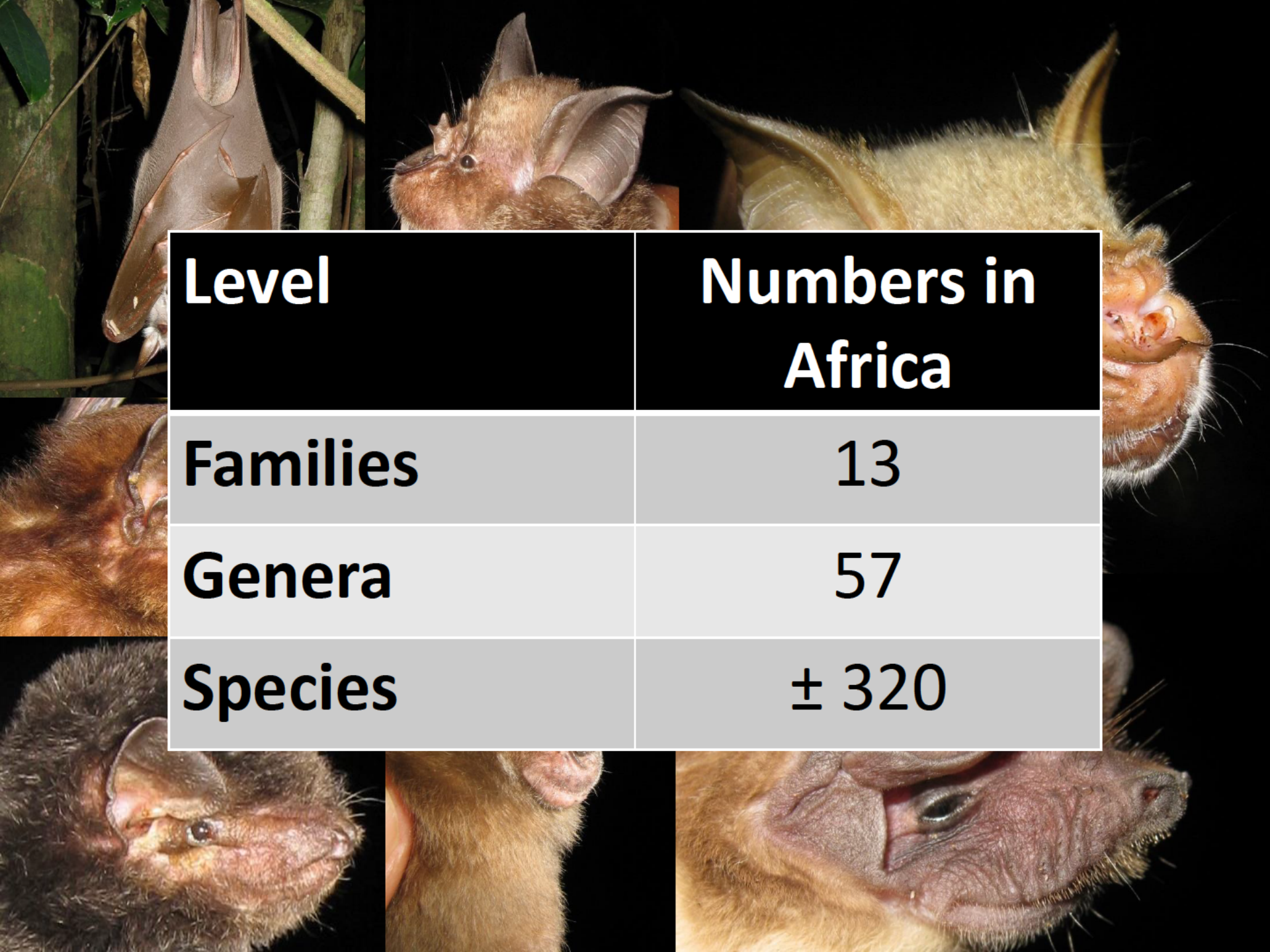
Bat identification workshop

UNIVERSITY OF ESWATINI

(b) (6) [redacted]



Level	Numbers in Africa
Families	13
Genera	57
Species	± 320

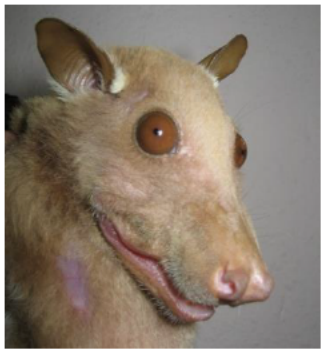
Can anyone identify this bat? Not easy!

How to proceed with identification?

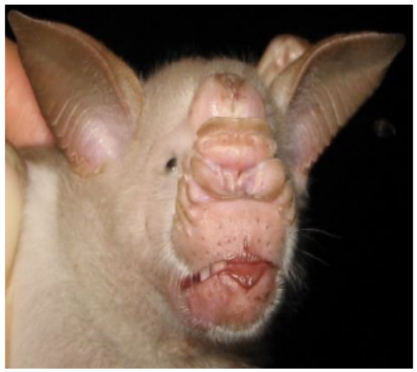
- So, what can we do?
- A useful approach is to identify the bat to a higher taxonomic level
- Class: Mammalia, Order: Chiroptera
- Family, Genus, Species

Southern African families

Pteropodidae



Hipposideridae



Rhinolophidae

Emballonuridae

Nycteridae

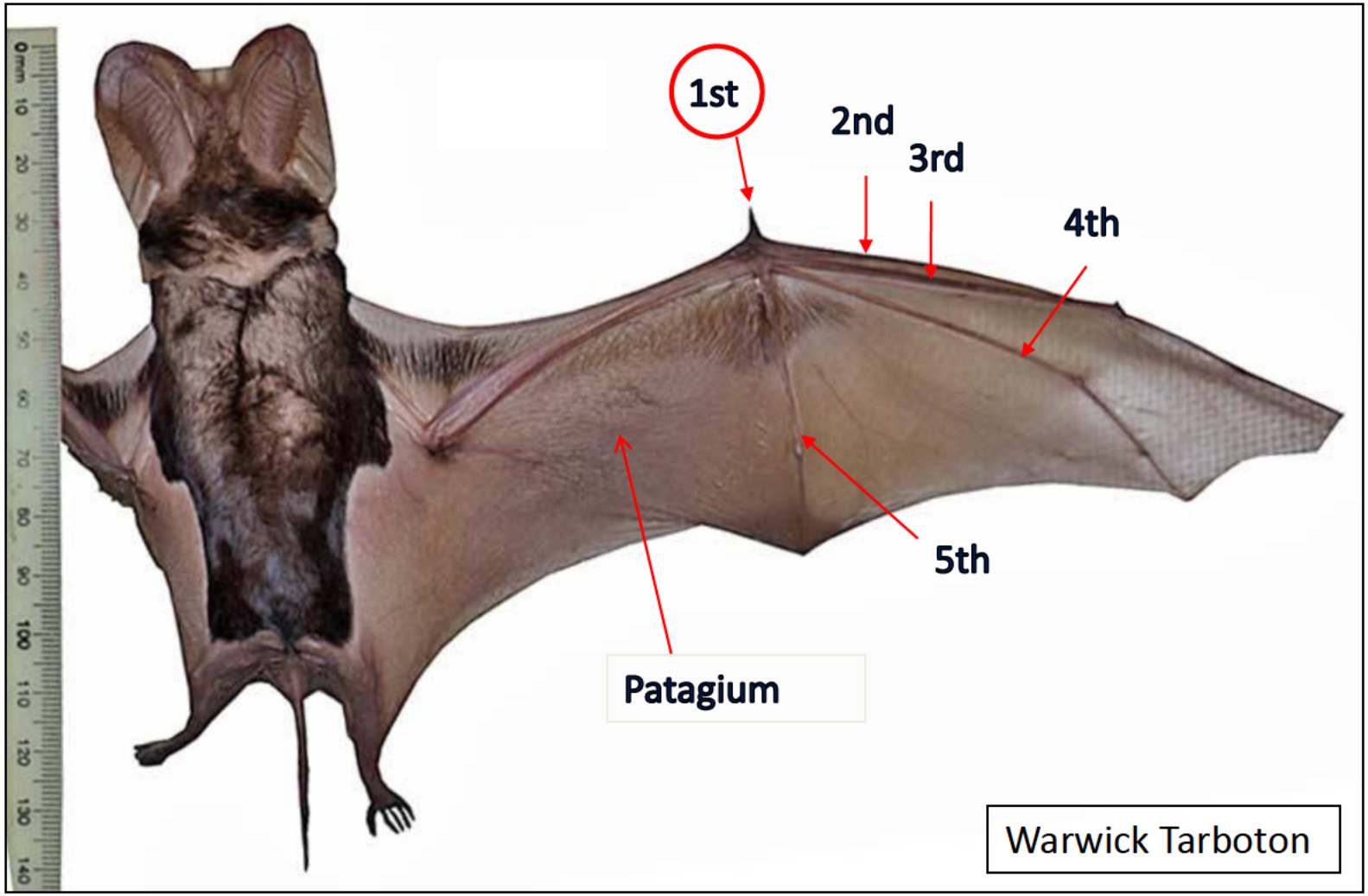
Molossidae

Vespertilionidae

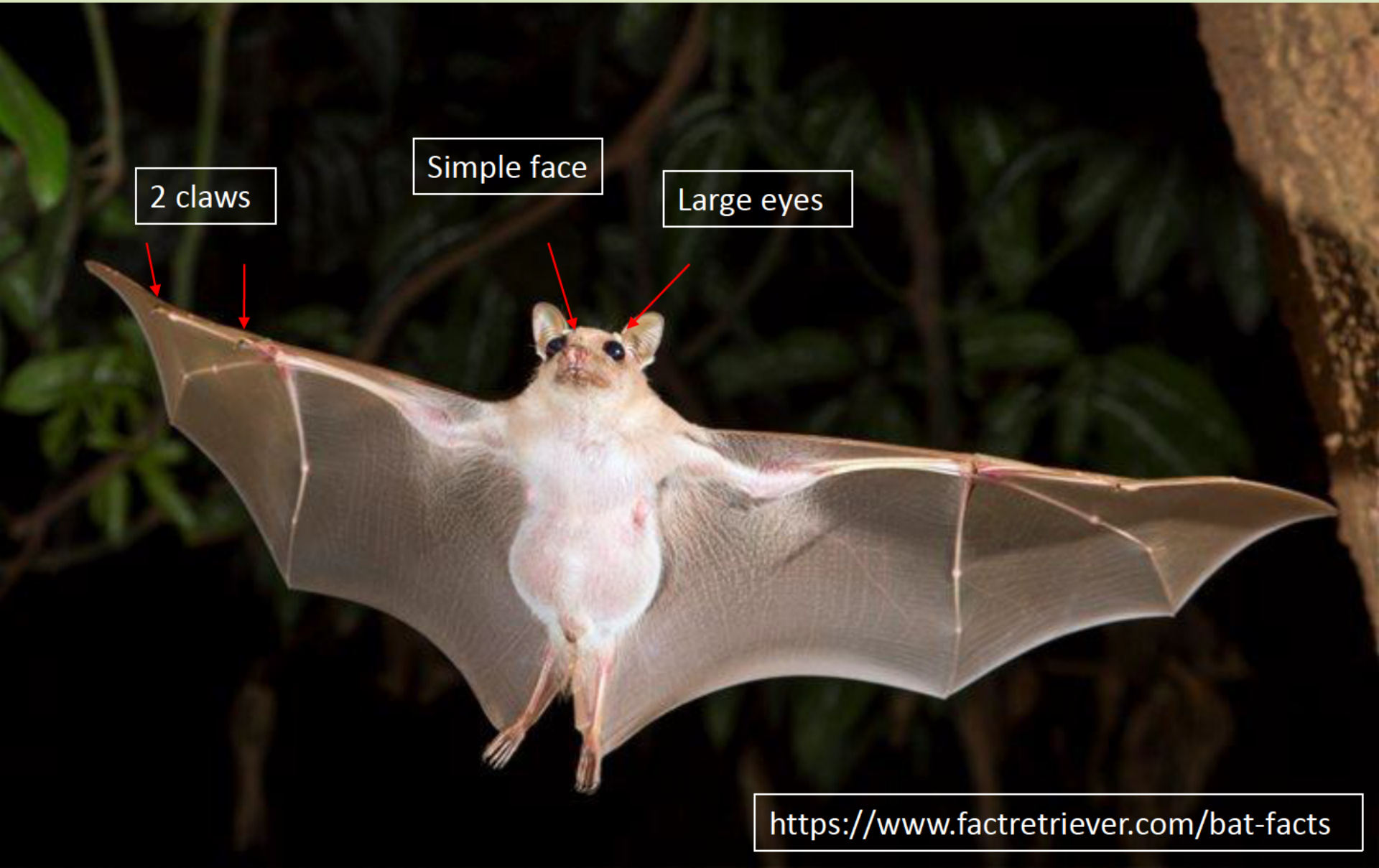
Miniopteridae

And two rare families:
Rhinonycteridae and Cistugidae

Note the claw on 1st digit



Pteropodidae (fruit bats)

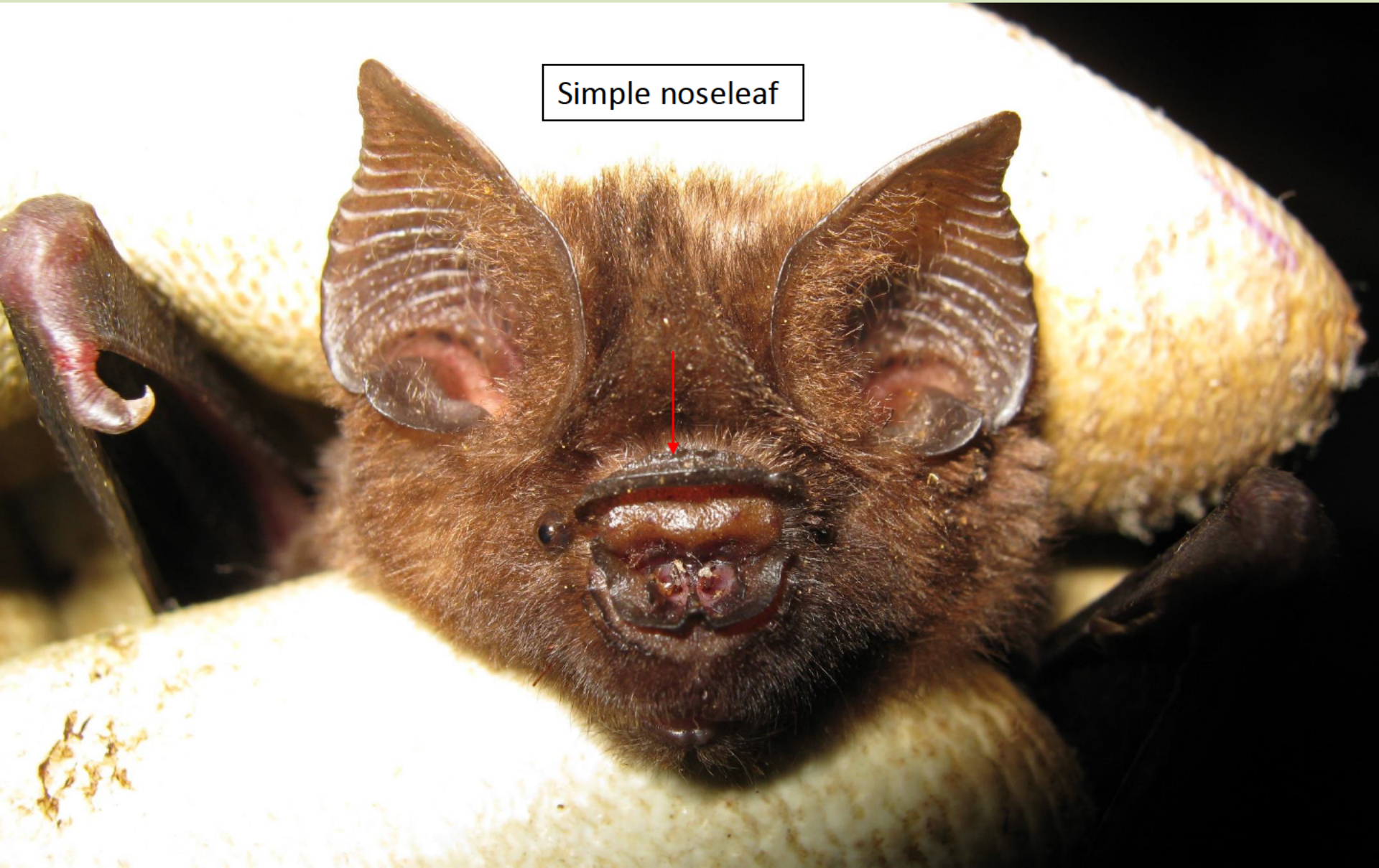


Typical genera/species

Epomophorus wahlbergi

Rousettus aegyptiaca

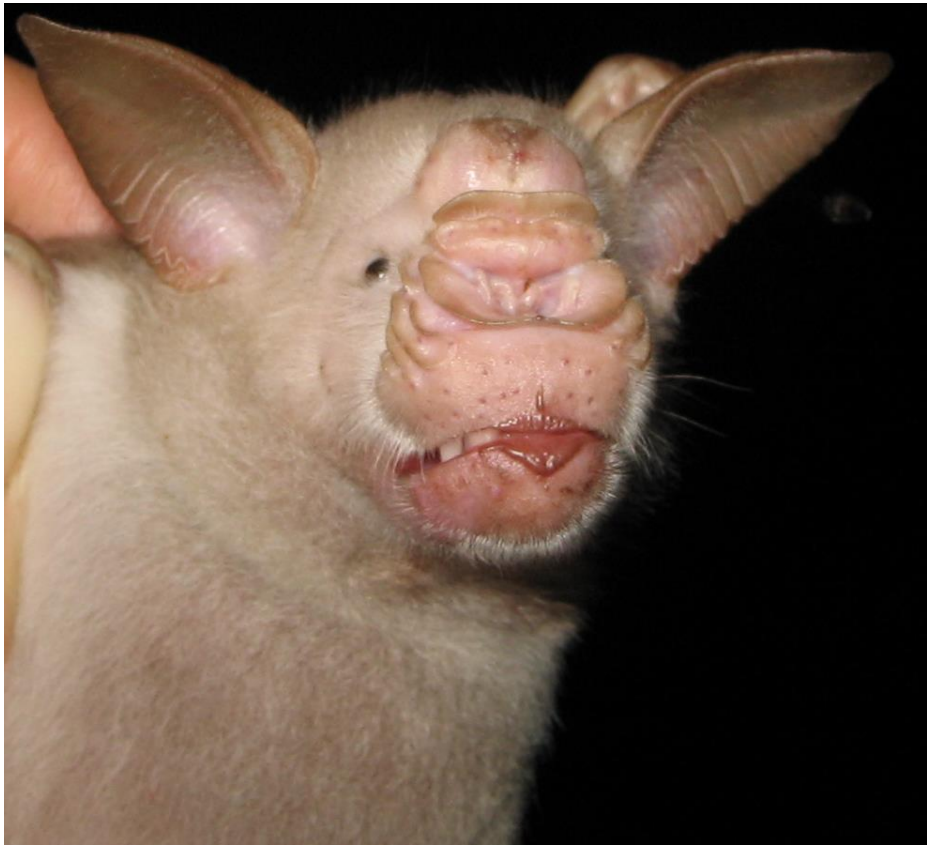
Hipposideridae (leafnose bats)



Typical genera/species

Hipposideros cafer

Macronycteris vittatus



Rhinolophidae (horseshoe bats)

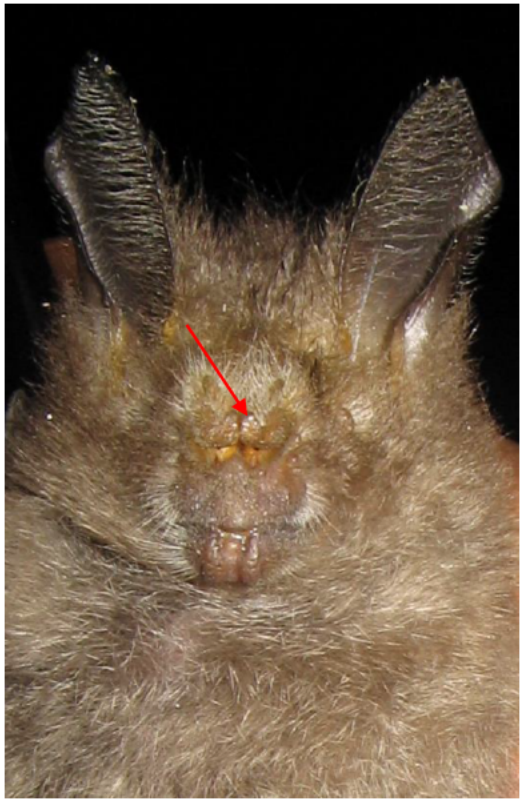
Complex noseleaf

Just one genus: *Rhinolophus* but many species!

Nycteridae (slit-faced bats)

T-shaped tail

Noseleaf covered



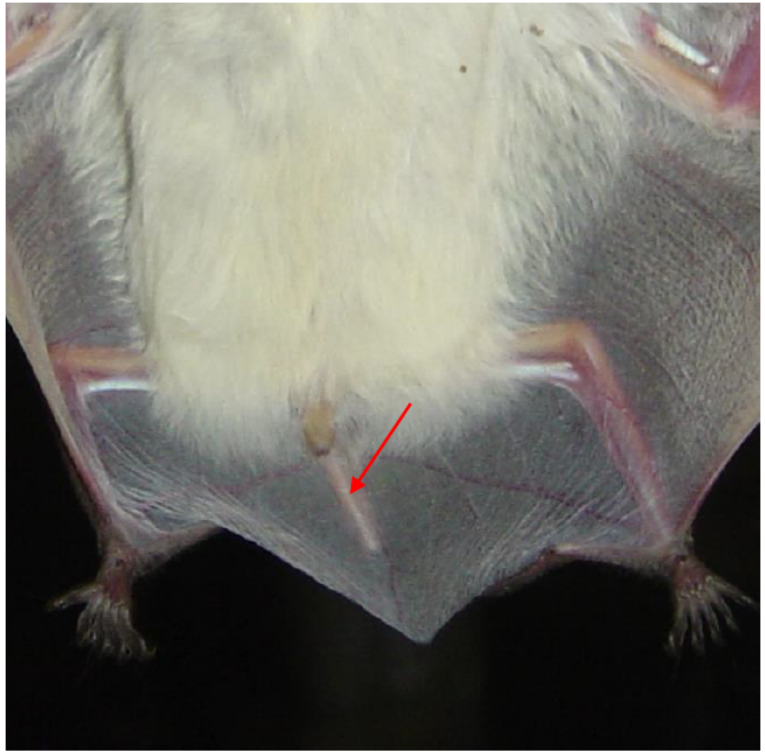
Just one genus: *Nycteris*

Emballonuridae (tomb bats)

Tail protrudes above tail membrane

Large eyes

No noseleaf



Just one genus: *Taphozous*

Molossidae (free-tailed bats)

Tail protrudes beyond membrane

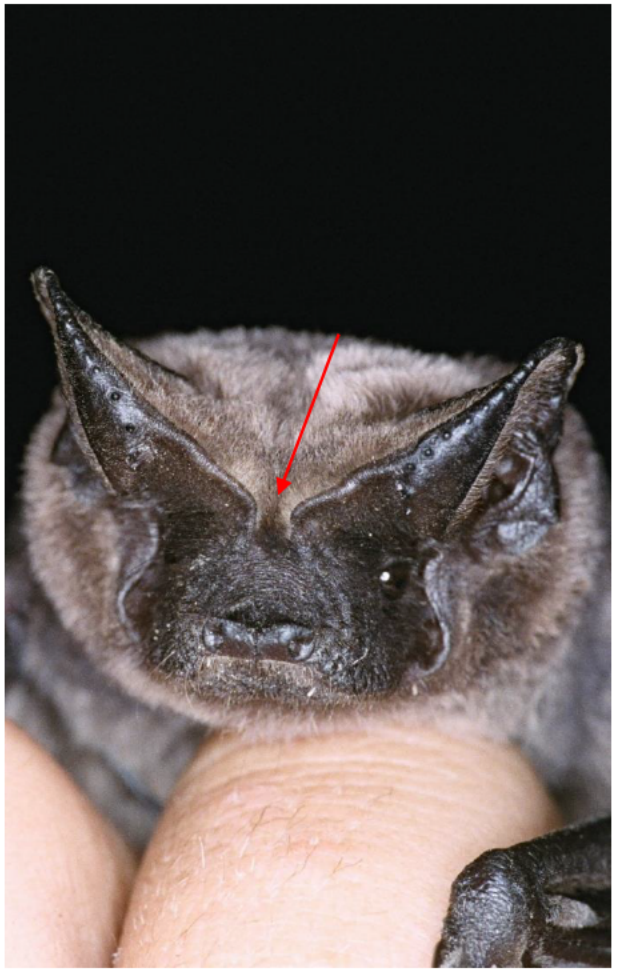
Bulldog-like face

Typical species (taxonomy not resolved)

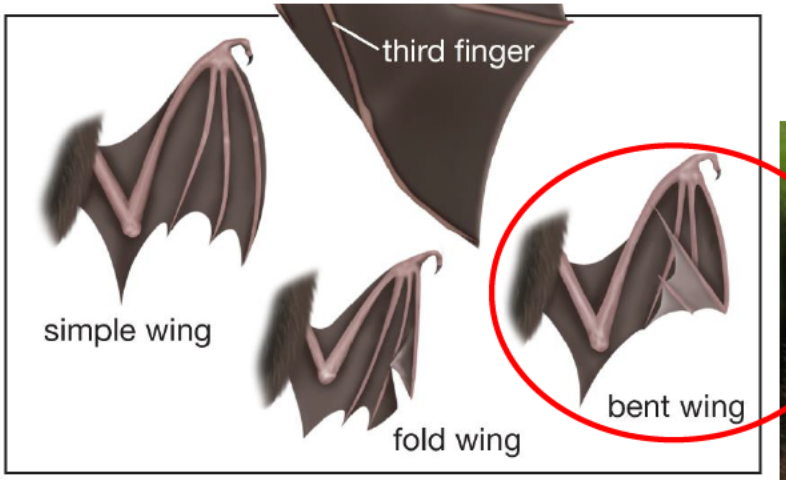
Chaerephon pumilus

Mops condylurus

Tadarida aegyptiaca



Miniopteridae (bent-winged bats)



'Bent' wings

No noseleaf

Domed head

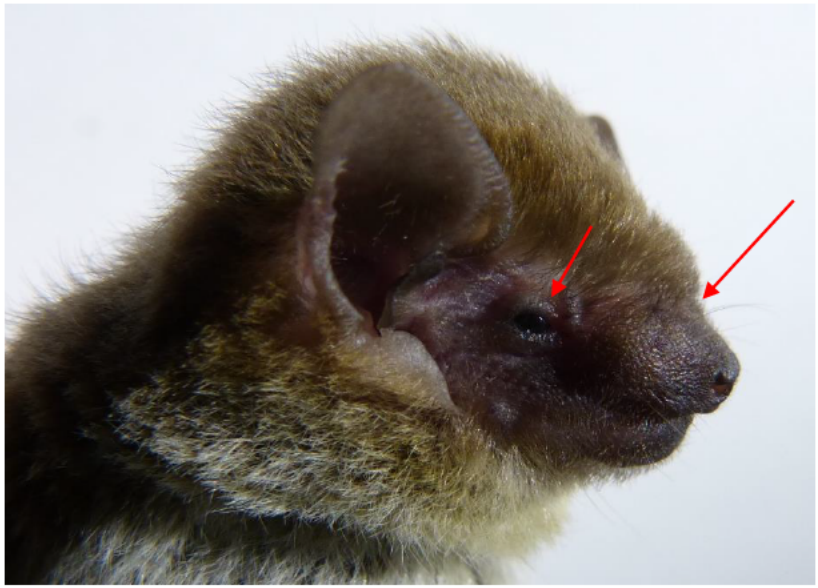
Just one genus: *Miniopterus*

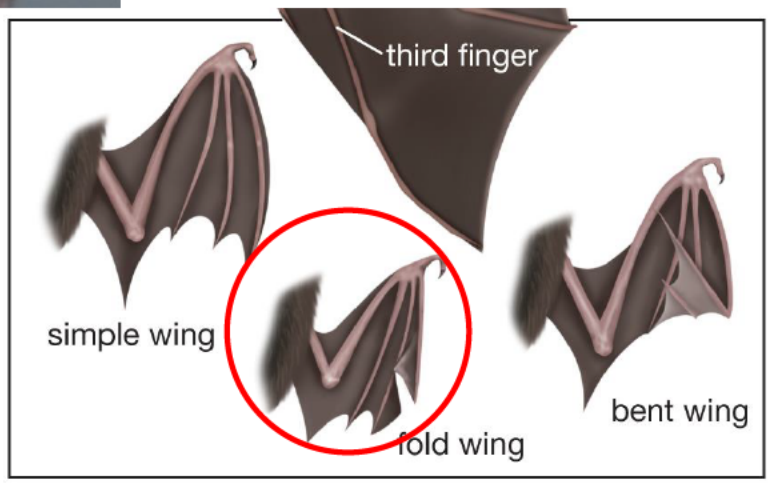
Vespertilionidae (vesper bats)

Folded wings

Small eyes

Simple face

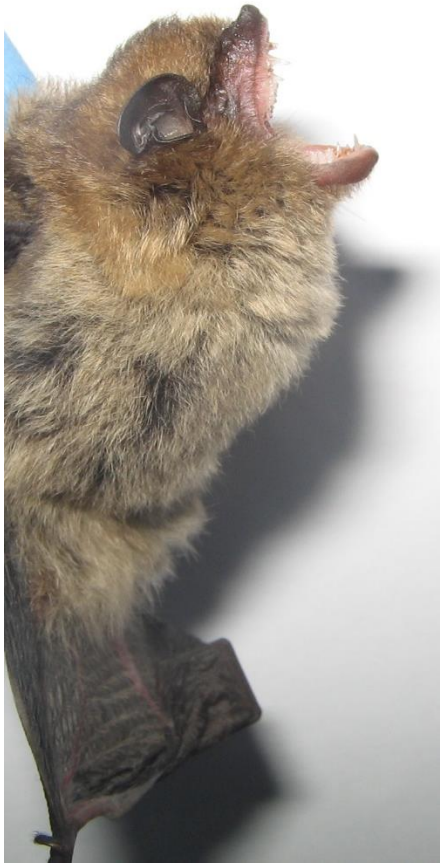




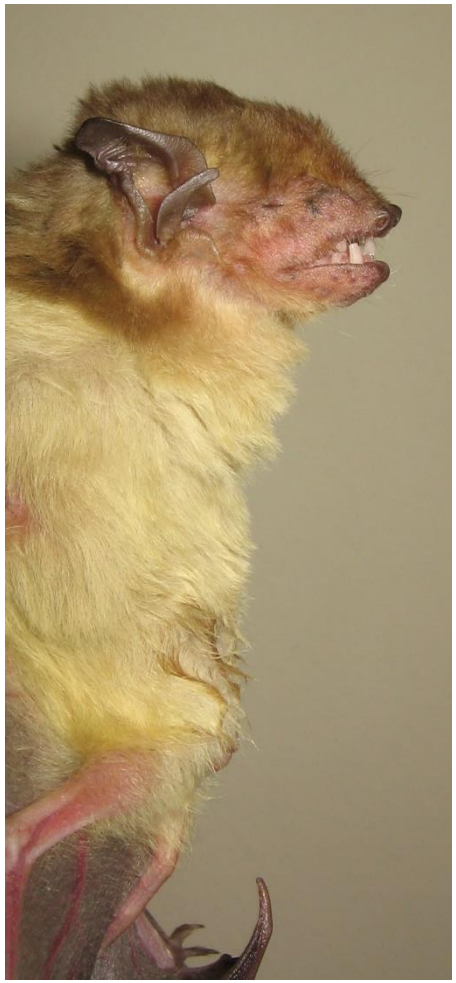
Many genera, and complicated!

Laephotis capensis

Afronycteris nana

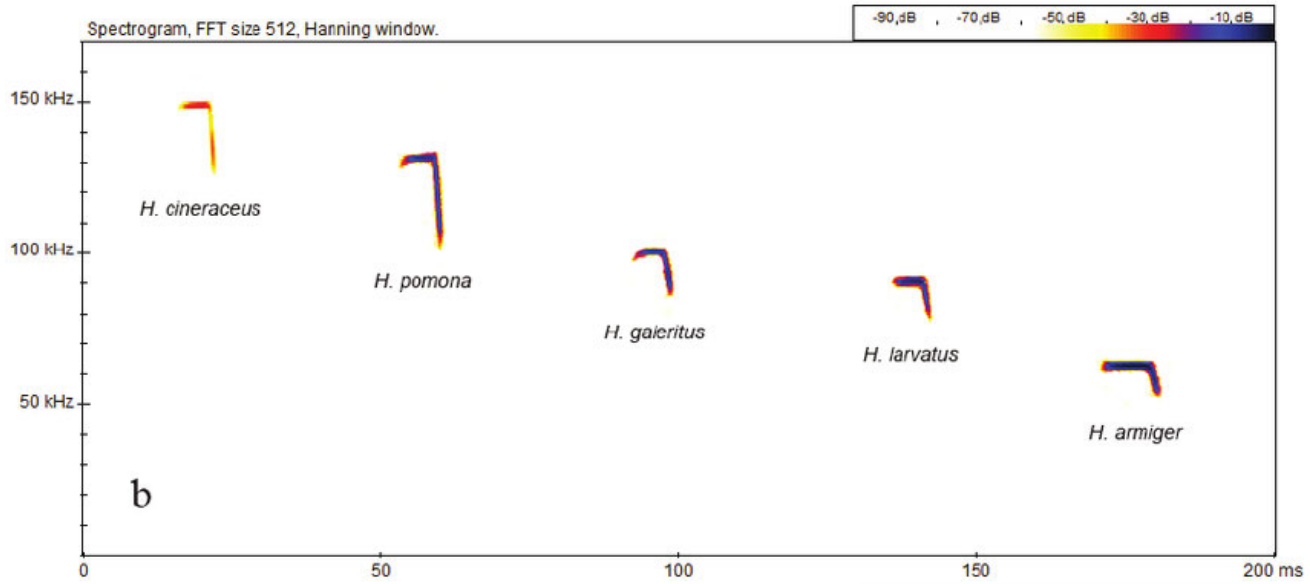


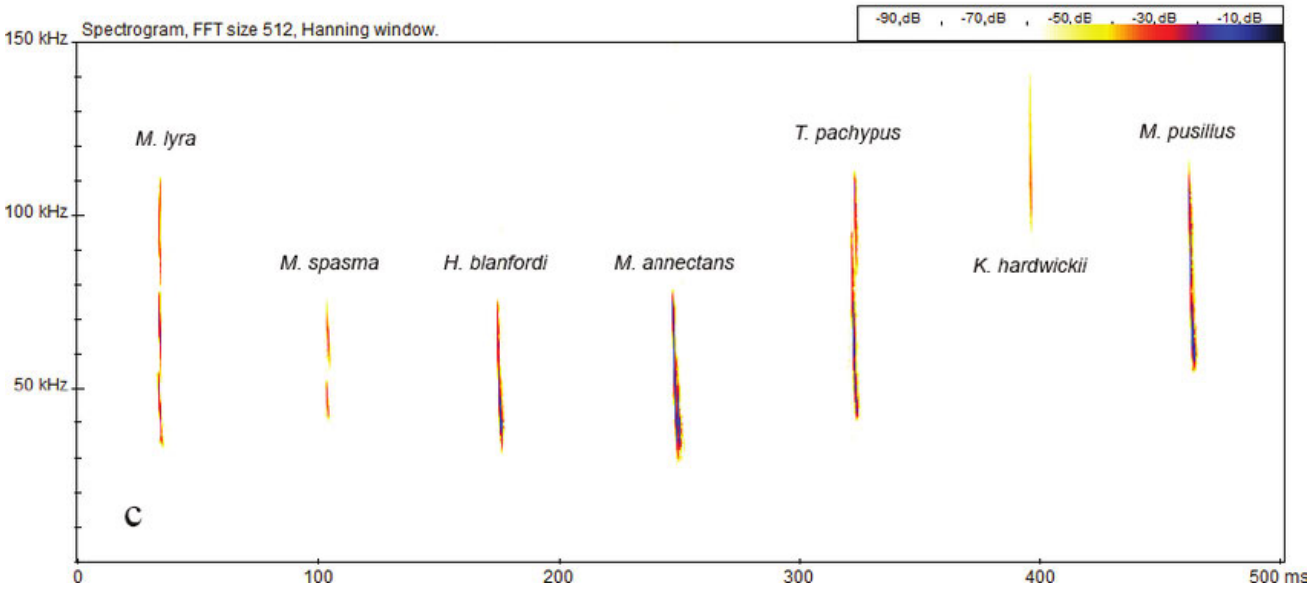
Scotophilus dinganii



Acoustic identification

Different species have different calls

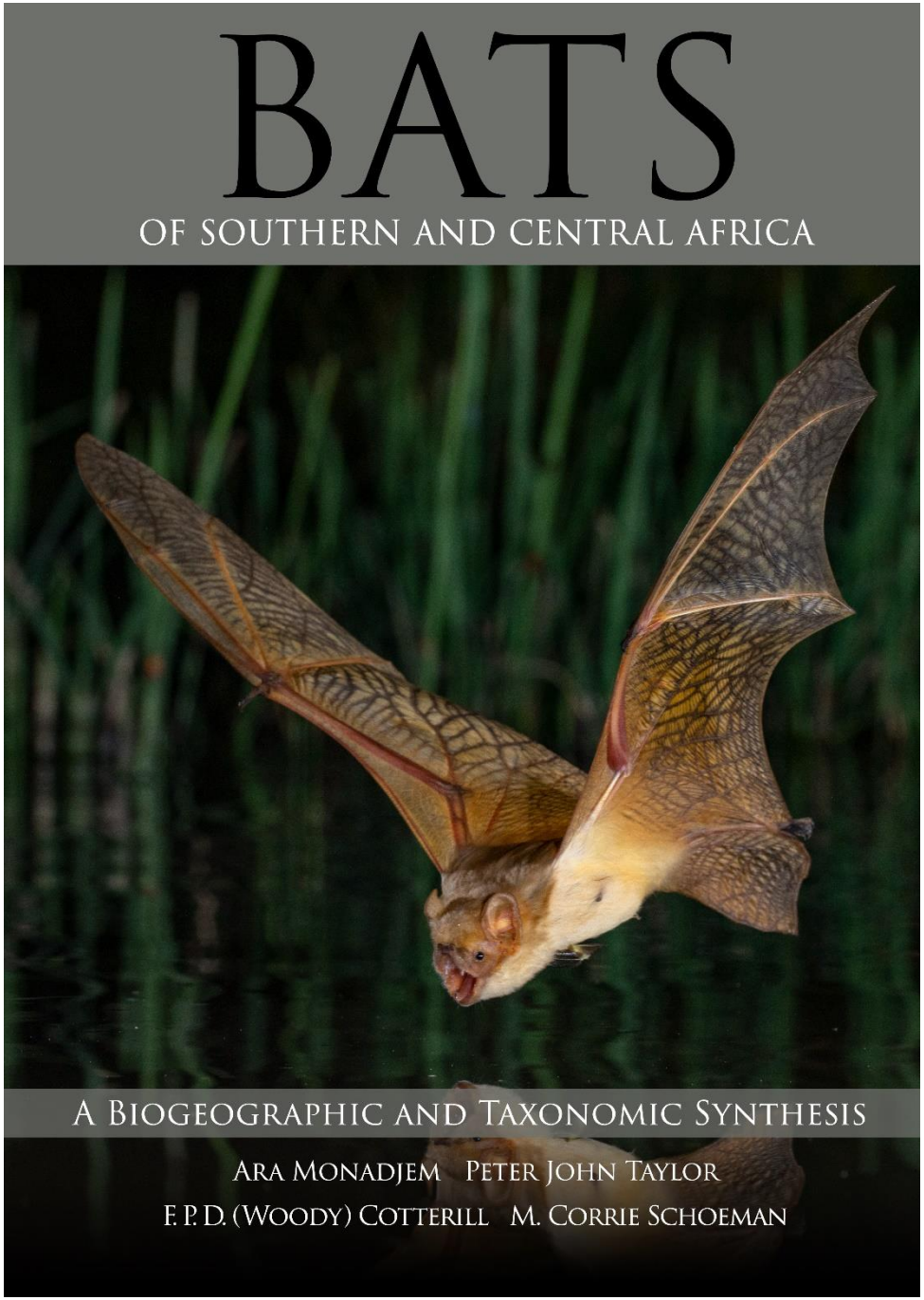




Phauk et al. 2013

Fully revised 2nd edition now available

(b) (6)



From: (b) (6)
To: (b) (6)
; Christopher Broder; (b) (6)
Eric
Laing; (b) (6)
[redacted]
Subject: SABRENet Bat identification workshop
Date: Tuesday, May 18, 2021 11:07:56 AM
Attachments: viruses-13-00936.pdf
Bat identification workshop_Monadjem.pdf

Dear all

Thank you for attending today's workshop. If you could not attend, the recording is on the drive at the following link;

(b) (5) [redacted]

I am also attaching a copy of (b) (6) 's slides

Attached is also our first official publication link to the DTRA project that was published today. It provides a good summary of coronavirus surveillance in Africa and suggestions for future work.

Kind regards

(b) (6)

[redacted]

(b) (6) [redacted]

(b) (5)

(b) (5)

(b) (5)

From: Broder, Christopher (b) (6)
To: (b) (6)
Subject: Fwd: Bat EID spillover risk research Zoom meeting
Date: Monday, May 3, 2021 12:52:34 PM
Attachments: Specific Aims Mavian bats museum.docx

---

hi (b) (6),

here is there grant SAs she sent before,
in case its lost in your inbox

new zoom meet tomorrow AM 10:00

(b) (6)

---------- Forwarded message ---------
From: (b) (6) >
Date: Tue, Apr 13, 2021 at 1:02 PM
Subject: Re: Bat EID spillover risk research Zoom meeting
To: Broder, Christopher (b) (6)
Cc: cc: (b) (6)

Dear (b) (6)

Sure, I'm attaching here a draft of the specific aims that (b) (6) and I drafted a while ago. As the project is evolving, they will change, but the main idea is there.

And thank you so much for connecting us to Dr. (b) (6).

Dr. (b) (6),

It's a pleasure to e-meet you. I'm looking forward to your meeting next week!

Best,

(b) (6)

(b) (5)

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From: (b) (6)
To: Laing, Eric
Cc: (b) (6) ; Broder, Christopher
Subject: Re: CREID TP
Date: Monday, March 15, 2021 9:01:59 PM
Attachments: EIDRC SE Asia Specific aims v6 FINAL_FINAL.docx
EIDRC Southeast Asia v7 FINAL_FINAL.docx
Project_Summary-Abstract_EIDRC_RFA-AI-19-028_(PI (b) (6))_v2 FINAL_FINAL.docx

---

Hi (b) (6),

Please see the attached.

Best, (b) (6)

[redacted]

On Mon, Mar 15, 2021 at 8:54 PM Laing, Eric (b) (6) wrote:

> Hi everyone,
>
> Could someone who has the technical proposal that was cleaned up and submitted easily on hand share it with me so I don't have to root through emails and drive folders?
>
> - (b) (6)
>
> [redacted]

CURRICULUM VITAE
Christopher C. Broder, Ph.D.

(b) (6)

BUSINESS ADDRESS: Department of Microbiology & Immunology
Uniformed Services University
4301 Jones Bridge Road
Bethesda, Maryland 20814-4799

Telephone: Office: (b) (6), Fax: 301-295-1545
Laboratories: (b) (6)
Mobil: (b) (6)
E-mail: (b) (6)

EDUCATION:

1983 BS, Biological Sciences, with honors. Florida Institute of Technology, Melbourne, Florida.
1985 MS, Molecular Biology, Florida Institute of Technology, Melbourne, Florida.
1989 PhD, Microbiology and Immunology. College of Medicine, University of Florida, Gainesville, Florida.

TRAINING / POSITIONS:

1983 - 1985 Graduate student, Florida Institute of Technology, Melbourne. (Adv: Kenneth L. Kasweck, PhD)

1985 - 1989 Graduate student, Department of Immunology and Medical Microbiology, University of Florida. Gainesville. (Adv: Michael D.P. Boyle, PhD)

4/89 - 10/89 Postdoctoral Associate, Department of Medicine, University of Florida. (Adv: Richard Lottenberg, MD)

11/89 - 1/90 Microbiologist (GS-11), Laboratory of Viral Diseases (LVD), National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD. (Adv: Bernard Moss, MD)

1990 - 1992 National Research Council Research Associate, LVD, NIAID, NIH. (Adv: Bernard Moss, MD)

1993 - 1996 IRTA Fellow, LVD, NIAID, NIH. (Adv: Bernard Moss, M.D. and Edward A. Berger, PhD)

1996 - 2000 Assistant Professor, Department of Microbiology and Immunology; and Molecular and Cell Biology (secondary), USU, School of Medicine, Bethesda, MD.

2000 - 2005 Associate Professor (Tenured), Department of Microbiology and Immunology; and Emerging Infectious Diseases and Molecular and Cell Biology (secondary) USU, School of Medicine, Bethesda, MD.

2005-present Professor (Tenured), Department of Microbiology and Immunology, Joint appointment, Emerging Infectious Diseases Graduate Program, USU, Bethesda, Maryland.

2006-2018 Director, Emerging Infectious Diseases Graduate Program, USU, Bethesda, Maryland.

2018-present Chair, Department of Microbiology and Immunology, USU, Bethesda, Maryland

HONORS / AWARDS:

1987, 88 National Institutes of Health Training Grant Award.
1989 Medical Guild Graduate Research Award.
1990-92 National Research Council Research Associateship Award.
1993-96 National Institutes of Health Intramural Research Training Award Fellowship.
1996 The Fellows Award for Research Excellence, Office of Science Education, NIH.
1996 Breakthrough of the Year, Science; American Association for the Advancement of Science.
1997 Newcomb Cleveland Prize, American Association for the Advancement of Science.
2001 Outstanding Instructor in Virology, USU, School of Medicine Class of 2003.
2008 The Henry Wu Award for Excellence in Basic Science Research.
2013 The 3rd Sidney Pestka Lecture; 22nd Annual Philadelphia Infection & Immunity Forum.

2013 The 2013 Federal Laboratory Consortium (FLC) Award for Excellence in Technology Transfer.

2013 The CSIRO Chairman's Medal. The Commonwealth Scientific and Industrial Research Organisation (CSIRO); Australia's national science agency.

2014 The Cinda Helke Award for Excellence in Graduate Student Advocacy.

2016 The James J. Leonard Award for Excellence in Translational/Clinical Research.

2017 USU, Impact Award for outstanding contributions to the School of Medicine.

2018 USU, Impact Award for outstanding contributions to the School of Medicine.

2019 The 2019 Federal Laboratory Consortium (FLC) Award for Excellence in Technology Transfer.

2019 USU Outstanding Biomedical Graduate Educator Award.

2019 Selection to the University of Florida, College of Medicine, "Wall of Fame".

2019 Dean's Award for Leadership, University of Florida, College of Medicine.

2020 Inaugural, 2020 Federal Laboratory Consortium (FLC) Impact Award.

2020 Military Health System Research Symposium (MHSRS) 2020 Outstanding Individual Research Accomplishment by an Individual Senior Researcher

PATENTS, INVENTIONS, LICENSES:

- Bacterial Plasmin Receptors as Fibrinolytic Agents: **U.S. Patent No. 5,237,050.**
- Oligomeric HIV-1 Envelope Glycoproteins (gp140): **U.S. Patent Nos. 6,039,957 and 6,171,596.** Methods for Production, Purification, and Use as an Immunogen in Mammals.
- CC Chemokine Receptor 5 (CCR5) DNA, New Animal Models and Therapeutic Agents for HIV Infection. **U.S. Patent No. 7,151,087.**
- Cells Expressing Both Human CD4 and a Human Fusion Accessory Factor (CXCR4) Associated with HIV Infection: **U.S. Patent No. 6,197,578.**
- 4G10, a Monoclonal Antibody against the Chemokine Receptor CXCR4, Raised against the N-terminal Sequence of CXCR4. DHHS Reference No. E-340-2002/0. **Licensed to Santa Cruz Biotechnology 2002.**
- Compositions and Methods for the Inhibition of Membrane Fusion by Paramyxoviruses: **U.S. Patent Nos. 7,666,431 and 8,114,410.**
- Soluble Forms of Hendra and Nipah Virus G Glycoprotein. **Australian Patent No. 2005327194. U.S. Patent Nos. 8,865,171; 9,045,532; 9,056,902; 9,533,038, 10,053,495.**
- HIV-1 Envelope Glycoprotein Oligomer and Methods of Use. **U.S. Patent No. 8,597658.**
- Soluble Forms of Hendra and Nipah Virus F Glycoprotein and Uses Thereof: **Australian Patent No. 2013276968. U.S. Patent Nos. 10,040,825; 10,590,172.**
- Human Monoclonal Antibodies against Hendra and Nipah viruses. **U.S. Patent Nos. 7,988,971; 8,313,746; 8,858,938**
- Antibodies against F glycoprotein of Hendra and Nipah viruses. **U.S. Patent Nos. 9,982,038 and 10,738,104.**
- Hendra sG: **Licensed to Zoetis, Inc. (formerly Pfizer Animal Health)**. Equivac ® HeV; Nov, 2012, Australia.
- Human antibody m102.4 therapy against Hendra and Nipah virus infection; Phase I clinical trial completed in May 2016, Queensland Health, Queensland, Australia.
- Hendra sG: **Licensed to Auro Vaccines, Aurobindo Pharma USA**. Nipah/Hendra virus human vaccine.
- Cedar Virus and Methods of Use: **U.S. Patent No., 10,227,664**.

PROFESSIONAL SOCIETIES:

American Society for Virology (ASV)
Association of Medical School Microbiology and Immunology Chairs (AMSMIC)

DEPARTMENTAL RESPONSIBILITIES (Teaching), Graduate and Medical Education:

Annual: Lecturer; Medical School Integrated Curriculum; Viral Zoonoses; Antiviral Drugs; Gastrointestinal Viral Diseases; Viral Vaccines; Viral Infections of the central nervous system.

Biannual: Lecturer; virus entry, virus receptors, negative-stranded RNA viruses, emerging viruses.

Current and Former Postdoctoral Trainees:

Krishnamurthy Govindaraj, PhD, Institute of Medical Sciences, Lucknow, India. 1999-2004. (Research Associate, Henry M. Jackson Foundation for the Advancement of Military Medicine)

Hong Chen, MD, Hunan Med. Uni. Hunan, China. 1997-00. (Scientist, AscentGene, Inc., College Park, MD)

Sanjay Phogat, PhD, University of Delhi South Campus, New Delhi, India. 2000-2001. (Principal Scientist, Immunogen

Design, International AIDS Vaccine Initiative (IAVI), New York)

Tzanko S. Stantchev, MD, Varna Institute of Medicine, Rousse, Bulgaria. 1998-2008. (Research Scientist, Division of Monoclonal Antibodies, CDER, FDA, Silver Spring, MD)

Anil Choudhary, PhD, University, Rohtak, India. 2001-2006. (Scientist, Profectus, Inc. Baltimore, MD).

Antony S. Dimitrov, PhD, The University of Tokyo, Japan. 2004-2006. (Senior Staff Scientist, Profectus BioSciences, Inc. Baltimore, MD; Department of Microbiology and Immunology, Unformed Services University)

Matthew I. Bonaparte, PhD, SUNY Upstate Medical University, NY, 2005-2007. (Scientist, Global Clinical Immunology Sanofi Pasteur, Swiftwater, PA)

Dimple Khetawat (Harit), PhD, University of Calcutta, India. 2003-2011. (Research Associate, UNC Eshelman School of Pharmacy, Division of Molecular Pharmaceutics, University of North Carolina at Chapel Hill)

Yee-Peng Chan, PhD, The University of Malaya, Kuala Lumpur, Malaysia, 2005-2014

Vidita Choudhry, PhD, Jawaharlal Nehru University, New Delhi, India 2006-2014 (NMRC, Silver Spring, MD)

Bang Vu, PhD, Free University of Brussels, Belgium, 2010-2017

Moushimi Amaya, PhD, George Mason University, VA, 2016-

Current and Former Graduate Students:

Donald J. Chabot, PhD (Microbiology and Immunology-97'; 2000), (Microbiologist, Clinical Research Management, Inc./ Team Akimeka, USAMRIID, Bacteriology Division, Fort Detrick, MD)

Agnes Jones-Trower, PhD (Molecular and Cellular Biology-97', 2001), (Staff Fellow, Division of Viral Products, CBER, FDA, Bethesda, MD (Ret.)

Katharine N. Bossart, PhD (Microbiology and Immunology-98'; 2003), (President & Owner; Integrated Research Associates, LLC. San Rafael, CA)

Jared Patch, PhD (Emerging Infectious Diseases-01'; 2007), (Research Scientist, Food Animal Vaccine Development, Elanco, Inc., Greenfield, IN)

Julie A. Pavlin, MD, PhD, MPH, COL, USA, Ret. (Emerging Infectious Diseases-00'; 2007), (Director, Board on Global Health, Health and Medicine Division, The National Academies of Sciences, Engineering, and Medicine, Washington, DC)

Kimberly Bishop, PhD (Emerging Infectious Diseases-02': 2007), (Deputy Head, Genomics Dept., Biological Defense Research Directorate (BDRD) Naval Medical Research Center (NMRC), Fort Detrick, MD)

Andrew Hickey, PhD, MPH, LCDR, USPHS (Emerging Infectious Diseases-03': 2009) (LT, United States Public Health Service, Chief, HIV/STD Laboratory Research Section, CDC-Thailand).

Stephanie Petzing, PhD (Emerging Infectious Diseases-05': 2012), (AAAS Science & Technology Policy Fellow, U.S. Department of Defense, Threat Reduction Program Oversight Office)

Dawn L. Weir, PhD, LCDR, USN (Emerging Infectious Diseases-07': 2013), (LCDR, United States Navy, Medical Services Corps), Presently; Naval Research Laboratories, Washington, DC)

Deborah L. Steffen, PhD (Emerging Infectious Diseases-07': 2013) (Faculty, Stone Ridge HS, Bethesda, MD; Ret.)

Eric Laing, PhD (Emerging Infectious Diseases-10:' 2016) (Research Assistant Professor, Department of Microbiology and Immunology, USU, 2019)

Chelsi Beauregard, PhD (Emerging Infectious Diseases-13': 2020), (Assistant Professor of Biology, Southern New Hampshire University, Manchester, NH)

Sofia Da Silva, PhD (Emerging Infectious Diseases-13': 2020), (ORISE fellowship; Division of Research, Innovation, and Ventures (DRIVe). Biomedical Advanced Research and Development Authority (BARDA))

Graduate Thesis Committees (other):

Uniformed Services University:

Emerging Infectious Diseases Graduate Program (USU)

Sharon Wen, PhD; 06'

Gabriel DeFang, PhD, LCDR, MSC, USN; 07'

Trupti Brahmbhatt, PhD, CAPT, MSC, USN; 07'

Shana Miles, MD, PhD, LT, MC, USN; 10'

Claire Wernly, PhD; 10'

Aura Garrison, PhD; 12'

Michael Washington, PhD, LTC, MEDCOM, USA; 14'

Tonia Zangari, PhD; 14'

Kate Mastraccio, PhD; 18'

Trung Ho, MD-PhD-candidate
Adrian Paskey, PhD; 20'
William Valiant, PhD; 19'

Molecular and Cellular Biology Graduate Program (USU)
Randall Merling, PhD; 07'
Mark Serkovich, MS; 06'
Mark Smith, VMD, Diplomate ACVP, PhD, LTC, VC, USA; 14'

External:
Philippa J. Miller, PhD; 04' (The University of Melbourne, Victoria, Australia)
Yee-Peng Chan, PhD; 05' (The University of Malaya, Kuala Lumpur, Malaysia)
Tonya Colpitts, PhD; 07' (University of Texas Medical Branch, Galveston, Texas, USA)
Stephanie L. Foster, PhD-candidate (University of Texas Medical Branch, Galveston, Texas, USA)

UNIVERSITY SERVICE:

1998-2014	Uniformed Services University Merit Review Committee (USU study section)
1997-1998	Research Committee for the LCME report to the Board of Regents
1997-2000	Chair, Bio-Instrumentation Center Committee, Uniformed Services University
2000-2001	Faculty Senator, Basic Sciences
1997-2001	Comparability and Faculty Welfare Committee
2006-2018	Graduate Program Director (Ph.D.): Emerging Infectious Diseases
2006-	EID, Executive Committee
2006-2018	Graduate Education Committee
2007-2015	MD/PhD Admissions and Curriculum Committee
2008-2009	USU, School of Medicine 5-year Evaluation
2009-10	University Space Committee
2009-	Basic Science Chairs Committee (Chair, 2010-11, 2015-16)
2009-10	USU School of Medicine Strategic Planning Committee
2010-11	USU School of Medicine Curriculum Reform Clerkship Committee
2011-14	Dean's Advisory Group
2011	Neuroscience Graduate Program Director Search Committee (Chair)
2011-	Board of Academic Counselors
2012-2015	Committee on Appointments, Promotions and Tenure (CAPT committee)
2014-	School of Medicine Endowment Committee Meeting
2016-	Joint Patent and Technology Review Group (JPTRG)
2018-	USU Building F "Emerging Infectious Disease/Global Health" Scientific Neighborhood Team
2019-	USU, Names and Honors Committee.
2019-20-	Department Chair Search Committees; Department of Anatomy, Physiology and Genetics; Psychiatry.
2019	Vice President for Research (VPR), USU, Search Committee.

OUTSIDE ACTIVITIES AND SERVICE:

National and International Committees and Boards:

1997	Board Member: Source Evaluation Board for Biotechnology of the National Institute of Standards and Technology, United States Department of Commerce, Advanced Technology Program.
1999	*Ad hoc* Member: Special Emphasis Panel on *HIV Neuropathogenesis* for the National Institute of Neurological Disorders and Stroke, National Institutes of Health.
2000	*Ad hoc* Member: Scientific Board of the Dutch Aids Fund, Netherlands.
2001	Program Reviewer, The Pasteur Institute: for the Unit of Viral Immunology, France.
2000-03	Member: Study Section: *Molecular Biology and Pathogenesis of HIV*. The University-wide AIDS Research Program. Office of the President of the University of California.
2003	*Ad hoc* Member: Experimental Virology (EVR) Study Section, NIAID, NIH.

2003 *Ad hoc* Member: AIDS Molecular and Cellular Biology Study Section, NIAID, NIH.

2003-14 Management and Oversight Committee Member. Middle Atlantic Regional Center of Excellence in Biodefense and Emerging Infectious Diseases Research.

2004 *Ad hoc* Member: Source Evaluation Board for Biotechnology of the National Institute of Standards and Technology, United States Department of Commerce, Advanced Technology Program.

2005 Review Committee Member; The National Screening Laboratory for the Regional Centers of Excellence for Biodefense and Emerging Infectious Disease, Harvard Medical School, Boston, MA.

2005 Program Reviewer, new research unit: "Host-Virus Relationships", in The Pasteur Institute: France.

2009 Member, National Veterinary Stockpile Nipah virus Countermeasures Workshop; United States Department of Agriculture; (Geelong, Australia; March 17-19).

2009 The Health Research Council of New Zealand, program reviewer.

2007- Editorial board, *Journal of Virology*.

2010- Editorial board, *Virology*.

2011- Editor, *Viruses*.

2011- Editorial board, *Pathogens*.

2012- Editor, *Virologica Sinica*

2011 Member, Discontools Nipah Virus Infection Panel Expert Group. Gap analysis. International Federation for Animal Health Europe, Brussels, Belgium.

2011 Invited expert for the National Academies. Evaluation of the updated site-specific risk assessment for the National Bio- and Agro-Defense Facility (NBAF) in Manhattan, Kansas.

2016 Netherlands Organisation for Scientific Res., Dutch national science council, Gravitation Programme review.

2017 Member, BSL4ZNet Expert Panel Meeting for Henipaviruses and Ebolaviruses; Canadian National Centre for Foreign Animal Disease, Winnipeg (November).

2017- World Health Organization (WHO) taskforce; Nipah virus research and development (R&D) roadmap; with University of Minnesota (CIDRAP) and Wellcome Trust. March 1-2, July 9-10, 2018.

2018- Nipah Therapeutics Protocol Team; ICMR, NIAID, WHO.

2018- Nipah Task Force (CEPI).

***Ad hoc* Reviewer for the Following Journals:** PNAS; J.Virol.; J.Infec.Dis.; Virology; J.Virol.Meth; Nat.Struc.Bio., Nat.Micro.Rev., PlosPath.; PlosNegTrop.Dis.; Viruses, Viro. J.; Anti.Agents and Chemo.; AntiviralRes., Monoclonal Antibodies, Virologica Sinica, Science, Sci.Trans.Med., Pathogens, Frontiers.

RESEARCH EXPERIENCE AND INTERESTS:

M.S. (85'): "Analysis of Thymidine Kinase mRNA and Construction of a cDNA Library from Mouse L5178Y Cells".

Ph.D. (89'): "Isolation and Characterization of a Group A Streptococcal Receptor for Human Plasmin".

Current: Interactions between pathogenic human and zoonotic enveloped animal viruses and host cells: virus receptors; envelope glycoprotein structure/function; vaccines; antivirals; virus assembly, bio-surveillance.

ACTIVE SUPPORT: (10)

Grant Title: "Advancement of Vaccines and Therapies for Henipaviruses"
Grant Number: U19 AI142764-01. Center of Excellence for Translational Research (CETR)
Grant Period: 03/20/19-02/29/24
Total Direct: $24,587,556 Agency: NIH/NIAID, Role: Overall Center PI, Director of the Administrative Core, and PI.
Partners: Profectus Biosciences, Inc. (Auro Vaccines, LLC), Mapp Biopharmaceuticals, Inc., Vanderbilt University, and University of Texas Medical Branch.

Grant Title: Serological Biosurveillance for Spillover of Henipaviruses and Filoviruses at Agricultural and Hunting Human-Animal Interfaces in Peninsular Malaysia
Grant Number: HDTRA1-17-10037
Grant Period: 05/01/17-04/30/22
Total Direct: $910,000 Agency: DTRA, DoD: With: Ecohealth Alliance, New York, NY. Co-PI, with J Epstein.

Grant Title: A Subunit Vaccine (HeV-sG) to Protect against Nipah and Hendra Diseases
Grant Period: 07/01/18-06/30/2028

Total Direct: CRADA to USU $538,046. (Total award to Profectus Biosciences 23 million). Agency: CEPI (Coalition for Epidemic Preparedness Innovations (CEPI): Role: Co-PI, with J Eldridge.

Grant Title: Collaborative development and evaluation of an equine vaccine against Hendra virus
Principal Investigator: Christopher C. Broder, Ph.D.
Agency: Pfizer (Zoetis)/CRADA Period: July 1, 2012 to September 30, 2040
(CRADA): Development and evaluation of an equine vaccine against Hendra virus.

DTRA BTRP (Ecohealth Alliance, New York, NY. Co-PI, with J Epstein.)
Title: Malaysia Partners Luminex Training and Research Preparedness
1 Year Requested, POP: 01/01/2020-11/30/2020
Subcontract, Total Award: $90,777. Role: Co-Principal Investigator

DHA IDCRP (Burgess, T.)
Epidemiology, Immunology, and Clinical Characteristics of Emerging Infectious Diseases with Pandemic Potential (EpICC-EID)
2 Years Requested, POP: 05/01/2020-04/30/2022
Subaward, Total Award: $1,078,273. Role: Associate Investigator

DARPA PREEMPT (A. Peel)
Preempting Spillover of Novel Coronaviruses from Bats to Humans
1 Year Requested, POP: 06/01/2020 – 05/31/2021
Subaward, Total Award: $120,318. Role: Collaborator

DTRA BTRP HDTRA12010025 (with W. Markotter, Uni Pretoria, S. Africa)
Title: Biosurveillance for Viral Zoonoses around Bat-Livestock-Human interfaces in Southern Africa
5 Years Requested, POP: 10/01/2020 – 09/30/2023
Subaward, Total Award: $1,116,971. Role: Co-Investigator

DTRA BTRP (Ecohealth Alliance, New York, NY. Co-PI, with J Epstein)
Title: Biosurveillance for Spillover of Henipaviruses and Filoviruses in Rural Communities in India
3 Years Requested, POP: 10/01/2020 – 09/30/2023
Subaward, Total Award: $888,721. Role: Co-Investigator

NIAID U01AI151797: Centers for Research in Emerging Infectious Diseases (CREID)
(Ecohealth Alliance, New York, NY. Co-PI, with P. Daszak.)
Emerging Infectious Diseases - South East Asia Research Collaboration Hub; 02/01/2020-03/31/2025
Subaward, $539,119. Role: Co-investigator

PENDING SUPPORT: (2)

(b) (5) [redacted]

PREVIOUS SUPPORT: (22)

Grant Title: HIV-1 Fusion Cofactors
Grant Number: R29 AI41411
Grant Period: 04/01/97-04/30/99
Total Direct: $512,407 Agency: NIH/NIAID Role: Principal Investigator

Grant Title: Structural and Functional Analysis of HIV-1 Entry Cofactors
Grant Number: R0 73FG-01
Grant Period: 10/01/96-09/30/99
Total Direct: $81,000 Agency: USUHS/DOD, Role: Principal Investigator

Grant Title: HIV-1 Fusion Cofactors
Grant Number: R01 AI043885
Grant Period: 07/15/98-01/31/11
Total Direct: $2,167,550 Agency: NIH/NIAID, Role: Principal Investigator

Grant Title: Analysis of Oligomeric HIV-1 Envelope Glycoproteins
Grant Number: R21 AI42599-01

Grant Period: 11/01/97-10/31/00-expiring
Total Direct: $300,000 Agency: NIH/NIAID, Role: Principal Investigator

Grant Title: HIV-1 Envelope-CD4-Coreceptor Complexes as Vaccines
Grant Number: R21 AI47697-01
Grant Period: 7/01/00-6/30/02
Total Direct: $300,000 Agency: NIH/NIAID, Role: Principal Investigator

Grant Title: Program title: Broadly Effective Neutralization and CTL against HIV-1
Project 2 title: HIV-1 gp140 Oligomers as Vaccine immunogens
Grant Number: PO1 AI48380
Grant Period: 09/01/01-06/31/06
Total Direct: $1,261,561 Agency: NIH/NIAID, Role: Principal Investigator, Project 2

Grant Title: Nipah Virus and Hendra Virus Subunit Vaccines
Grant Number: R21 AI065597
Grant Period: 07/01/05-06/30/07
Total Direct: $275,000 Agency: NIH/NIAID, Role: Principal Investigator

Grant Title: Nipah Virus and Hendra Virus Peptide Therapeutics
Grant Number: U01 AI056423
Grant Period: 09/15/03 – 08/01/08
Total Direct: $2,025,326 Agency: NIH/NIAID, Role: Principal Investigator

Grant Title: Biodefense and Emerging Infectious Diseases Research: (MARCE)
Program II: Emerging Virus Entry into Host Cells: Strategies for Inhibition
Project II-3: Hemorrhagic Fever Viruses / Australian Bat Lyssavirus Tropism Entry and Host Factor Dependence
Grant Number: 2U54 AI057168
Grant Period: 08/01/03 – 02/28/14
Total Direct: $1,661,546 Agency: NIH/NIAID, Role: Principal Investigator, Project II-3

Grant Title: Characterization of the Envelope Glycoproteins of Beilong and J-virus
Grant Number: R073NN
Grant Period: 10/01/06-09/30/09
Total Direct: $60,000 Agency: USUHS/DOD, Role: Principal Investigator

Grant Title: Emerging Viruses and Host Cell Interactions in Bats
Grant Number: R073SA
Grant Period: 10/01/09-09/30/12
Total Direct: $60,000 Agency: USUHS/DOD, Role: Principal Investigator

Grant Title: High Potency HIV-1 Broadly Cross-Reactive Neutralization
Grant Number: U01AI078412
Grant Period: 04/01/2008 – 03/31/2013
Total Direct: $3,000,000 Agency: NIH/NIAID, Role: Co-Principal Investigator

Grant Title: Vaccines and Therapeutics for Nipah and Hendra virus
Grant Number: U01AI077995
Grant Period: 06/01/2008 – 8/31/2014
Total Direct: $5,617,562; Agency: NIH/NIAID, Role: Principal Investigator

Grant title: Development of sG as a human vaccine against Nipah/Hendra
Principal Investigator: T. Fouts, A. Dimitrov / Co-PI: Christopher C. Broder, Ph.D., Subaward.
Period: May 1, 2012 to April 30, 2017
Total Direct: $225,000; Agency: NIH/NIAID, Type: 1R01AI098760-02.

Grant title: Preclinical Development of m102.4, a Human Anti-Hendra and Nipah Antibody
Principal Investigator: T. Fouts, A. Dimitrov / Co-PI: Christopher C. Broder, Ph.D., Subaward.
Period: May 1, 2011 to April 30, 2016
Total Direct: $225,000; Agency: NIH/NIAID, Type: 1R01AI093346-03.

Grant Title: Nipah & Hendra virus Nonhuman Primate Model & Therapeutics Development
Grant Number: U01 AI182121
Grant Period: 03/15/2009 – 02/28/2015
Total Direct: $6,940,076. Agency: NIH/NIAID, Role: Co-Principal Investigator (with T. Geisbert, UTMB).

Grant Title: Analysis of the entry and egress of Cedar virus a new species of Henipavirus
Grant Number: R0732012
Grant Period: 10/01/12-09/30/15
Total Direct: $60,000 Agency: USUHS/DOD, Role: Principal Investigator

Grant Title: Identification, Countermeasures, and New Therapies Toward Biological Threat Agents;
Component Project: Soluble Trimeric Filovirus Envelope Glycoproteins
Grant Number: #HT9404-13-1-0021
Grant Period: 10/01/14-03/31/17

Total Direct: $78,085. Agency: BDRD/NMRC (DoD), Role: Component Project PI (with J. Czarnecki, NMRC)

Grant Title: Therapies for Neurotropic Viral Biothreat Pathogens
Grant Number: PPG. HT9404-13-1-0008
Grant Period: 10/01/13 – 9/30/17
Total Direct: $1,950,882.
Agency: USUHS, Role: Component Project PI (with B. Schaefer, USU)

Grant Title: Nipah Virus and Hendra Virus Entry and Virion Assembly
Grant Number: R01 AI054715
Grant Period: 04/01/06-09/30/18
Total Direct: $2,225,000 Agency: NIH/NIAID, Role: Principal Investigator

Grant Title: A Recombinant Cedar Virus-based Henipavirus Replication Platform for High-throughput Inhibitor Screening
Grant Number: R21 AI137813
Grant Period: 04/01/18-03/31/20
Total Direct: $275,000 Agency: NIH/NIAID, Role: PI

Grant Title: Chulalongkorn Luminex Training and Research Preparedness
Grant Number: DTRA STEP HDTRA1-17-C-0019
Grant Period: 01/05/19 – 06/01/19
Total Direct: $195,178: Agency: DTRA, DoD, Role: PI

INVITED LECTURES (>100):

1994. Department of Pathology and Lab Med., University of Pennsylvania, Jan 13. ***"HIV-1 Envelope Glycoprotein Mediated Cell Fusion: Structural Features of CD4 and Involvement of Accessory Components"***.

1994. GSF-Forschungszentrum für Umwelt und Gesundheit, GmbH, Neuherberg. Institut für MolekulareVirologie, Oberschleißheim, Germany. Current Advances In Molecular Biology Seminar Series. Aug 10. ***"Factors Associated with the Selective Fusogenic Activities of HIV-1 Envelope Glycoproteins for Specific CD4$^+$ Cell Types"***.

1995. Department of Microbiology, campus-wide series. University of Pennsylvania Feb 22. **"*Molecular Characterization of Viral Glycoprotein Mediated Membrane Fusion"***.

1996. 3rd International Workshop on HIV and Cells of Macrophage Lineage. Villa Monastero, Piazza Venini, Varenna, Italy. Oct17. ***"HIV Tropism: Distinct Accessory Fusion Factors for Different CD4$^+$ Cell Types"*.**

1998. Molecular Basis of Disease / Molecular and Cellular Biology Program, Medical College of Ohio, Health Education Building, Toledo, Ohio. February 17. ***"HIV: Envelope Glycoprotein and Membrane Receptors"***.

1999. Division of Retrovirology, WRAIR, Dec 10. ***"HIV Envelope and Virus Entry"***.

2000. Division of Viral Products, seminar series-CME credit approved, CBER, FDA, NIH, Bethesda, MD. February 24. ***"HIV-1 Envelope Glycoprotein: Receptor Interactions and Refined Subunit Immunogens"***.

2000. Center for Immunology & Microbial Disease, CME approved. Albany Medical College, Albany New York. March 27. ***"HIV-1 Envelope Glycoprotein-Receptor Interactions and new Subunit Immunogens"***.

2000. 2nd Frederick Workshop on the Cell Biology of Viral Entry. May 7-10, NCI-FCRDC, Frederick, MD, Invited Chair, Session I: ***"Virus-Receptor Interactions and Entry"***.

2001. Indiana University School of Medicine, Department of Microbiology, Indianapolis, IN, Mar 15. ***"Virus-Receptor Interactions: Tropism, Entry, and Refined Subunit Immunogens"***.

2001. Department of Microbiology, campus-wide seminar series-CME credit approved. University of Pennsylvania School of Medicine, Philadelphia, PA. October 3. ***"Functional and Structural Studies on Hendra and Nipah viruses - Newly Emerging and Highly Lethal Zoonotic Paramyxoviruses".***

2002. 2nd Collaborative Research Seminar on HIV and other Viral Entry Inhibitors. New York, NY. May 5. ***"Hendra and Nipah Viruses – Newly Emerging and Highly Lethal, Zoonotic Paramyxovirus Threats".***

2002. 3rd Frederick Workshop on the Cell Biology of Viral Entry. NCI-Frederick Cancer Research and Development Center, MD. May 7. ***"Hendra and Nipah Virus Envelope Glycoprotein-mediated Fusion.***

2002. Department of Microbiology and Immunology, Georgetown University, Washington, DC. Nov. 8. ***"Nipah and Hendra Viruses Emerging Zoonotic Paramyxovirus Threats".***

2002. Division of Viral Products, seminar series-CME approved, CBER, FDA, NIH, Bethesda, MD. Nov. 14. ***"Nipah and Hendra Viruses Emerging Zoonotic Paramyxovirus Threats".***

2003. 2003-Biodefense Vaccines, Therapeutics and Diagnostics: *Policy, Funding,* Development, Testing, Production, and Distribution. Biodefense Vaccines: The State of the Science. June 2-4, Washington, D.C. "***Hemorrhagic Fever and Emerging Viruses: Vaccines and Antiviral Agents".***

2003. NIH Research Festival. Mini-Symposia. Virus Entry – Virus Receptor Interactions. NIH, Bethesda, MD. Oct. 15. "***Nipah Virus and Hendra Virus Fusion, Entry, and its Inhibition".***

2003. Norman P. Salzman Fourth Annual Symposium in Virology: **Highly Pathogenic Viruses: Potential Agents of Bioterrorism.** FDA and the Foundation for the National Institutes of Health. ***"Nipah Virus and Hendra Virus: Emerging Zoonotic Paramyxovirus Threats".*** Nov 20, Cloisters Chapel, Building 60, NIH Campus, Bethesda, MD.

2003. 6th Asia Pacific Congress of Medical Virology. "***Nipah Virus and Hendra Virus Fusion, Entry, and its Inhibition".*** Dec. 6-10. Kuala Lumpur, Malaysia.

2004. USAMRIID, Fort Detrick, MD. Mar 9. **"*Nipah and Hendra: Emerging Viral Threats".***

2004. First Annual Regional Centers for Biodefense and Emerging Infectious Diseases Research meeting. Bethesda, MD. April 19-20. ***"Middle-Atlantic RCE Research Program 2: Emerging Viruses".***

2004. Department of Microbiology and Immunology, SUNY Upstate Medical University, Syracuse, New York. April 29. **"*Nipah and Hendra: Emerging Viral Threats".***

2004. 4th Frederick Workshop on the Cell Biology of Viral Entry. NCI-Frederick, MD. May 4. ***"A Soluble Hendra Virus Attachment Envelope Glycoprotein Blocks Fusion".***

2005. Second Annual Regional Centers for Biodefense and Emerging Infectious Diseases Research meeting. Galveston, TX. March 13-15. ***"Receptor Binding, Fusion Inhibition, and Induction of Cross-Reactive Neutralizing Antibodies by a Soluble G Glycoprotein of Hendra Virus".***

2005. 2005 ASM Biodefense Research: Symposium: Advances in Molecular Pathogenesis of Threat Agents Baltimore, MD. March 23. ***"Biology of Nipah and Hendra Viruses: Implications for Development of Vaccines and Therapeutics".***

2005. 2005-Biodefense Vaccines & Therapeutics Symposium: State of the Science. Arlington, VA. ***"Antibodies, Vaccines and Therapeutics for Emerging Virus Threats".***

2006. University of Virginia, Jan 10. Charlottesville**. *"Hendra and Nipah Viruses: Different and Dangerous"*.**

2006. Third Annual Regional Centers for Biodefense and Emerging Infectious Diseases Research meeting. New York City, NY. Mar 28. ***"A feline model of acute Nipah virus infection and protective vaccination with a soluble G glycoprotein".***

2006. University of Kentucky, Department of Molecular and Cellular Biochemistry *Lexington, Kentucky, Oct10.* ***"Hendra and Nipah viruses: From membrane fusion and receptors to potential therapeutic strategies"***

2006. Filoviruses: Recent Advances and Future Challenges: (ICID Global Symposia), Winnipeg, Canada. Sept 17-19. ***"Henipaviruses: From membrane fusion and receptors to Therapeutic Strategies".*** Plenary.

2006. 7th Asia Pacific Congress of Medical Virology. "***Nipah Virus and Hendra Virus Fusion, Entry, and its Inhibition".*** Nov 12-15. New Delhi, India. Plenary session.

2007. University of Texas Medical Branch. August 12. ***"The Envelope Glycoproteins of Hendra and Nipah viruses: Multifunctional molecules, vaccine immunogens and therapeutic targets***".

2007. University of Pittsburg, Center for Vaccine Research Seminar Series. September 26. "***The Envelope Glycoproteins of Hendra and Nipah Viruses: Multifunctional Molecules, Vaccine Immunogens and Therapeutic Targets".***

2007. University of Maryland, Department of Microbiology and Immunology. October 3. ***"The Envelope Glycoproteins of Hendra and Nipah viruses: Multifunctional molecules, vaccine immunogens and therapeutic targets".***

2008. The 3rd International Symposium of Emerging Viral Diseases. Oct 26-28. ***"Nipah and Hendra Virus Glycoproteins and Receptor Interactions."*** Plenary. Wuhan Institute of Virology, Chinese Academy of Sciences. Wuhan, China.

2008. American Society of Tropical Medicine and Hygiene (ASTMH) annual meeting. Session co-organizer and co-chair. Henipaviruses. ***"Nipah and Hendra Virus Receptor Binding and Entry."*** December 7-11. New Orleans, Louisiana.

2009. National Veterinary Stockpile Nipah virus Countermeasures Workshop; United States Department of Agriculture; (Australian Animal Health Laboratory, CSIRO, Geelong, Australia; March 17-19 2009). ***"Status of Vaccines and Therapeutic Countermeasures against Hendra and Nipah viruses."***

2009. Division of Viral Products Seminar Series-CME approved, March 26, CBER, FDA, NIH, Bethesda, MD. ***"Nipah and Hendra Virus Entry and New Animal Models of Infection and Pathogenesis."***

2009. NIH, Virology Interest Group seminar series. May 7th, NIH, Bethesda, MD. Nipah and Hendra Virus: ***"Receptor Binding and Entry, and New Animal Models of Infection."***

2009. WHO/FAO/OIE Workshop on Henipaviruses and Ebola-Reston Virus. Twin Waters, Queensland, Australia, Oct. 12-16, 2009 ***"Status of Vaccines and Therapeutic Countermeasures against Hendra and Nipah viruses."***

2009. Penn State, Bortree Lecture Series, October 7, ***"Nipah and Hendra viruses: From Receptor Binding and Entry to New Animal Models of Infection".***

2009. Juniata College, Huntingdon, PA. Oct 8, ***"Emerging Infectious Diseases: Graduate Education, USU".***

2009. New England Regional Center of Excellence in Biodefense and Emerging Infectious Diseases (NERCE-BEID) Workshop on Primate Infectious Diseases. Oct 28, ***"Hendra and Nipah virus –Therapeutics and new Primate Models".***

2009. Chemical, Biological, Radiological and Nuclear Countermeasures seminar series. BARDA, HHS, Nov. 17. ***"Vaccines and Therapeutic Countermeasures against Hendra and Nipah viruses".***

2009. IBC's 7th Annual International Conference: Antibody Therapeutics, San Diego, CA, Dec 8-10. ***"A Neutralizing Human Monoclonal Antibody Therapy for Nipah and Hendra Virus Infection".***

2010. Department of Microbiology and Immunology, Uni. of Illinois at Chicago, Mar 15. ***"Nipah and Hendra viruses: Receptor Binding and Virus Entry Studies Lead to New Therapeutics and Animal Models".***

2010. Dept of Pediatrics and Dept of Micro&Immun, Emory University School of Medicine, Atlanta, GA, Mar22. ***"Nipah and Hendra viruses: Studies on Receptor Binding and Entry Lead to New Therapeutics and Animal Models".***

2010. Dep. of Molecular and Microbiology, National Center for Biodefense & Infectious Diseases, George Mason University, VA. ***"New Animal Models and Countermeasures against Nipah and Hendra Virus".***

2010. 4th International Symposium of Emerging Viral Diseases. Oct 26-28. ***"Nipah and Hendra viruses: Studies on Receptor Binding and Entry Lead to New Therapeutics and Animal Models"*** Plenary. Wuhan Institute of Virology, Chinese Academy of Sciences. Wuhan, China.

2010. The 2nd International Conference on Infections of the Nervous System. Dec 2-6. ***"The New Non-Human Primate and Ferret Models for Nipah and Hendra Virus Pathogenesis and the Evaluation of Vaccine and Therapeutic Countermeasures"*** Plenary. St. Denis, Reunion Island.

2011. National Cancer Institute, Antibody Interest Group Seminar Series. NIH. Feb 25. ***"A Cross-Reactive Human Monoclonal Antibody Therapeutic for Hendra Virus and Nipah Virus".***

2011. 14th Annual Conference on Vaccine Research; the National Foundation for Infectious Diseases. May 16 ***"Bridging Animal and Human Health in the Search for Countermeasures for Henipaviruses"*** Plenary. Baltimore, MD.

2011. Colloquium Series on Infectious Disease; Institute for Infectious Diseases and Zoonoses, University of Munich Ludwig-Maximilians-Universität, June 1. ***"Bridging Animal & Human Health in the Development of Vaccines & Therapeutics against Hendra and Nipah Virus"***, Munich, Germany.

2011. Canadian Science Centre for Human and Animal Health. ***"Bridging Human and Animal Health in Developing Henipavirus Countermeasures"***. Oct 4. National Microbiology Lab, Public Health Agency of Canada, Winnipeg.

2012. Informa's Empowered Antibodies Congress 2012. June 13. ***"Successful Recombinant Human Monoclonal Antibody Therapy against Nipah and Hendra Virus Disease"***, Berlin, Germany.

2012. Vaccines and Diagnostics for Transboundary Animal Diseases," Sept 17-19, ***"Status of Passive and Active Vaccination Strategies against Hendra and Nipah viruses"***, Center for Food Security and Public Health, College of Veterinary Medicine, Iowa State University, Ames, Iowa.

2012. Infectious Disease & Immunity Colloquium, Sept 25 ***"Henipavirus Envelope Glycoproteins: Structural Studies and Ephrin Receptor Mediated Entry"***, Center for Biodefense and Emerging Infectious Diseases, UTMB, Galveston.

2012. Seminars at Huazhong Agricultural University, Oct 24, ***"The Present Status of Passive and Active Vaccination against Hendra and Nipah viruses"***, Wuhan, China.

2012. 5th International Symp.on Emerging Viral Diseases, Oct 25, ***"Henipavirus Envelope Glycoproteins: Structural Studies and Ephrin Mediated Entry"***. Plenary. Wuhan Institute of Virology, Chinese Academy of Sciences. Wuhan, China.

2012. 1st Ann. Host Pathogen Interactions in Biodefense and Emerging Infectious Disease, Nov 13, ***"Equine Hendra vaccine on the market-and a human monoclonal antibody therapy against Hendra and Nipah virus progresses further"***. George Mason Uni. VA.

2013. Annual meeting, Association of Medical School Microbiology and Immunology Chairs (AMSMIC), Jan 24, ***"Hendra and Nipah viruses: From Discovery to a Vaccine (From Bench to Bed- and Paddock-side)"***. Marco Island, FL.

2013. 11th ASM Biodefense and Emerging Diseases Research, ***"Tackling the Henipavirus Transboundary Threats by***

Passive and Active Immunization Approaches" Feb 25-27, Washington, DC.

2013. 3rd annual Sidney Pestka Lecturer, Annual Philadelphia Infection and Immunity Forum. ***"Immunization approaches succeed against the transboundary Hendra and Nipah virus threats"*** May 10.

2013. Program in Emerging Infectious Disease, Duke-NUS Medical School, Singapore. ***"Henipavirus Envelope Glycoproteins and Receptor Interactions: Structure, Function, and Therapeutic Targets"*** Jul5.

2013. Ruijin Hospital, School of Medicine Shanghai Jiao Tong University, Shanghai, China. ***"Passive and Active Immunization Approaches Succeed against the Nipah and Hendra virus Transboundary Threats"*** July 12.

2013. Infections of the Nervous System Pathogenesis and Worldwide Impact, Chinese University of Hong Kong. Gordon Research Conferences. *"**Combating the Hendra Virus and Nipah Virus encephalitic zoonoses by passive and active immunization"*** July 7-12.

2013. Basic Microbiology and Infectious Disease Training Program and the Department of Molecular Genetics & Microbiology Distinguished Lectures. University of Florida College of Medicine. Gainesville, FL. ***"Immunization strategies succeed against the transboundary Hendra and Nipah virus threats"*** Nov 25.

2014. The Hendra virus Team and Vaccine Retreat, Mornington Peninsula, Victoria, Australia. ***"From Virus Entry Studies to Hendra then Nipah then therapeutics-- What Next?"*** Feb 24.

2014. Division of Biotechnology Products CDER/FDA, Bethesda, MD. ***"Henipavirus Envelope Glycoproteins and Receptor Interactions: Structure, Function, and Countermeasure Targets"*** Apr 14.

2014. Informa; Antibodies Congress 2014: Recombinant and Bi-specific antibodies. Jun 18. ***"Combating the Hendra and Nipah virus emerging biothreats: A human monoclonal antibody therapy advances to clinical trial"***, Barcelona, Spain.

2014. 6th International Symposium on Emerging Viral Diseases, Oct 30, ***"Envelope glycoproteins of henipaviruses, Australian bat lyssavirus and rabies virus as targets of neutralizing human monoclonal antibodies"***. Wuhan Institute of Virology, Chinese Academy of Sciences. Wuhan, China.

2014. Norman P. Salzman 16th Annual Symposium in Virology. Foundation for the National Institutes of Health. *"**Next at Bat: A licensed vaccine and human monoclonal antibody therapy to combat the Hendra and Nipah virus Threats"*.** Nov 13, The Natcher Conference Center, Ruth Kirchstein Auditorium, NIH Campus, Bethesda, MD.

2014. ASTMH 63rd Annual Meeting Symposium: Bats and emerging viruses. ***"Development of the Hendra virus vaccine: A One-Health approach to Hendra virus control in Australia"*,** November 2-6, New Orleans.

2015. 18th Annual Conference on Vaccine Research; the National Foundation for Infectious Diseases. ***"Nipah virus and Hendra virus Animal Vaccines"*** April 13-15, Bethesda, MD.

2015. National Cancer Institute, National Institutes of Health, WebEx meeting, Apr 22. ***"A Human Monoclonal Antibody Therapy for People and a 'One Health' Vaccine for Horses as Countermeasures against the Hendra virus and Nipah Virus Threats"*** Bethesda, Maryland.

2016. Northwestern University Feinberg School of Medicine, Feb 8 ***"A 'One Health' Vaccine Approach and Human Antibody Therapy against Hendra and Nipah Viruses".*** Chicago.

2016. Division of Viral Products, CBER/FDA, June 23. ***"A human antibody therapy and a 'One Health' vaccine approach against Hendra virus and Nipah Virus"*** White Oak, MD.

2016. College of Pharmacy, Shandong University of Traditional Chinese Medicine. Oct 18. ***"Hendra Virus and Nipah Virus Active and Passive Vaccines"*,** Jinan, China.

2016. 7th International Symposium on Emerging Viral Diseases Oct 19-21, ***"Hendra and Nipah Virus: Passive and Active Vaccines"***, Wuhan Institute of Virology, Chinese Academy of Sciences. Wuhan, China.

2016. 4th International One Health Congress & 6th Biennial Congress of the International Association of Ecology and Health. Dec 3-7. Invited Keynote presentation: ***"A human antibody therapy and a 'One Health' vaccine approach against Hendra virus and Nipah Virus"***, Melbourne, Australia.

2017. Dept. of Tropical Medicine, Medical Microbiology and Pharmacology, University of Hawaii at Manoa; ***"A Vaccine and Therapy for Nipah virus and Hendra virus"***, October 10, 2017.

2017. NIAID, Division of Microbiology and Infectious Diseases, Rockville, MD; ***"A Nipah Virus and Hendra virus Vaccine and Therapy"***, October 19, 2017.

2018. Dept. Microbiology and Immunology, University of Illinois at Chicago; ***"Vaccines, Therapies and New Research Platforms for Nipah and Hendra Viruses"***, March 30, 2018.

2018. Aug 6-8, Indian Council for Medical Research / World Health Organization (ICMR-WHO): Workshop on Research

Roadmap for Nipah Virus Disease for India. ***"Monoclonal antibody therapeutic for Nipah and Hendra virus infection"***

2018. Aug 23, NIAID Technology Transfer Office, Rockville, MD. ***"Hendra-Nipah countermeasures"***.

2018. Oct 2-4, ***"Nipah Virus Returns: progress towards treatment and prevention"***. Infectious Disease Society of America (IDSA); IDWEEK-2018, San Francisco.

2018. Oct 18, ***"Nipah and Hendra Viruses: Countermeasures, New Tools and Surveillance"***. College of Pharmaceutical Science, Shandong University of Traditional Chinese Medicine, Jinan, China.

2018. Oct 21, ***"Nipah Virus Returns: progress towards treatment and prevention"***, 8th International Symposium on Emerging Viral Diseases, Wuhan, China.

2018. Oct 30, ***"Nipah and Hendra Viruses: Countermeasures, New Tools and Surveillance"***. Department of Microbiology, Perelman School of Medicine, University of Pennsylvania.

2019. Mar 4, ***"Vaccines and Therapeutics for Nipah Virus and Hendra Virus".*** Disease X: Advanced Diagnostics for Emerging Threats, Bangkok, Thailand.

2019. Mar 6, ***"An Antibody Therapeutic and a Nipah virus and Hendra Vaccine for Human Use"*** Thailand Ministry of Public Health (MOPH) – US, CDC, Bangkok.

2019. Apr 19, ***"An Antibody Therapeutic and a Vaccine for Nipah virus and Hendra virus"*** Cell Biology & Molecular Genetics Department, University of Maryland. College Park, MD.

2019. Apr 24, ***"Nipah and Hendra Viruses: Countermeasures, New Tools and Surveillance"*** COE seminar series FDA, White Oak Campus, MD.

2019. May 9, ***"Tackling Nipah and Hendra Virus: Countermeasures, New Tools, and Surveillance"*** WRAIR, Silver Spring, MD.

2019. Sept 25, ***"Bats, Pigs, Horses, and People...One Health Approaches against Nipah Virus and Hendra Virus"*** Department of Microbiology and Immunology, University of Maryland School of Medicine. Baltimore, MD.

2019. Oct 4, **"Bats, Pigs, Horses, Oh My! Battling emerging zoonotic viruses"** Notable Alumnus Lecture, University of Florida College of Medicine, Alumni Weekend. George T. Harrell, M.D., Medical Education Building. Gainesville, FL.

2019. Oct 31, ***"Bats, Horses, Pigs, People....One Health Countermeasures against Hendra and Nipah Viruses"*** World Vaccine Congress, Barcelona, Spain.

2019. Nov 18, ***"Bats, Horses, Pigs, and People....One Health Countermeasures against Nipah and Hendra Viruses"*** National Health Research Institutes (NHRI), Taiwan.

2019. Dec 10, ***"Monoclonal Antibody Countermeasures for Pathogenic Henipaviruses"*** Nipah virus international Conference; Nipah@20. Singapore.

2020. Oct 22, ***"A One-Health approach against Hendra Virus in Australia Leads to a Nipah virus vaccine for people"*** Department of Veterinary Medicine, VA-MD College of Veterinary Medicine, University of Maryland.

PUBLICATIONS: (total citations; >22,000; h-index, 68)

1. Lottenberg, R, **CC Broder**, and MDP Boyle. Identification of a Specific Receptor for Plasmin on a Group A Streptococcus. Infection and Immunity. 55(8):1914-1918, 1987.
2. **Broder, CC**, R Lottenberg, and MDP Boyle. Mapping of the Domain of Human Plasmin Recognized by its Unique Group A Streptococcal Receptor. Infection and Immunity. 57(9): 2597-2605, 1989.
3. Appelgate, ML, MM Moore, **CC Broder**, A Burrell, G Juhn, K.L. Kasweck, P-F Lin, A Wadhams, and JC Hoizer. Molecular Dissection of Mutations at the Heterozygous Thymidine Kinase Locus in Mouse Lymphoma Cells. Proc. Natl. Acad. Sci. USA. 87(1):51-55, 1990.
4. **Broder, CC**, R Lottenberg, GO vonMering, K Johnston and MDP Boyle. Isolation of a prokaryotic plasmin receptor: relationship to a plasminogen activator produced by the same microorganism. J. Biol. Chem. 266:4922-28, 1991.
5. McCoy, HE, **CC Broder**, and R Lottenberg. Strepokinases Produced by Pathogenic Group C Streptococci Demonstrate Species-Specific Plasminogen Activation. J. Infect. Dis. 164:515-521, 1991.
6. Lottenberg, R, **CC Broder**, MDP Boyle, SJ Kain, BL Schroeder, and R Curtiss III. Cloning, Sequence Analysis, and Expression in *Escherichia coli* of a Streptococcal Plasmin Receptor. J. Bacteriology. 174:5204-5210, 1992.
7. **Broder, CC**, DS Dimitrov, R Blumenthal, and EA Berger. The block to HIV-1 envelope glycoprotein-mediated membrane fusion in animal cells expressing human CD4 can be overcome by a human cell component(s). Virology. 193:483-491, 1993.
8. Dimitrov, DS, **CC Broder**, EA Berger, and R Blumenthal. Calcium Ions are required for Cell Fusion Mediated by the CD4-HIV-1 Envelope Interaction. J.Virol.67:1647-52, 1993.

9. **Broder, CC**, and EA Berger. CD4 Molecules with a Diversity of Mutations Encompassing the CDR3 Region Efficiently Support HIV-1 Env Glycoprotein-mediated Cell Fusion. J.Virol. 67:913-926, 1993.
10. Nussbaum, O, **CC Broder**, and EA Berger. HIV-1 Envelope Glycoprotein/CD4 Mediated Cell Fusion: A Novel Recombinant Vaccinia Virus-Based Assay Measuring Activation of a Reporter Gene by Bacterio-phage T7 RNA Polymerase Selectively In Fused Cells. J.Virol. 68:5411-5422, 1994.
11. **Broder, CC**, O Nussbaum, WG Gutheil, WW Bachovchin, and EA Berger. Evidence against CD26 Involvement in HIV-1 Envelope Glycoprotein/CD4-Mediated Cell Fusion. Science. 264:1156-1159, 1994.
12. **Broder, CC**, PL Earl, D Long, B Moss, and RW Doms. Antigenic Implications of HIV-1 Envelope Glycoprotein Quaternary Structure: Oligomer-Specific and -Sensitive mAbs. Proc Natl Acad Sci USA. 91:11699-11703, 1994.
13. **Broder, CC**, PE Kennedy, F Michaels, and EA Berger. Expression of Foreign Genes in Cultured Human Primary Macrophages Using Vaccinia Virus Vectors. Gene. 142:167-4, 1994.
14. Earl, PL, **CC Broder**, D Long, S Lee, J Peterson, S Chakrabarti, RW Doms and B Moss. Native oligomeric forms of HIV-1 envelope glycoprotein elicit a diverse array of mAb reactivities. J. Virol. 68: 3015-26, 1994.
15. Nussbaum, O, **CC Broder**, L Bar-Lev Stern, S Rozenblatt, B Moss, and EA Berger. Functional and Structural Interaction between Measles Virus Hemagglutinin and CD46. J. Virol. 69:3341-3349, 1995.
16. **Broder, CC** and EA Berger. Fusogenic Selectivity of the Envelope Glycoprotein is a Major Determinant of HIV-1 Tropism for CD4+ T-Cell Lines vs. Macrophages. Proc Natl Acad Sci USA. 92:9004-08, 1995.
17. Golding, H, DS Dimitrov, J Manischewitz, **CC Broder**, J Robinson, S Fabian, D Littman, and C Lapham. PMA-induced down modulation of tailless CD4 Receptors Requires Prior Binding of gp120 and Suggests a Role for Accessory Molecules. J.Virol. 69:6140-6148, 1995.
18. Richardson, TM, BL Stryjewski, **CC Broder**, JA Hoxie, JR Mascola, PL Earl, and RW Doms. The Humoral Response to Oligomeric HIV-1 Envelope Protein. J.Virol.70:753-62, 1996.
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ANNEXURE: 3-RV-1/2019 (SOP-IEC-KGMU)

ONE PAGE CV FOR NON-KGMU INVESTIGATORS

Broder	Christopher	C.
Date of Birth (b) (6) **):**		**Sex: Male**

Study Site Affiliation (Co-Investigator)

Professional Mailing Address	**Study Sited Address (Include institution name)**
Department of Microbiology and Immunology **Uniformed Services University,** (b) (6) **4301 Jones Bridge Rd, Bethesda, MD 20814-4799, USA**	
Telephone (office): (b) (6)	**Mobile Number:** (b) (6)
Telephone (Residence): Mobile	**Email:** (b) (6)

Academic Qualifications (Most current qualification first)

Degree / Certificate	Year	Institution, Country
BS, Biological Sciences	1983	Florida Institute of Technology, Melbourne, Florida, USA
MS, Molecular Biology	1985	Florida Institute of Technology, Melbourne, Florida, USA
PhD, Microbiology and Immunology	1989	University of Florida, Gainesville, Florida, USA

Current and Previous Relevant Positions Including Academic Appointments (Most current position first)

Month and Year	Title	Institution / Company, Country
August, 2018	Chair	Uniformed Services University, USA
January, 2005	Professor	Uniformed Services University, USA

Brief Summary of Relevant Clinical Research Experience:

I have been a in the area of enveloped virus-host cell interactions for over the past 32 years. I developed the first oligomeric HIV-1 gp140 envelope glycoprotein, established a vaccinia virus-based reporter gene assay for measuring viral glycoprotein-mediated membrane fusion, and defined the membrane fusion tropism of HIV-1 followed by the discovery of the HIV-1 coreceptors (CXCR4 and CCR5). In 1999, I established an international group of experts in Hendra virus and Nipah virus research. My lab's work includes the discovery of the entry receptors for Hendra and Nipah (ephrin-B2/B3), developed the feline, ferret and African green monkey models of Hendra and Nipah pathogenesis, the structural solutions of the F glycoprotein and the G-ephrin receptor interactions; the discovery and development of antiviral human monoclonal antibodies including one having a Phase I clinical trial completed in May, 2016, known as m102.4 that has been used by compassionate emergency protocol in 15 people in Australia and one in the United States because of significant exposure risk to Hendra or Nipah infection. I also developed the Hendra/Nipah subunit vaccine based on soluble Hendra G (HeV-sG); called Equivac® HeV (Zoetis, Inc.) which is the first commercialized vaccine to a BSL-4 agent. We have developed reverse genetics platforms for Australian bat lyssavirus and Cedar henipavirus that can be utilized at BSL-2. My lab is now doing virus surveillance using multiplex assays with native-like viral membrane proteins that measure antiviral humoral responses in animals and humans

Signature: (b) (6) **(Signature Re**	**Date:** (b) (6)

ANNEXURE: 3-RV-1/2019 (SOP-IEC-KGMU)

ONE PAGE CV FOR NON-KGMU INVESTIGATORS

Broder	Christopher	C.
Date of Birth (b) (6)):		**Sex: Male**

Study Site Affiliation (Co-Investigator)

Professional Mailing Address **Department of Microbiology and Immunology** **Uniformed Services University, (b) (6)** **4301 Jones Bridge Rd, Bethesda, MD 20814-4799, USA**	**Study Sited Address** **(Include institution name)**
Telephone (office): (b) (6)	**Mobile Number: (b) (6)**
Telephone (Residence): Mobile	**Email: (b) (6)**

Academic Qualifications (Most current qualification first)

Degree / Certificate	Year	Institution, Country
BS, Biological Sciences	**1983**	**Florida Institute of Technology, Melbourne, Florida, USA**
MS, Molecular Biology	**1985**	**Florida Institute of Technology, Melbourne, Florida, USA**
PhD, Microbiology and Immunology	**1989**	**University of Florida, Gainesville, Florida, USA**

Current and Previous Relevant Positions Including Academic Appointments (Most current position first)

Month and Year	Title	Institution / Company, Country
August, 2018	**Chair**	**Uniformed Services University, USA**
January, 2005	**Professor**	**Uniformed Services University, USA**

Brief Summary of Relevant Clinical Research Experience:

I have been a in the area of enveloped virus-host cell interactions for over the past 32 years. I developed the first oligomeric HIV-1 gp140 envelope glycoprotein, established a vaccinia virus-based reporter gene assay for measuring viral glycoprotein-mediated membrane fusion, and defined the membrane fusion tropism of HIV-1 followed by the discovery of the HIV-1 coreceptors (CXCR4 and CCR5). In 1999, I established an international group of experts in Hendra virus and Nipah virus research. My lab's work includes the discovery of the entry receptors for Hendra and Nipah (ephrin-B2/B3), developed the feline, ferret and African green monkey models of Hendra and Nipah pathogenesis, the structural solutions of the F glycoprotein and the G-ephrin receptor interactions; the discovery and development of antiviral human monoclonal antibodies including one having a Phase I clinical trial completed in May, 2016, known as m102.4 that has been used by compassionate emergency protocol in 15 people in Australia and one in the United States because of significant exposure risk to Hendra or Nipah infection. I also developed the Hendra/Nipah subunit vaccine based on soluble Hendra G (HeV-sG); called Equivac® HeV (Zoetis, Inc.) which is the first commercialized vaccine to a BSL-4 agent. We have developed reverse genetics platforms for Australian bat lyssavirus and Cedar henipavirus that can be utilized at BSL-2. My lab is now doing virus surveillance using multiplex assays with native-like viral membrane proteins that measure antiviral humoral responses in animals and humans

Signature: **(Signature Required)**	**Date:**

From: Broder, Christopher (b) (6)
To: (b) (6)
Subject: Re: DTRA India: CV and form needed for ethical approvals
Date: Wednesday, January 20, 2021 6:01:06 PM
Attachments: (b) (6) CV-full-01202021-Signed.pdf
Annexure 3_KGMU_IEC(b) (6) r-signed.pdf
Annexure 3_KGMU_IEC(b) (6) .docx

(b) (6)

not sure about what was needed in the last yellow box

On Wed, Jan 20, 2021 at 5:37 PM (b) (6) wrote:

> Hi (b) (6) ,
> A gentle reminder. I hope to move forward with the as soon as possible,
> Thanks,
> (b) (6)
>
> On Jan 14, 2021, at 3:24 PM, (b) (6) wrote:
>
> Hi (b) (6) ,
> Ethical Approval documents through King George Medical University require all listed Co-PI's of the DTRA-India project to:
>
> 1) Provide a signed copy of their CV
> 2) Fill a one-page summary CV (attached here in Word)
>
> Could you please provide these two docs to me in the next week, so we can move forward with this important step?
> Thanks, and please write me with questions or concerns,
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Complementary regulation of caspase-1 and IL-1β reveals additional mechanisms of dampened inflammation in bats

Geraldine Goh[a,1], Matae Ahn[a,1], Feng Zhu[a], Lim Beng Lee[a], Dahai Luo[b,c], Aaron T. Irving[a,d,2], and Lin-Fa Wang[a,e,2]

[a]Programme in Emerging Infectious Diseases, Duke–National University of Singapore Medical School, 169857, Singapore; [b]Lee Kong Chian School of Medicine, Nanyang Technological University, 636921, Singapore; [c]NTU Institute of Structural Biology, Nanyang Technological University, 636921, Singapore; [d]Zhejiang University–University of Edinburgh Institute, Zhejiang University School of Medicine, Zhejiang University International Campus, Haining, 314400, China; and [e]Singhealth Duke–NUS Global Health Institute, 169857, Singapore

Edited by Vishva M. Dixit, Genentech, San Francisco, CA, and approved September 14, 2020 (received for review February 21, 2020)

Bats have emerged as unique mammalian vectors harboring a diverse range of highly lethal zoonotic viruses with minimal clinical disease. Despite having sustained complete genomic loss of AIM2, regulation of the downstream inflammasome response in bats is unknown. AIM2 sensing of cytoplasmic DNA triggers ASC aggregation and recruits caspase-1, the central inflammasome effector enzyme, triggering cleavage of cytokines such as IL-1β and inducing GSDMD-mediated pyroptotic cell death. Restoration of AIM2 in bat cells led to intact ASC speck formation, but intriguingly resulted in a lack of caspase-1 or consequent IL-1β activation. We further identified two residues undergoing positive selection pressures in *Pteropus alecto* caspase-1 that abrogate its enzymatic function and are crucial in human caspase-1 activity. Functional analysis of another bat lineage revealed a targeted mechanism for loss of *Myotis davidii* IL-1β cleavage and elucidated an inverse complementary relationship between caspase-1 and IL-1β, resulting in overall diminished signaling across bats of both suborders. Thus we report strategies that additionally undermine downstream inflammasome signaling in bats, limiting an overactive immune response against pathogens while potentially producing an antiinflammatory state resistant to diseases such as atherosclerosis, aging, and neurodegeneration.

bats | AIM2 | caspase-1 | inflammasome | IL-1β

Bats are placental mammals which uniquely utilize powered flight for locomotion, harbor a diverse viral repertoire, and possess longevity exceptional to their body size. In recent years, bats have been implicated in major outbreaks caused by fatal zoonotic viruses, such as SARS-CoV and MERS-CoV, henipaviruses, filoviruses including Ebola and Marburg virus, and a high likelihood of the currently circulating SARS-CoV-2 (1–4). Ongoing outbreaks with significant mortality and morbidity in human and livestock have driven a targeted search for the originating hosts of these spillover pathogens and pivotal studies have identified bats as significant reservoir and ancestral hosts to more zoonotic diseases per species, against all other mammalian orders (2, 5). Of key interest is the bat innate and adaptive immune system, due to evolutionarily driven or yet undiscovered, altered interactions between the host–pathogen interface, leading to their tolerance of viral diseases.

Genomic and transcriptomic studies have identified disparities between bats and other mammals. Positive selection has been shown in critical innate immune, tumor suppressive, and DNA damage checkpoint genes of bats (6), including NLRP3, TP53, and ATM. Altered natural killer (NK) cell repertoires were found among *Pteropus alecto*, *Myotis davidii*, and *Rousettus aegyptiacus* bats, along with differential contraction of IFN-α genes and expansion of IFN-ω genes in *Pteropus*, *Myotis*, and *Rousettus* bat species (7, 8). Despite mounting genomic evidence that bats have unique alterations in innate immune pathways, experimental confirmation is rare. We recently demonstrated that NLRP3 is dampened in bats as a result of loss-of-function bat-specific isoforms and impaired transcriptional priming (9). The stimulator of IFN genes (STING), a key adaptor to the DNA-sensing cGAS protein, is also exclusively mutated at S358 in bats, resulting in a reduced IFN response to HSV1 (10). We previously reported a complete absence of Absent in melanoma 2 (AIM2)-like receptor (ALR) genes across all available bat genomes from both Yinpterochiroptera and Yangochiroptera suborders (11). As these modifications in bats signify shifts in cell signaling and immune regulation, we thus investigated the loss of the PYHIN or ALR gene family for implications on the bat DNA-sensing inflammasome response.

The ALRs are an essential group of germline-encoded pattern recognition receptors (PRRs) comprising 5 members in humans and 14 members in mice, with the most well studied being AIM2 (12, 13). AIM2 is the prototypical member of the ALR family and was shown to mediate intracellular dsDNA-responsive inflammasome signaling, typically of invading pathogenic origin or aberrant host cytosolic DNA (14, 15). There is extensive

Significance

Bats have been shown to dampen several key upstream pathogen and danger-associated molecular patterns, yet much of the downstream signaling is yet unknown. Here, we identify residues in caspase-1 which are critical for enzymatic activity and have been targeted for inhibition in *Pteropus* bats. Further, we discover cleavage-site flanking residues which lead to loss of IL-1β cleavage in *Myotis* bats. Thus, we report an inverse relationship between caspase-1 function and IL-1β cleavage, resulting in a consistent reduction of downstream signaling by the inflammasome across bats within the two suborders. In sum, we confirm that bats have targeted the inflammasome pathway at multiple levels and via heterogeneous strategies to reduce proinflammatory responses, thus mitigating potential immune-mediated tissue damage and disease.

Author contributions: G.G., M.A., A.T.I., and L.-F.W. conceived the study; M.A., A.T.I., and L.-F.W. provided resources and materials; G.G., L.B.L., and M.A. performed experiments; G.G., M.A., F.Z., and D.L. analyzed the data; and G.G., M.A., D.L., A.T.I., and L.-F.W. wrote the manuscript with input from all authors.

The authors declare no competing interest.

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[1]G.G. and M.A. contributed equally to this work.

[2]To whom correspondence may be addressed. Email: aaronirving@intl.zju.edu.cn or linfa.wang@duke-nus.edu.sg.

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diversity across mammalian ALR families and AIM2 is the only member with preserved orthology among species (12, 16). Upon recognizing exposed intracellular DNA, it binds to the major turn of the DNA helix and recruits its adaptor apoptosis-associated speck-like protein containing a CARD (ASC), which forms aggregates (ASC specks) to complex with downstream procaspase-1 (15, 17). The caspase-1 proenzyme undergoes homodimerization and autocleavage for activation, conferring it the ability to bind, cleave, and mature IL-1β, IL-18, and gasdermin D (GSDMD) and trigger pyroptotic cell death (18–22). The mammalian AIM2 is crucial in its role in sensing intracellular foreign DNA, accompanied by a potential for either a pathological or protective inflammatory response in the host (23–25). Yet the consequence of its absence in bats, a unique animal model shown to down-regulate components of its inflammasome pathway, is poorly understood.

Caspase-1, or cysteine aspartic protease 1, is the central inflammasome effector for pyroptosis and cytokine secretion, playing roles in diverse cellular processes, including apoptosis/necrosis, metabolism, mitophagy, and autophagy (26–29). The propeptide consists of a caspase-recruitment domain (CARD), a p20 and p10 polypeptide sequence, and undergoes sequential autoproteolysis at aspartic acid residues into p20/p10 subunits which dimerize to achieve the activated conformation for substrate binding and cleavage (18, 21, 30). It is converged upon by all canonical inflammasome receptors, including NLRP3, NAIP/NLRC4, NLRP1, AIM2, and pyrin, and many other members of the NLR family such as NLRP6, NLRP7, and NLRP12, mediating critical proinflammatory host responses against microbes or autoimmune and autoinflammatory sequelae (31). Further, it is involved in multiple age-related diseases, including amyloid β accumulation in Alzheimer's disease and cardiac injury during acute myocardial infarction (32, 33). While intensive study of human caspase-1 inhibitors are currently ongoing, given its therapeutic potential, still little is known about the downstream activation of caspase-1 in bats, especially given their altered immune landscape and dampened inflammasome function.

Here, we confirm that genomic loss of AIM2 in bats dismantles the inflammasome adaptor recruitment responsive to dsDNA. Additionally, reconstitution of the human gene in a bat in vitro environment is sufficient to partly restore this intracellular pathway up to ASC level. However, we discovered an absence of the downstream cytokine release or cell-death initiation despite robust ASC speck formation in bat primary macrophages. We identify key residues in the bat caspase-1 responsible for dampened IL-1β cleavage, or altered IL-1β cleavage sites which significantly reduce its processing and maturation in bats. Importantly, we have elucidated multiple levels of disengagement within the bat inflammasome pathway with key implications in their response toward cellular stress, inflammation, and pathogenic detection.

Results

Absence of ASC Speck Induced by DNA Stimulation in Bat Kidney and Immune Cells Is Restored by Human AIM2. Given the ALR family members, including AIM2, are the only DNA sensors mediating the intracellular sensing of pathogenic and aberrant host DNA to activate the inflammasome, we hypothesized that absence of all ALR genes in bats would result in the inability for bats to trigger inflammasome signaling. Indeed, with exogenous dsDNA ligand PolydA:dT stimulation of bat bone-marrow-derived macrophages (BMDMs), we observed a lack of recruitment of ASC into aggregates (ASC speck), which could be seen in dsDNA-treated murine BMDMs (Fig. 1*A*). Further, using high-throughput image-based flow cytometry (Imagestream), we observed that the cytosolic ASC remained diffusely distributed in bat cells, unlike their aggregation into a speck-like morphology in mouse macrophages (Fig. 1*B*).

To investigate whether lack of ASC speck formation was attributable to the absence of gene and protein expression from the ALR family, we generated a human AIM2-mCitrine fusion construct cloned into a mammalian expression vector and rescued the gene in *Pteropus alecto* kidney-derived (PaKiS) immortalized cells (34). We selected the human AIM2 protein, as AIM2 is the only ALR gene with conserved evolutionary and functional orthology across species (16) and the human ortholog is closest in homology to the only nonfunctional PYHIN peptide fragment identified in bats (*Pteronotus parnelli*) (11). Transient overexpression of human AIM2 alone was sufficient to restore ASC speck formation in PaKiS cells stably expressing bat ASC (Fig. 1*C* and *SI Appendix*, Fig. S1 *A* and *B*), resulting in organization into a perinuclear inflammasome complex with colocalization of AIM2 and downstream ASC (Fig. 1*D*). To evaluate the activation in response to intracellular DNA, lentiviral delivery was used to generate PaKiS cells stably expressing both human AIM2 and bat ASC at low copy number. ASC speck formation was induced with addition of PolydA:dT DNA ligand and increased in a dose-dependent manner in AIM2-positive cells only (12-fold increase at 2.0 μg/mL compared to 0 μg/mL) (Fig. 1*E*). This was accompanied by increasing detection of AIM2 oligomerization (15-fold), signaling intact sensing of dsDNA by AIM2 and consequent formation of the inflammasome recruitment platform (Fig. 1*F*). Importantly, interaction of the adaptor ASC with the AIM2 sensor demonstrates the highly conserved nature of bat ASC to retain ability for recruitment to oligomerized human AIM2. This supports our previous observation whereby bat ASC is also conserved with the human ASC in its function and speck properties, including size, density, and shape in response to NLRP3 (9).

Human AIM2 Restores ASC Speck Formation but Not Caspase-1 Activation or IL-1β Release in Bat Macrophages. As the *P. alecto* kidney in vitro immortalized cell system lacks classic inflammasome machinery, we examined primary in vitro differentiated bat BMDMs for activation of the inflammasome as a consequence of AIM2 restoration. Transduction of lentivirus carrying a control vector or human AIM2 was performed in *P. alecto* BMDMs (PaBMDMs), and ASC speck formation was similarly quantified by Imagestream. Only AIM2 reconstituted ($AIM2^+$) bat macrophages treated with PolydA:dT were able to induce endogenous bat ASC aggregation, whereas minimal induction was observed in the mock (vehicle)-treated control vector or $AIM2^+$, or PolydA:dT-treated control vector, conditions (Fig. 2*A*). Imagestream analysis supported this finding with visible ASC speck in PolydA:dT-treated $AIM2^+$ BMDMs compared to vehicle-only (mock) controls (Fig. 2*B*). This suggested AIM2-dependent restoration of ASC speck induction in response to the DNA ligands.

Thus, we next looked for downstream IL-1β cleavage or induction of pyroptosis. Unexpectedly, mature IL-1β was unable to be detected in the supernatant despite ASC speck induction in DNA-treated AIM2-reconstituted bat BMDMs, in contrast to the mouse BMDMs (Fig. 2*C*). This was accompanied by minimal lytic cell death and low lactate dehydrogenase (LDH) activity levels in the cell supernatant (Fig. 2*D*). To measure the caspase-1 activity specifically in bat BMDMs, we utilized the 660-YVAD-fmk fluorescent-labeled inhibitor of caspase activation (FLICA) assay with relative specificity for the caspase-1 active site. We observed lack of substrate binding even in DNA-treated and $AIM2^+$ bat BMDMs, compared to the robust activity in treated mouse BMDMs which possess endogenous AIM2 (Fig. 2*E*). As a previous study similarly identified minimal secretion of IL-1β upon NLRP3 inflammasome activation in bat primary immune cells, we decided to investigate the downstream convergence of both sensing platforms onto caspase-1.

Failure in IL-1β Production Is Due to Substitution of Two Residues in *P. alecto* Bat Caspase-1. Lack of downstream activation in the presence of ASC specks can be attributed to either caspase-1, or

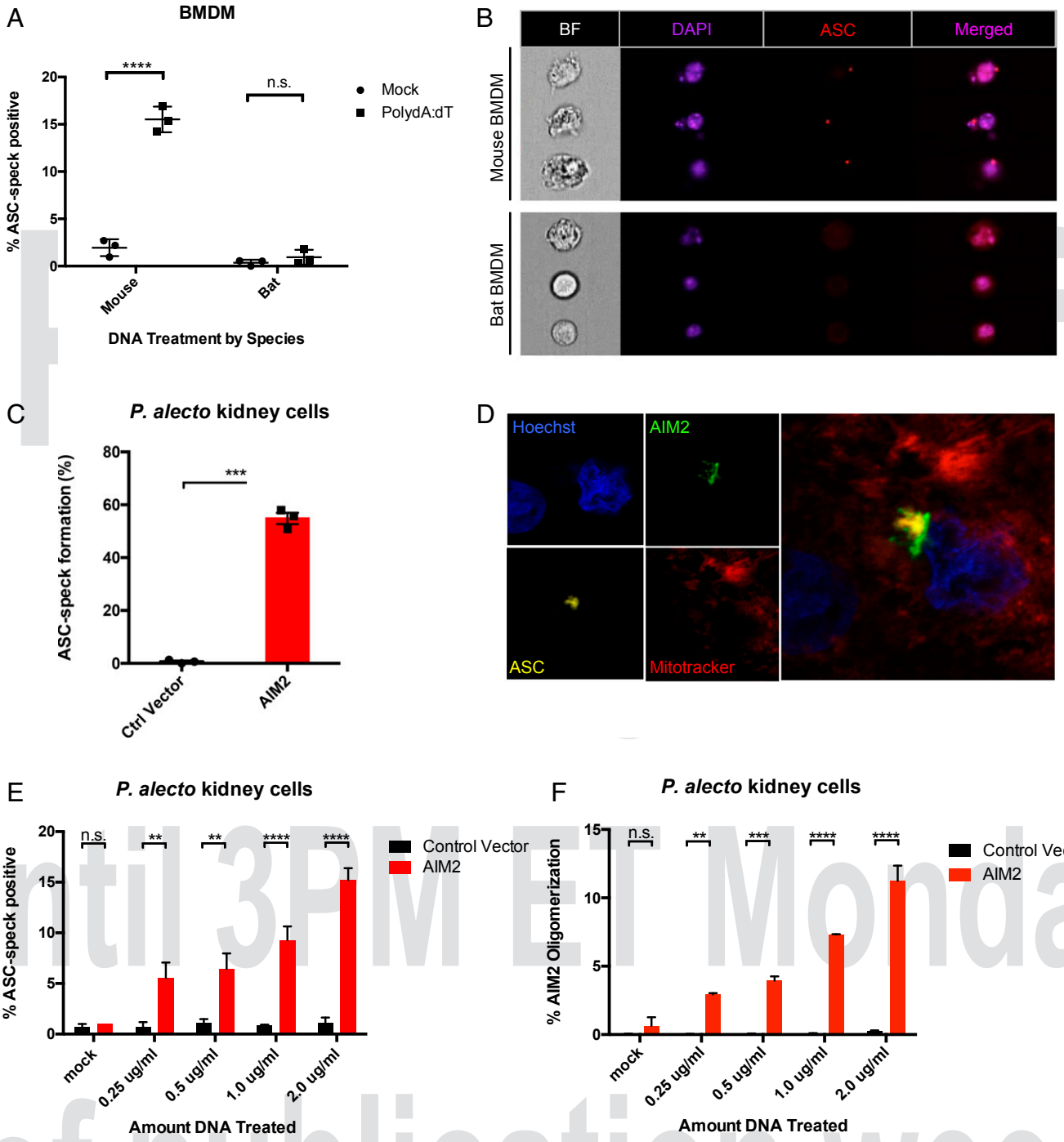

Fig. 1. Reconstitution of AIM2 restores DNA-triggered ASC speck in bat cells. (*A*) Measurement of ASC speck formation in mouse and bat BMDMs treated with either vehicle (mock) or transfected dsDNA (PolydA:dT, 1 μg/mL) for 4 h after 3 h LPS (mouse) or CL264 (bat) priming. (*B*) Single-cell imaging of mouse or bat BMDMs collected on Imagestream to visualize ASC speck aggregation or diffuse intracellular distribution, shown as bright field (BF), DAPI, and ASC signals. (*C*) ASC speck formation was quantified on Imagestream flow cytometry in *P. alecto* kidney cells (PaKiS) stably expressing bat ASC-mPlum and transiently expressing human AIM2. (*D*) Single-plane confocal imaging of transfected PaKiS cells showing ASC speck aggregation and association with cotransfected AIM2. Hoechst 33342 nuclear staining (blue), AIM2-mCitrine (green), ASC-mPlum (yellow), and Mitotracker (red). (*E*) Retroviral transduction of control vector or AIM2 was performed in ASC-mPlum stably expressing PaKiS cells, and DNA transfected in a dose-curve (mock, 0.25 to 2.0 μg/mL PolydA:dT). Imagestream flow cytometry was performed for triggered ASC speck. (*F*) AIM2 oligomerization induced in a dose-dependent manner quantified by Imagestream. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$, n.s., nonsignificant; linear regression and two-tailed unpaired *t* test. Data are presented as mean ± SEM of three biological replicates (*A* and *B*) or three independent experiments (*C*–*F*).

IL-1β, or both. To dissect the mechanism, we reconstituted the entire AIM2 inflammasome axis in human embryonic kidney (HEK293T) cells, coexpressing AIM2, ASC, caspase-1, and IL-1β. Human genes were used for the upstream components (AIM2 and ASC) to standardize the system in a human cell line. Either control (empty vector), human, or *P. alecto* caspase-1 (HsCASP1, PaCASP1) proteins were expressed in increasing concentrations and the cell lysates were immunoblotted for

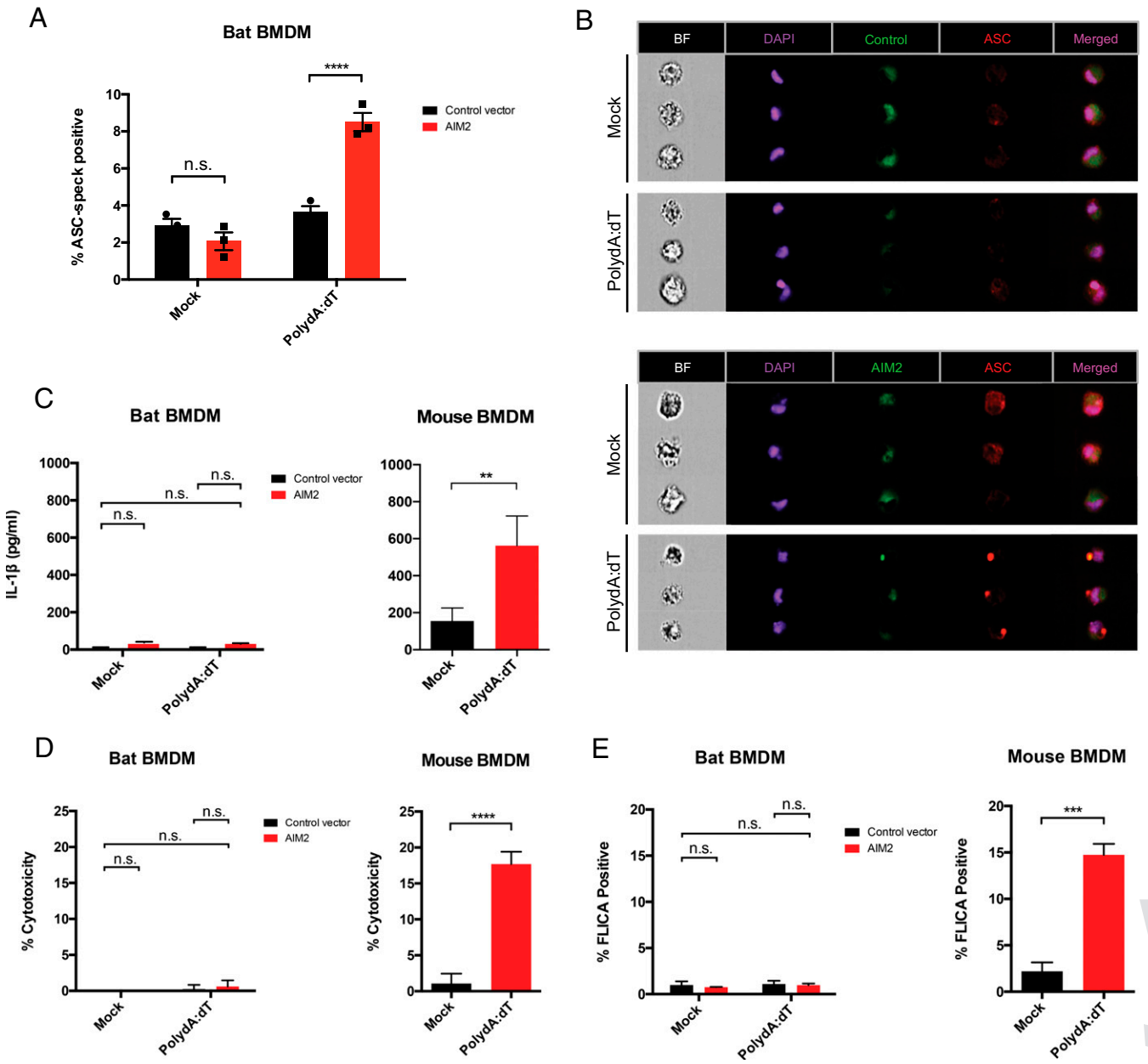

Fig. 2. Lack of caspase-1 or IL-1β signaling despite ASC speck restoration in AIM2-reconstituted bat macrophages. (*A*) Mouse or *P. alecto* BMDMs (PaBMDM) were differentiated in CSF-1 for 5 d and transduced with AIM2 or control vector lentivirus. At 48 h posttransduction, macrophages were primed with CL264 or LPS and treated with vehicle (mock) or DNA (PolydA:dT, 1 μg/mL) for 4 h and ASC speck was quantified via Imagestream. (*B*) Representative images for PaBMDMs stained for DAPI and anti-PaASC, shown is HsAIM2-mCitrine reconstitution and PaASC speck formation with either mock or PolydA:dT treatment. (*C*) LDH assay was performed on supernatant to measure cytolytic cell death in PolydA:dT-treated bat control or AIM2$^+$ BMDM (*Left*) or mouse BMDM (*Right*). (*D*) Similarly, IL-1β secretion was quantified in the supernatant of treated bat and mouse BMDMs using in-house bat ELISA protocol as previously published (14) and mouse IL-1β ELISA kit. (*E*) Fluorescent-labeled inhibitor of caspase activation (FLICA) assay was performed on bat and mouse BMDMs for caspase-1 activation upon DNA treatment, with staining for 1 h and flow cytometry analysis. Statistical analysis was performed using two-way ANOVA with Bonferroni's multiple comparisons test (*A* and *C*–*E*) and two-tailed unpaired *t* test (*C*–*E*). $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$, n.s., nonsignificant. Data are representative of three biological replicates ($n = 3$) in *B* or mean ± SEM of three biological replicates ($n = 3$) in (*A*, *C*, and *E*).

cleaved IL-1β p17, representing the product of inflammasome activation. Notably, there was minimal detection of mature bat IL-1β p17 in the cell lysates despite increasing expression of PaCASP1 (Fig. 3*A*). In contrast, human caspase-1 showed robust cleavage of bat IL-1β, suggesting intact cleavage of bat IL-1β. We observed an overall decrease in pro-PaCASP1 expression in contrast to human protein expression, and an absence of PaCASP1 p32 or other intermediate self-cleavage products. Importantly, a clear reduction in PaCASP1 activity compared to HsCASP1 was observed, which suggested decreased functionality of bat caspase-1.

To confirm the loss of caspase-1 activity, we interrogated PaCASP1 for intact activation by FLICA assay with specificity to caspase-1 to detect for active site binding. Similarly, although HsCASP1 exhibited 10-fold times higher FLICA substrate retention and fluorescence (11.03% ± 0.201), PaCASP1 had minimal detection in activity (2.855% ± 0.991), with levels similar to the control vector (1.58% ± 0.218) (Fig. 3 *B* and *C*). To

further confirm that loss of inflammasome signaling was at the bat caspase-1 and not IL-1β level, we paired expression of either HsCASP1 or PaCASP1 with human or bat IL-1β. Assay of the lysates for either HsIL-1β or PaIL-1β indicated that HsCASP1, but not PaCASP1, was able to successfully cleave the IL-1β from both species (Fig. 3*D*). Altogether, this indicates that caspase-1, and not IL-1β, is dampened in the *Pteropus* bat inflammasome pathway, resulting in failure of IL-1β maturation and cleavage.

Structural studies in human caspase-1 have identified C285 and H237 as essential residues for catalytic activity, among others which form the substrate-binding active site (35, 36). Additional residues within the p10 and p20 fragments have been shown to mediate crucial interactions in dimerization, and mutations result in inability to bind and cleave cytokines for secretion (37). A recent study established that homodimerization of two p20/p10 subunits forms the active conformation of caspase-1, whereby autoprocessing of the caspase-1 p10 fragment is required for GSDMD cleavage and initiation of pyroptosis (22). Given the existing understanding of caspase-1 activation, we analyzed the caspase-1 gene sequence to determine the potential mechanism of its reduced activity in bats. Sequences of available bat caspase-1 genes across Yinpterochiroptera and Yangochiroptera suborders, and 10 nonbat mammalian species were aligned, and phylogenetic analysis by maximum likelihood (PAML) performed to identify lineages and sites acted on by selection pressures (38) (*SI Appendix*, Fig. S2*A*). We found one ancestral branch of the bats to have undergone positive selection pressures, and branch-site testing identified greater positive selection pressure exerted on two residue sites in its subsequent branch (*SI Appendix*, Fig. S2*B*). Compared to human, mouse, and other included species, the *P. alecto* and *Pteropus vampyrus* caspase-1 sequence showed alterations at residue 365 from Asp to Asn, and at 371 from Arg to Gln (red boxes). Both residues are localized within the p10 polypeptide and span five amino acids apart.

To understand the consequence of these substitutions, we performed site-directed reverse mutagenesis of the specific sites, replacing either one, or both residues in the bat with the equivalent human residues, resulting in N365D-only, Q371R-only, or double-mutant (DM) PaCASP1 (Fig. 3*E*). Likewise, the human HsCASP1 gene was mutated to individually or simultaneously replace both residues with those of *Pteropus* bat amino acid sites (D365N-only, R371Q-only, and HsCasp1 DM). Only the PaCASP1 DM, but not the single mutants, provoked reversal of the inactive PaCASP1 phenotype to rescue cleavage of IL-1β (Fig. 3*F*). Conversely for HsCASP1, single mutation of either site to the bat residues was sufficient to abrogate its ability to cleave IL-1β (Fig. 3*G*). Further, HEK293T cells expressing either HsCASP1 WT or mutant PaCASP1 DM showed increased cellular stress and death morphologically, while HsCASP1 DM and PaCASP1 WT exhibited increased viability regardless of ASC speck formation (*SI Appendix*, Fig. S3).

Bats also possess the GSDMD gene with conserved pore-forming N-terminal subunits and a caspase-1 recognition site $_{266}$FLSD$_{269}$; yet its function and cleavage potential by bat caspase-1 are still unknown. Thus, PaGSDMD was cloned and compared with HsGSDMD for cleavage by human and *P. alecto* wild-type (WT) or mutant caspase-1 variants. Similar to the pattern of IL-1β processing by PaCASP1, cleavage of PaGSDMD was undetected in WT PaCASP1, while PaCASP1-DM successfully restored cleavage as observed by the 31-kDa PaGSDMD-N fragment (Fig. 3*H*). Conversely, HsCASP1-D365N, R371Q, or the combined HsCASP1-DM led to abrogration of GSDMD cleavage (Fig. 3*I*). To provide the molecular basis of the loss-of-function mutations of D365N and R371Q, we examined the structure of human caspase-1 bound to GSDMD (Protein Data Bank [PDB] code: 6VIE) (39). In the substrate-bound caspase-1 state (p20/p10 dimer), R371 participates in electrostatic interactions with E367 of the opposite p10 subunit, therefore R371Q mutation results in unfavorable interaction across the interface (*SI Appendix*, Fig. S4). D365 forms part of the dimer interface and may stabilize the homodimeric interactions. As such, similar to R371Q, D365N mutation would weaken the caspase 1 dimer–dimer coordination, leading to inactivation of the enzyme (22, 39, 40). Taken together, our findings demonstrate that both sites in the p10 fragment are necessary for caspase-1 activation by the inflammasome complex, and both IL-1β and GSDMD substrate maturation is dampened in *P. alecto* bats in a caspase-1-dependent manner.

Complementation between Caspase-1 Activity and IL-1β Cleavage Results in Consistent Inflammasome Dampening across Bats. Bats, belonging to the order Chiroptera, are the second largest group of mammals with more than 1,000 species. Within the order Chiroptera, *Pteropus* bats are part of the Pteropodidae family in the suborder Yinpterochiroptera, which are distinct from the suborder Yangochiroptera, containing the rest of the microbat families (41, 42). To better understand if downstream inflammasome dampening is a consistent pattern across bats, we extended our study to include both the *Eonycteris spelaea* bat (cave nectar bat), also from Yinpterochiroptera, and the *M. davidii* (David's myotis) species from the Yangochiroptera suborder. Due to the ability of caspase-1 to signal via multiple upstream sensors, we reconstituted the NLRP3 inflammasome axis in HEK293T cells and expressed either human or relevant species of bat caspase-1 in a dose-dependent manner. We observed that *E. spelaea* caspase-1 (EsCASP1) retained the ability to cleave *P. alecto* IL-1β at reduced levels, and *M. davidii* caspase-1 (MdCASP1) demonstrated intact activity comparable to that of human at low-dose expression (Fig. 4*A*).

Next, we reconstituted the NLRP3 inflammasome axis and varied IL-1β of three different bat species in either a HsCASP1, EsCASP1, or MdCASP1 system (Fig. 4*B* and *SI Appendix*, Fig. S5*A*). Interestingly, we observed that *M. davidii* IL-1β possessed the least capacity for cleavage and *P. alecto* the highest, in contrast to their respective caspase-1 activity (highest in *M. davidii* and lowest in *P. alecto*). We hypothesized that efficient targeting of MdIL-1β for reduced cleavage may occur at or near its cleavage site. Therefore, we aligned the IL-1β amino acid sequence of *M. davidii* against eight other species of bats (*Myotis lucifugus*, *Eptesicus fuscus*, *Miniopterus natalensis*, *Desmodus rotundus*, *P. alecto*, and *P. vampyrus*, *R. aegyptiacus*, *Hipposideros armiger*) and six other model mammalian species (*Sus scrofa*, *Canis lupus*, *Pan troglodytes*, *Mus musculus*, and *Rattus norvegicus*) (*SI Appendix*, Fig. S5*B*). Among the 15 total species analyzed using branch site modeling in PAML, we discovered a Ser117 residue immediately adjacent to the cleavage site Asp^{115}-Ala^{116} which was under higher selection in the *Myotis* branch, which was a proline residue in all except three other species (*M. natalensis*, *S. scrofa*, and *C. lupus*).

We next hypothesized that residues conserved between *Homo sapiens*, *P. alecto*, and *E. spelaea* but differing in *M. davidii* may be responsible for the impaired cleavability of MdIL-1β. We thus additionally identified G110, S111, E113, and Q122 in MdIL-1β as distinct from equivalent IL-1β residues of the other three species. For a direct comparison of MdIL-1β with PaIL-1β (which is fully cleavable), we performed site-directed mutagenesis replacing each of the respective MdIL-1β residues into their *P. alecto* counterparts, along with a combined mutation containing all amino acid site substitutions (Fig. 4*C*). Notably, the MdIL-1β mutant S117P was able to strongly restore IL-1β cleavage, along with a partial restoration by a double mutant GS110/111DG; however, the combined mutant demonstrated strongest cleavage ability (Md > Pa 110 to 112) (Fig. 4*D*). Conversely, the PaIL-1β mutants DG110/111GS and P117S resulted in defective IL-1β cleavage (Fig. 4*E*). Further, both residues appear to have a partial effect either in restoration (MdIL-1β) or abrogation (PaIL-1β) of function, whereby the

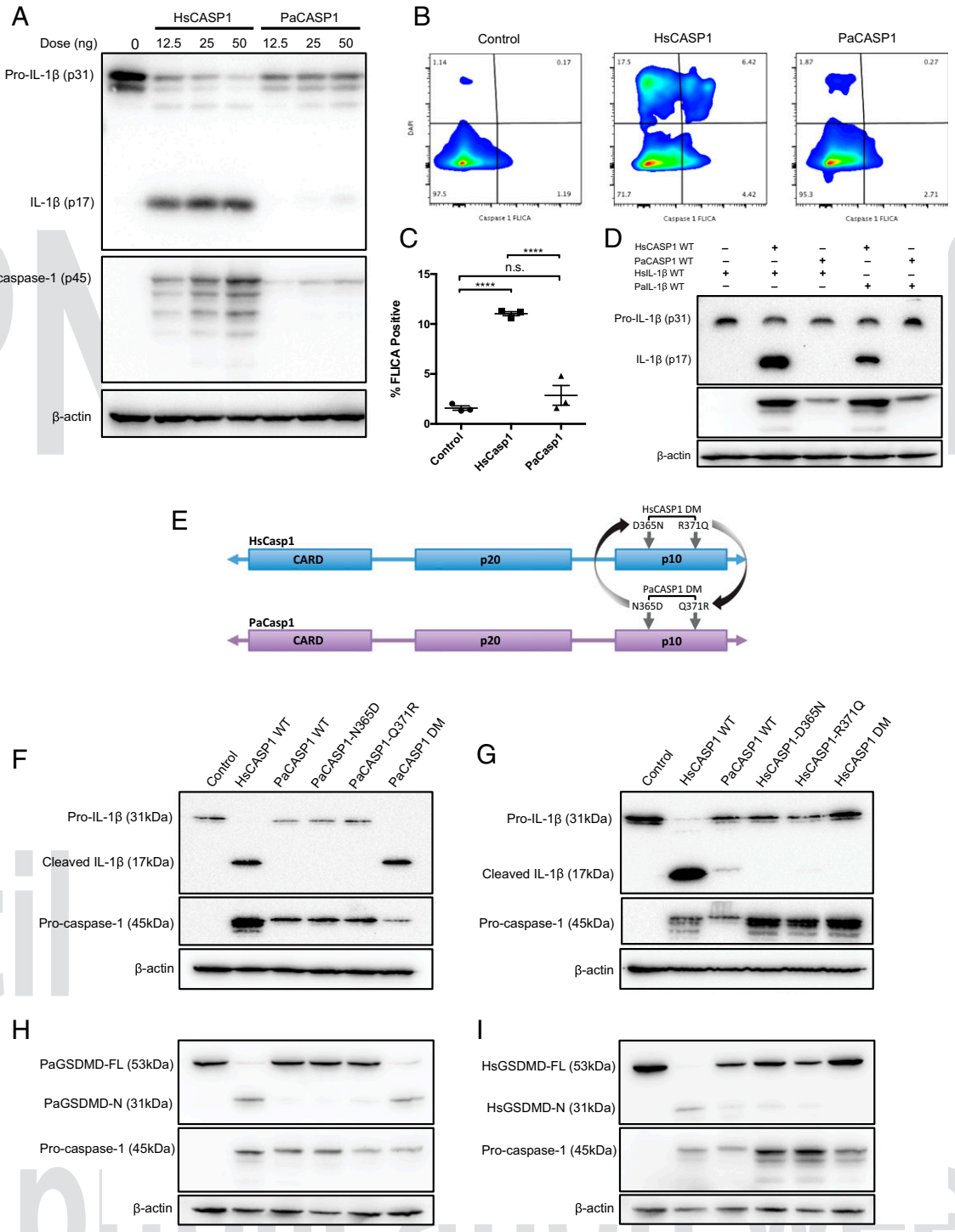

Fig. 3. Inactive PaCasp1 is rescued by substitution of N365D and Q371R in the p10 domain. (*A*) The AIM2 inflammasome axis was reconstituted in HEK293T cells using either HsCASP1 or PaCASP1 in a dose-curve. Cell lysates were stained for pro-IL-1β (p31) or mature IL-1β (p17). Procaspase-1 (p45) was assayed to compare expression, and lysates were normalized by β-actin. (*B*) Cells were reconstituted with the AIM2 inflammasome axis genes and incubated for 48 h, and stained with 660-YVAD-fmk caspase-1 FLICA substrate (Immunochemistry). Flow cytometry was performed to detect caspase-1 activation in control vector, HsCASP1 or PaCASP1 expressing cells, and data analyzed in FlowJo. (*C*) Quantification of FLICA-positive cells measured via flow cytometry. (*D*) Immunoblot of cell lysates crossing HsCASP1-3XFLAG with HsIL-1β-HA or PaIL-1β-HA, and PaCasp1-3XFLAG with HsIL-1β-HA or PaIL-1β-HA, coexpressed with human AIM2 and ASC. Cleaved human or bat IL-1β was measured with anti-HA antibody. (*E*) Schematic of site-derived reverse mutagenesis conducted in the p10 domain of PaCASP1 or HsCASP1. Targeted residues are shown, either single bat N365D or Q371R mutation, or containing both mutations (PaCASP1 DM); and D365N, R371Q, or HsCASP1 DM for the human gene. (*F*) AIM2 inflammasome axis was reconstituted in HEK283T cells, varying the PaCASP1 for WT, single-mutant N365D, Q371R, or double mutant DM. Cleaved IL-1β p17 was assayed via immunoblotting 48 h posttransfection. (*G*) Similarly, HsCASP1 WT, D365N, R371Q, or DM was reconstituted, and cleavage of IL-1β was analyzed by immunoblot. Shown are the pro-IL-1β (31 kDa), cleaved IL-1β (17 kda), and human or bat procaspase-1 (45 kDa). (*H* and *I*) Similarly, PaGSDMD was coexpressed with PaCASP1 (*H*) and HsGSDMD with cognate HsCASP1 (*I*) WT, single-mutants or double-mutant variants, in conjunction with upstream AIM2 inflammasome axis. Cleavage of GSDMD was detected by immunoblotting for the GSDMD-N-2xMyc domain after 48 h incubation in vitro as per *D*. Data are representative of three independent experiments in *A*–*G*. ****$P < 0.0001$, n.s., nonsignificant. Statistics were performed using unpaired Student's *t* test and presented as mean ± SEM of three independent replicates (*C*).

effect was strongest in the combined mutants. Thus, we observe that given a certain level of caspase-1 activity in any of the bat species (*P. alecto* low, *E. spelaea* medium, and *M. davidii* high), the cleavage potential of IL-1β occurs in opposite direction (*P. alecto* high, *E. spelaea* medium, and *M. davidii* low) (Fig. 4*F*). Taken together, this demonstrates a complementary mechanism whereby full caspase-1 activity is balanced by diminished cleavage potential of IL-1β, and vice versa, resulting in an overall, equivalent dampening of inflammasome signaling across multiple bat species from both suborders.

Discussion

We have confirmed that loss of AIM2/ALRs in bats results in inactive initiation of the inflammasome cascade in response to cytosolic DNA in vitro. Crucially, we reveal another layer of dampening through bat caspase-1, the principal cysteine protease responsible for cleaving inflammatory cytokines such as IL-1β and IL-18. We identified two inactivating alterations N365D and Q371R localized in the p10 sequence of wild-type *P. alecto* bat caspase-1 which when rescued by substitution of the human residues at equivalent sites, restored caspase-1 enzyme functionality. Simultaneously, when human caspase-1 was replaced with the equivalent bat amino acids, either residue change resulted in abrogation of IL-1β cleavage. Despite retention of caspase-1 activity in other bat species, corresponding reduction in IL-1β cleavage mitigated downstream inflammasome signaling. Thus, we have experimentally validated two additional residues of mammalian caspase-1, which are integral to its activity for substrate maturation, and demonstrated a proof of concept whereby downstream inflammasome activation in bats is dampened through a unique inverse relationship involving bat caspase-1 and IL-1β.

The discovery of dampened caspase-1 in bats has particular significance in their response to infection and immunity. As the classical inflammatory effector of the inflammasome complex, caspase-1 is converged upon by multiple upstream sensors, including NLRP3, NLRP1, AIM2, NLRC4, and others (43–47). These sensors are activated by a diverse array of cell- and pathogen-derived stimuli, including viral and bacterial nucleic acids, flagellin, ATP, and MSU crystals, and reactive oxidative species (ROS). Such signals trigger a systemic activation of alert and defense mechanisms, including pyroptosis, cytokine signaling, and the recruitment of neutrophils and macrophages into the affected tissue area (17, 48, 49). As caspase-1 cleaves cGMP-AMP (cGAMP) synthase (cGAS) to enhance host resistance to DNA viruses, and MAVS and TRIF to abolish IFN signaling (50), it is possible that bats may have evolved other compensatory mechanisms to resist viral pathogenesis by biasing cross-regulation of these pathways (7). Indeed, there is increasing evidence that AIM2 and other inflammasomes oppose type I IFN sensors, including cGAS, STING, and MyD88/IRF7 (51–54). As such, constitutive IFN expression in bats may, in part, be both an outcome and compensatory mechanism of inflammasome dampening, allowing them to mitigate viral pathogenesis. Further, as noncanonical caspase-1 substrates range from cytoskeletal components, enzymes in cell metabolism, and diverse other proteins involved in cellular stress responses and cell death pathway, it is possible that caspase-1 may retain residual baseline activity for regulation of these processes, thus conserving its expression in bats albeit at reduced function. Thus the effect of our findings on these nonimmune substrates would also warrant investigation.

It is notable that both identified caspase-1 residues acted on by high positive selection pressures possess noncharged (Asn, Gln) instead of charged (Asp, Arg) side chains, representing a substantial decrease in capacity for ionic bond formation. Both amino acids are situated within the p10-p10 interface of the caspase-1 $(p20p10)_2$ homodimer and are shown to participate in critical electrostatic interactions across the interface of the active conformation of caspase-1 (*SI Appendix*, Fig. S4). Loss of these charged residues likely results in weakened caspase-1 dimerization, preventing robust autoprocessing and substrate cleavage. To our knowledge, no prior study has identified either D365 and R371 in α6 as essential for caspase-1 activity in humans or other mammalian species, with early structural studies reporting the interface to only consist of residues 318 to 322 and 386 to 396 (35). Two noncompetitive inhibitors discovered decades earlier, gold thiomalate and auranofin, closely mimic this interface-disruptive mechanism but differ in residue specificity (35). While most widely used caspase-1 inhibitors to date utilize active site-specific mechanisms to impede function (18, 32, 35, 55–57), none of the marketable inhibitors yet allosterically perturb caspase-1 at the p10 dimer–dimer interface despite efforts (58, 59). Thus, our findings may prove to facilitate additional specificity in inhibitor design. Importantly, our findings provide evidence of evolutionary drivers of inhibitory mechanisms in nature informing insight into human caspase-1 and suggest another potential alternative strategy for caspase-1 targeting in human therapeutics.

Positive selection pressure occurred in both caspase-1 residues of the *Pteropus* bat genus but not in any other bat species. We observed retention of caspase-1 activity by *E. spelaea* and *M. davidii* bats from both bat suborders Yinpterochiroptera and Yangochiroptera, which may be explained by a lack of D365 and R371 substitutions in caspase-1 of both bats. Intriguingly, further downstream investigation for IL-1β maturation elucidated an inverse relationship between bat caspase-1 activity and IL-1β cleavage potential, whereby the cleavage of IL-1β within a bat species occurs in the opposite direction to its caspase-1 activity (e.g., in *M. davidii*, caspase-1 high and IL-1β low). The mechanism by which MdIL-1β cleavage is diminished was shown to occur through the S117 site immediately adjacent to the ^{115}Asp-Ala^{116} cleavage site, which when restored by mutation to S117P permitted cleavage of the MdIL-1β mutant into its 17-kDa fragment. This was followed to a smaller extent by the double mutant GS110-111DG of MdIL-1β, suggesting that all three residues might play a role in dampening maturation of the cytokine in *Myotis* bats. We demonstrate that by targeting either caspase-1 or IL-1β, bat species of both suborders possess dampening of important downstream inflammasome components.

Therefore, it is clear that differing strategies have been coopted by bats to dampen either caspase-1 activation, IL-1β cleavage, or both in a complementary manner, highlighting the importance of this phenotype across bats. Indeed, previous genomic or functional studies have found that different bat species exhibited varying or independent genomic strategies to dampen the AIM2 or NLRP3 inflammasome sensors, which culminated in an equivalent level of loss or reduction of activity (9, 11). However, cleavage of IL-1β alone is not sufficient to generate biological inflammasome functioning, and recent studies have demonstrated requirement of GSDMD for IL-1β and IL-18 secretion and activation of pyroptotic cell death (60–62). Thus, in bat species with intact caspase-1 activity, other significant functions may be retained such as GSDMD-mediated pyroptosis and secretion of IL-18, or activation of IL-37 and inactivation of IL-33, which may confer higher regulatory control of pro/antiinflammatory responses (63–65). As such, multiple other indicators of inflammasome functioning in bats remain unknown and require further investigation in the context of these findings.

In conclusion, we find strong experimental evidence pointing to diminished multisensor inflammasome signaling in bats, suggesting high selection pressures acting not only on single, but multiple levels in this pathway. Given the inflammasome functions at the forefront of innate immune signaling, such alteration of inflammasome signaling in bats has a critical role in viral disease tolerance and asymptomaticity (66). Inflammasome activation has been implicated in multiple coronavirus infections, including MERS-CoV, SARS-CoV, and SARS-CoV-2 (67), possibly

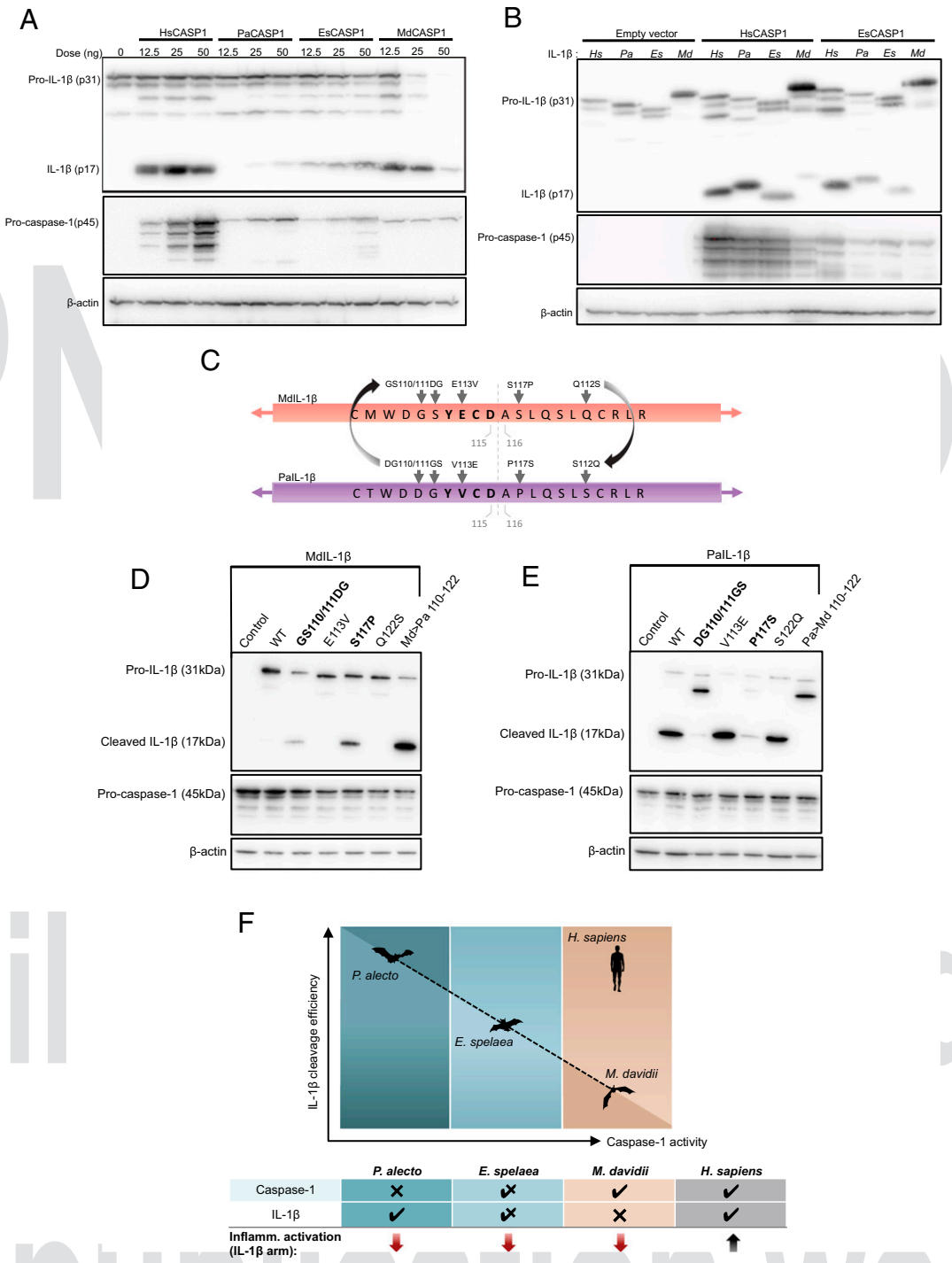

Fig. 4. Complementary relationship between *M. davidii*, *E. spelaea*, *and P. alecto* caspase-1 and IL-1β. (*A*) HEK293T cells were transfected with human NLRP3, ASC, pro-IL-1β for inflammasome axis reconstitution, and coexpressed with either *H. sapiens*, *P. alecto*, *E. spelaea*, or *M. davidii* caspase-1 in increasing concentrations (12.5 to 50 ng/well). Cells were incubated for 48 h and the cell lysate was harvested for Western blot. The amount of mature/cleaved IL-1β (p17) was compared between HsCASP1 and the three bat species and normalized by β-actin. (*B*) Coexpression of human, *P. alecto*, *E. spelaea*, or *M. davidii* IL-1β in HEK283T with reconstituted human NLRP3 and ASC. Cells were transfected with either empty vector, HsCASP1, or EsCASP1 (as indicated). Cell lysate was immunoblotted for pro-IL-1β (p31) and cleaved IL-1β (p17) with anti-HA, procaspase-1 (p45) with anti-FLAG, and normalized by β-actin. Figures are representative of three independent experiments (*A* and *B*). (*C*) Alignment of full-length IL-1β sequences was performed for *H. sapiens*, *P. alecto*, *E. spelaea*, and *M. davidii*, and site-directed mutagenesis was performed substituting identified MdIL-1β amino acid residues with equivalent sites from PaIL-1β near the Asp-Ala cleavage site (gray arrows). In bold, cleavage sites YECD and YVCD for MdIL-1β and PaIL-1β, respectively. (*D*) Full-length WT or mutant MdIL-1β was coexpressed with HsCASP1, AIM2, and ASC and incubated for 48 h; and lysates were assayed for successful cleavage of 17 kDa IL-1β via staining of C-terminal HA-tag. Md → Pa 110 to 122 denotes combined introduction of all identified *P. alecto* residues expressed by the MdIL-1β mutant protein. (*E*) Various mutant PaIL-1β was similarly expressed within the AIM2 inflammasome axis and incubated in vitro for 48 h, and various levels of cleaved PaIL-1β were detected by Western blot. (*F*) Diagram of inverse reciprocal relationship between *P. alecto*, *E. spelaea*, and *M. davidii* caspase-1 activity and IL-1β cleavage efficiency, displayed on the *x* axis and (*Left*) *y* axis, respectively. *Top* showing increasing pattern of caspase-1 activity (*P. alecto* < *E. spelaea* < *M. davidii* < *H. sapiens*) is countered by decreasing IL-1β cleavability (*M. davidii* < *E. spelaea* < *P. alecto* < *H. sapiens*). Table shows vertical summation of either caspase-1 or IL-1β cleavage and resultant function of the downstream inflammasome axis (IL-1β arm).

affecting the ability of bats to function as a reservoir host. With unique capacity for metabolically costly flight, bats could have adapted to elevated metabolic states by dispensing with this inflammatory arm (68–70). Further, inflammasome suppression improves longevity or prevents age-related decline in mice and promotes longevity in humans (71–75), which is in line with bats' long-lived mammalian phenotype. Taken together, our study contributes significant mechanistic understanding for strategies targeting inflammasome dampening in bats, offers potential insight in regulation of human inflammation, and further elucidates the ability of bats to harbor and transmit zoonotic pathogens without sustaining detrimental costs of immune activation.

Materials and Methods

Reagents. Reagents are as previously described (9). Ultrapure LPS-B5, CL264, and Hygromycin B Gold were obtained from InvivoGen. *P. alecto* ASC-specific monoclonal antibody (mouse IgG2b) was generated by GenScript's monoclonal antibody service. Rabbit polyclonal anti-ASC (AL177) (human/mouse) was purchased from Adipogen. Goat polyclonal anti-dog IL-1β (ab193852) (cross-reactive to *P. alecto*) and rabbit polyclonal anti-mouse IL-1β (ab9722) were from Abcam. mAb to β-actin (A2228) was from Sigma-Aldrich and mAb to GFP and variants (including mCitrine) were from Roche (11814460001). Anti-mouse/rabbit/goat horseradish peroxidase (HRP)-conjugated secondary antibodies were from Santa Cruz.

Plasmids. Generation of expression constructs for NLRP3, ASC-mPlum, IL-1β-HA, and empty vectors (control vectors) are as previously described (34, 76). Human AIM2 was cloned from human peripheral blood mononuclear cells (PBMC) cDNA using Q5 Polymerase (NEB) with *Age*I and *Not*I flanking primers (NUS-IRB reference code H-18–029). AIM2 was digested and ligated into pQCXIH (Clontech) vector containing C-terminal mCitrine or 3× FLAG. Procaspase-1 was cloned from the human pCI-caspase-1 construct (Addgene plasmid 41552) or Omniscript (Qiagen)-generated cDNA of *P. alecto* spleen. Caspase-1 was inserted into pQCXIH-mCitrine and pQCXIH-3× FLAG vectors. *P. alecto* or human caspase-1 mutants were generated by overlap extension PCR with primers containing the respective mutations. Similarly, *P. alecto*, *E. spelaea*, and *M. davidii* IL-1β were cloned by PCR of *bat* spleen cDNA into pQCXIH (Clontech) backbones containing C-terminal HA-tag. *P. alecto* and *M. davidii* IL-1β mutants were also generated by overlap extension PCR. Gasdermin D was cloned from human PBMC cDNA and *P. alecto* spleen cDNA with 2× MYC-tag on the N terminus into pQCXIH (Clontech) backbones containing C- terminal HA-tag. Primer sequences are listed in *SI Appendix*, Tables S1 and S2. All constructs were prepared with endotoxin-free plasmid maxi-prep kits (Omega Bio-tek).

Cells. All procedures utilizing animal samples in this study were performed in compliance with all relevant ethical regulations. Capturing and processing of bats (*P. alecto*) in Australia was approved by the Queensland Animal Science Precinct and University of Queensland Animal Ethics Committee (AEC#SVS/073/16/USGMS) and the Australian Animal Health Laboratory Animal Ethics Committee (AEC#1389 and AEC#1557). Where possible, wild bats with irreparable physical damage (torn wings) already scheduled to be killed were utilized. Processing of bats has been described previously (9, 69). Wild-type C57BL/6 mice were obtained with permission from the Singhealth institutional animal care and use committee. Harvesting and differentiation of bone marrow from *P. alecto* bats has been described previously and performed according to identical protocols (76, 77). Mouse bone marrow was harvested from C57BL/6 mice and frozen once in liquid nitrogen, thawed, and differentiated over 7 d in 10 ng/mLmacrophage colony-stimulating factor (M-CSF) as described previously (34). GP2-293 retroviral packaging cells were obtained from Clontech. GP2-293, HEK293T, and PaKiS cells were cultured in Dulbecco's modified Eagle medium (DMEM) (Gibco 11965092) medium supplemented with 10% fetal bovine serum (FBS). PaKiS (CVCL_YM14) is a spontaneous-immortalized kidney epithelial cell line with identical origin from the parental primary cells of PakiT03 (RRID: CVCL_DR89) (34). GP2–293 cell culture medium was supplemented with sodium pyruvate and nonessential amino acid (NEAA) cell culture supplement (Life Technologies) during retroviral packaging.

Reconstitution of AIM2 in Bat Macrophages. Retrovirus was generated by cotransfecting pVSV-G envelope protein with the plasmid containing the gene of interest (AIM2-mCitrine or mCitrine-only) at 1:1 ratio in GP293 cells grown at 70% confluency. Cells were incubated for 48 to 72 h in DMEM containing 10% FBS at 37 °C, and supernatant centrifuged and filtered through 0.45-μm PVDF sterile filters (Millipore). To further concentrate the retrovirus, either 100,000 MW Vivaspin columns (Sartorius) in a benchtop centrifuge or ultracentrifugation at 125,000 × *g* for 90 min (Optima X, Beckman Coulter) in a SW41-TI rotor was performed to 75 to 100× dilution. Retrovirus was titrated on HEK293T cells and added at multiplicity of infection 5 into PaBMDM media at day 5 of differentiation. Cells were incubated for 48 h, supernatant was removed, and cells were recovered in additional 24 h of PaCSF-1 RPMI with 10% FBS before treatment.

In Vitro dsDNA Stimulation. *P. alecto* immortalized kidney cells have been described previously (9). Cells stably expressing *P. alecto* ASC were transduced with mCitrine-only or AIM2-mCitrine retrovirus generated from GP2-293 cells. Cells were selected with puromycin for 5 d and recovered in 10% FBS DMEM media. AIM2-mCitrine/ASC double-positive cells were sorted by fluorescence activated cell sorting (FACS) using BD FACSAria for medium-to-low expression. Cells were grown to 70% confluency and transfected with increasing doses of PolydA:dT (Invivogen). For BMDM stimulation, differentiated cells were primed with 1 μg/mL CL264 (PaBMDM) or LPS B5 (MmBMDM) (Invivogen) for 3 h, washed with FBS-free RPMI (Gibco), and transfected with 1 mg/mL PolydA:dT using Lipofectamine 3000 (Thermo Fisher) in RPMI for 4 h. Supernatant was collected for LDH assay, IL-1β ELISA, and cells were stained for flow cytometry by Imagestream of FACS.

Imagestream Imaging Flow Cytometry. Cells were harvested for Imagestream imaging flow cytometry as previously described (9). Briefly, BMDM cells were harvested with 5 mM ethylenediaminetetraacetic acid (EDTA), washed once with phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde, and permeabilized with 0.3% Triton-X + 2% FBS for 10 min at 4 °C. Cells were stained with primary ASC and prelabeled fluorescent anti-mouse antibodies for 1 h at room temperature, with DAPI for 15 min, and then washed twice with PBS and resuspended in FACS buffer. PaKiS were harvested by trypsinization and resuspended in FACS buffer directly. Events on the Imagestream X were acquired using using INSPIRE software on an Amnis ImageStreamX Mk II imaging flow cytometer using 40× magnification. At least 5,000 events were acquired per sample and analyzed with the inbuilt IDEAS software. Cells in focus were gated by brightfield r.m.s. values, single cells by aspect ratio by area values, and intact nuclei using DAPI staining. Double positive cells (AIM2-mCitrine, ASC-mPlum) were gated and analyzed for ASC speck formation plotted via mean fluorescence intensity by max-pixel intensity.

Confocal Microscopy. PaKiS cells were seeded into 24-well plates containing coverslips (#1.5 thickness). Cells stably expressing *P. alecto* ASC-mPlum were transiently transfected with human AIM2-mCitrine for 4 h, washed, and incubated for 48 h. Cells were stained for 30 min at 37 °C incubation with working concentration of Mitotracker according to manufacturer's instructions (Thermo Fisher). Mitotracker probe solution was removed and cells were washed with PBS 2× before fixing with 4% paraformaldehyde. Nuclear staining was performed with DAPI. Coverslips were mounted onto glass slides with Mowiol 4.88 and images acquired on a Leica TCS SP8 machine at 100× resolution. Images were processed using ImageJ 2.0.0 software.

IL-1β ELISA and LDH Release Assay. Supernatant collected from DNA-treated PaBMDMs was centrifuged to remove debris and frozen once at −80 °C. The supernatant was then measured by a sandwich ELISA protocol as previously described (9). Briefly, purified recombinant PaIL-1β protein was utilized for the standard curve, with goat anti-canine IL-1β primary antibody and rabbit anti-mouse IL-1β antibody used as capture and detection antibodies, respectively. IL-1β in mBMDM supernatants was detected using the BioLegend IL-1β Standard ELISA kit. LDH release assay was performed as previously described using a Cytotoxicity Detection Kit PLUS (LDH) from Roche (9). Calculations were performed as per manufacter's instructions, with low and high controls included for normalization of individual biological replicates.

Evolutionary Analysis of Mammalian Caspase-1 and IL-1β. Caspase-1 coding sequences (CDSs) were retrieved from National Center for Biotechnology Information (NCBI) for one armadillo (*Dasypus novemcinctus*) and many Boreoeutheria species, including Euarchontoglires and Laurasiatheria. Euarchontoglires species include two primates (human and *Pan troglodytes*), two rodents (rat and mouse), and one tree shrew (Chinese tree shrew, *Tupaia belangeri chinensis*) (*SI Appendix*, Table S3). Homologs of caspase-1 in the 15 bat genomes were identified by discontiguous MegaBLAST (BLAST + 2.7.1) with max e-value of 1e-5 and word size of 11. Similarly, IL-1β sequences for *M. davidii* and eight other species of bats (*M. lucifugus*, *E. fuscus*, *M. natalensis*,

D. rotundus, *P. alecto*, and *P. vampyrus*, *R. aegyptiacus*, and *H. armiger*) and six other model mammalian species (*S. scrofa*, *C. lupus*, *P. troglodytes*, *M. musculus*, *R. norvegicus*) were retrieved from NCBI or PCR-cloning and gene sequencing performed on bat cDNA (*SI Appendix*, Table S4). Alignment of the CDSs was generated by MAFFT (78) and used to plot the phylogeny tree by the maximum-likelihood method with the general-time-reversible (GTR) model and 1,000 bootstrap replicates in PHYML 3.0 software (38). The phylogeny tree and alignment file then served as input for performance of positive selection analysis on CodeML from the PAML package (version 4.9) (79), and branch-site models with relevant branches were marked on the tree. LRTs were performed in different substitution models, including 1) M0 (one-ratio), M1a (nearly neutral), M2a (positive selection), M3 (discrete), M7 (beta), M8 (beta and $\omega > 1$), and M8a (beta and $\omega = 1$) in site mod; 2) M0 (one-ratio) and two-ratio model assuming different ω for background and foreground branches in branch mode; and 3) positive selection along specified branches (model A) against a null model (model A null) that allows neutral evolution and negative selection for branch-site mode. Positive selection sites were scored by the Bayes empirical Bayes (BEB) method (80).

Caspase-1 Western Blot and FLICA Assay. The AIM2 inflammasome axis (HsAIM2, HsASC, human caspase-1, and IL-1β) was reconstituted into HEK293T cells at increasing doses using Fugene 6 (Promega) at 3:1 ratio with total DNA. Cells seeded into 96-well plates (Corning) were incubated for 48 h posttransfection and lysed in lysis buffer (79). cOmplete ULTRA protease inhibitor mixture and PhosSTOP phosphatase inhibitors (Roche) were added to lysis buffer before use. Proteins were separated on 12 to 15% SDS/PAGE gels and transferred onto 0.45-μm polyvinylidene difluoride (PVDF) membrane with a Trans-Blot Turbo transfer system (Biorad). Membranes were blocked in 5% bovine serum albumin (BSA) for 1 h and stained with primary antibody followed by HRP-conjugated secondary antibody. Membranes were developed with Amersham ECL Prime Western blotting detection reagent (GE Healthcare) on a myECL Imager (Thermo Scientific). For FLICA detection, cells were trypsinized, washed once in PBS, and stained with 660-Caspase-1 FLICA substrate (Immunochemistry) with occasional agitation for 1 h. Cells were washed three times with cellular wash buffer (provided), resuspended in PBS with 2% BSA, and analyzed via flow cytometry (LSRFortessa Cell Analyzer, BD Biosciences). Live/dead gating was performed using DAPI and subgated for AIM2-mCitrine and ASC-mPlum positivity, before gated for FLICA-660 positive staining (*SI Appendix*, Fig. S6). At least 10,000 cell events were collected per replicate and independently analyzed on FlowJo.

Data Availability. All study data are included in the article text and *SI Appendix*.

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From: (b) (6)
To: (b) (6) christopher.brode (b) (6)
(b) (6)
Subject: re: bats, viruses - request for story comment
Date: Thursday, October 22, 2020 5:34:12 PM
Attachments: pnas.202003352.pdf

Hello, my name is (b) (6), and I'm a science reporter in New York writing for science news service Inside Science, which is syndicated by MSNBC, Fox News and Discovery News, among others.

I'm interested in interviewing you about (b) (6) et al.'s research on bats and viruses appearing in the next issue of the Proceedings of the National Academy of Sciences. I've spoken with several of you before regarding bats and viruses — good to contact you again.

My deadline is 2 p.m. ET on Sunday. Could you contact me via email to set up an appointment as soon as possible or call me at (b) (6)?

I understand when the embargo on your research is -- my deadline allows my editor time to actually work on the story, as opposed to doing a rush job that can introduce inaccuracies into the piece. The story naturally will not get published until after the embargo lifts.

It would really help me out if you could answer a few questions:

x) What do you personally find most surprising or exciting about these results? What do you feel is the most important implication of these findings?

x) What specific potential clinical applications might this research hold? Perhaps by helping bodies not react in potentially harmful way to infections and other disorders?

x) Obviously COVID-19 is of concern right now. Might you talk about how we might think about these findings in relation to the current pandemic?

x) Are there any specific questions or criticisms you have about these findings?

x) What specific directions do you think research might or should go from here? What obstacles do you foresee in future research or development?

x) This shouldn't have to be asked, but can you for a lay audience talk about how the discovery that bats are reservoirs for so many diseases shouldn't mean that people should go out of their way to cull bats?

x) Are there any questions you would have liked to answer that I didn't ask you? Is there anything we didn't cover that you feel is important?

x) Since editorial style says I not refer to people by their institutional title (e.g. assistant professor of chemical engineering), what specialty might I refer to you by (e.g. virologist)?

x) Is there any research of yours that journalists have not reported on yet that might be interesting for a story?

Hope to hear from you soon! Thanks --

(b) (6)

Stories I've written:

Danger in the Forest:
http://www.sciam.com/article.cfm?id=drug-traffickers-endanger-preservation
At Trading Crossroads, Permafrost Yields Siberian Secrets:
http://www.nytimes.com/2004/01/06/science/06MUMM.html
The Worst Nuclear Plant Accident in History: Live from Chernobyl
http://blogs.scientificamerican.com/guest-blog/2011/03/15/the-worst-nuclear-plant-accident-in-history-live-from-chernobyl/

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From: (b) (6)
To: (b) (6)
Christopher Broder; (b) (6)
Eric Laing (b) (6)
Cc: (b) (6)
Subject: DTRA funding application
Date: Wednesday, October 7, 2020 9:18:54 AM
Attachments: DTRA Markotter (b) (6) Technical proposal.pdf

Dear colleagues

Hope you are well. As you may recall, we submitted a proposal to DTRA in 2019 for funding and you were listed as partners (See attached). The good news is that the funding was finally awarded. We would like to schedule an orientation project meeting on 19 October from 3-5 pm (Virtual). This will focus on an introduction to the project and activities proposed. We will have a discussion on the work plan proposed, responsibilities and any deviations that we need to make, keeping the current restrictions of the COVID -19 pandemic in mind.

I will also send a separate meeting invite. The meeting will also be recorded and if you cannot join, we will also make this recording available.

Almost a year has passed since the submission of this proposal, please let me know who needs to be included/excluded in this invite since there may have been staff changes since.

This is really an exciting project and we are looking forward to starting the conversations, activities and building capacity in Southern Africa.

Kind regards

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From: (b) (6)
To: (b) (6)
Christopher Broder; (b) (6)
Cc: (b) (6)
Subject: PROOFS: PNAS paper - please respond by Friday 10 AM.
Date: Wednesday, September 30, 2020 5:42:06 PM
Attachments: ProofPDF_PNAS_202000429.pdf

All,
Attached are the author proofs for our PNAS paper. Please check that your name and affiliation, including department and postal code, are correctly written.

I'll address the other questions listed.

Please send me any necessary corrections by Friday morning, 10 AM EDT.

Cheers,
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From: (b) (6)
To: Christopher Broder; (b) (6) ; Eric Laing; (b) (6)
Subject: DTRA documents
Date: Wednesday, September 2, 2020 9:57:15 AM
Attachments: DTRA (b) (6) Statement of Work.pdf
DTRA (b) (6) Technical proposal.pdf

Dear all

See attached the DTRA technical proposal and Work statement that will form part of our meeting tomorrow.

Talk soon.

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From: (b) (6)
To: (b) (6)
Christopher Broder; (b) (6)
Subject: Update on PNAS Nipah paper: resubmitted
Date: Tuesday, July 21, 2020 4:27:06 PM
Attachments: Nipah dynamics in bats (b) (6) et al 2020_revised w figs.docx
Nipah dynamics in bats et al 2020_response letter_final.pdf

Hi everyone,
I hope everyone is staying safe and healthy. I wanted to update you on the status of our PNAS paper. I was finally able to finish responding to the reviewers' substantive though positive comments, and have resubmitted the revised manuscript. Thank you to those of you who helped with specific questions the reviewers had. I've attached the revised manuscript and the response letter to the Editor, for your records.

I don't know what the timing of review will be given that PNAS says they're backed up and prioritizing COVID-19 papers, but hopefully it won't be too long. Mostly, thank you all for your continued patience, and here's hoping for a positive response.

Cheers.
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From: (b) (6)
To: (b) (6) ; Broder, Christopher; (b) (6) eric.laing (b) (6)
Cc: (b) (6)
Subject: Re: CREID Meeting - EHA, UNC, & USU_Action Items
Date: Sunday, July 19, 2020 4:18:43 PM
Attachments: Project Summary EIDRC SEA (b) (6) .pdf
EIDRC SEA Research Strategy FINAL.pdf
(b) (6) appointments.xlsx
COVID-related projects to collect ideas.docx

Dear Members of EID-SEARCH (Emerging Infectious Diseases - South East Asia Research Collaboration Hub) Center,

It was very nice to speak to you on Friday. Sorry, we have missed some of you on the call. (please feel free to add your team members who will work on this project into this email chain)

Attached please find the project summary and proposal in PDF if some of you haven't got a chance to read the details before.

Now we would like to ask everyone's input on two items:

1) **COVID-19 related side projects: please send paragraphs about any ideas you might be able to rapidly working on through the CREID collaborations re. COVID-19 for potential extra fundings from NIAID (by Monday, July 21).** NIAID asked about this, and (b) (6) is talking to the program officers on Tuesday morning, so the goal is to get ~10 ideas to propose to them, to find out what they are interested to fund. Attached in the Word document are some ideas we captured from previous calls with (b) (6) , please feel free to add on and/or flash out any of the bullet points.

2) **Working Groups: please volunteer or recommend one person from your team on the Working Groups in the attached Excel sheet (by Wednesday, July 23).** These Working Groups have been identified by DMID and the Coordinating Center as the initial Groups to establish as the Network gets up and running. The Working Groups are expected to meet monthly, and 4 of them will be convened initially if possible, in August, and will meet again in September during the NIAID-facilitated Network kickoff meeting. Each center will appoint one person for each Working Group, but we are considering a second appointment as well in case of the schedule conflicts.

Apologies for this short notice. Please let me know if you have any questions or any further information I can provide. Look forward to hearing from you.

Best regards,
(b) (6)

(b) (6)

[redacted]

On Fri, Jul 17, 2020 at 10:50 AM (b) (6) [redacted] wrote:

> Dear All,
>
> During the call today, we would like to talk about the CREID Working Groups.
>
> Please see below a brief description of the functions for each to help inform your appointments. The attached Excel file provides additional information on the Working Groups.
>
> **Working Groups:** These Working Groups have been identified by DMID and the Coordinating Center as the initial Groups to establish as the Network gets up and running. The Working Groups are expected to meet monthly, and 4 of them will be convened initially if possible, in August, and will meet again in September during the NIAID-facilitated Network kickoff meeting. Research Centers can appoint one person for each Working Group but can choose not to make appointments for all Working Groups.
>
> Look forward to speaking with you soon.
>
> Kind regards,
> (b) (6)
>
> [redacted]
>
> On Wed, Jul 8, 2020 at 7:37 AM (b) (6) [redacted] wrote:
>
> > (b) (6) is inviting you to a scheduled Zoom meeting.
> >
> > Topic: CREID Meeting - EHA, UNC, & USU
> >
> > Time: Jul 17, 2020 01:00 PM Eastern Time (US and Canada)
> >
> > Join Zoom Meeting

> https://zoom.us/j/91143369261
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> Meeting ID: 911 4336 9261
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From: (b) (6)
To: (b) (6)
Christopher Broder; (b) (6)
Subject: RE: Nipah dynamics in P medius draft for PNAS
Date: Friday, January 10, 2020 9:55:11 PM

Well done and good luck to all!

(b) (6)

From: (b) (6)
Sent: Saturday, 11 January 2020 1:36 AM
To: (b) (6)
Christopher Broder (b) (6)
Subject: Re: Nipah dynamics in P medius draft for PNAS

- External Email -

Dear co-authors,
Our paper has been submitted to PNAS! Thank you all for your thoughtful and helpful comments.
I think you've each received a link to the submission from PNAS, but if not, here's the version that was submitted.

I'll be in touch when I hear anything.

Cheers,
(b) (6)

On Wed, Dec 18, 2019 at 3:00 PM (b) (6) wrote:

> All,
> I hope this email finds you well. Attached is a draft of our manuscript entitled Nipah virus dynamics in bats and implications for spillover to humans. After multiple protracted periods of review in Science Advances, and ultimately a rejection, I've prepared this draft for submission to PNAS. I've revised the manuscript and updated figures and supplemental data in response to the last round of review (comments below, if you're interested).
>
> I have an editor at PNAS willing to send this for review, so please send me any input you may have by December 28th so that I can submit by the 31st. Unless I hear otherwise, I will assume you still consent to co-authorship.
>
> Thank you for your patience, and happy holidays!
>
> Cheers,
> (b) (6)
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From: (b) (6)
To: (b) (6) Christopher Broder; (b) (6)
Subject: RE: Nipah dynamics in P medius draft for PNAS
Date: Friday, January 10, 2020 7:04:35 PM

Well done – finger's crossed…

Cheers,

(b) (6)

[redacted]

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From: (b) (6)
Sent: Friday, January 10, 2020 12:36 PM
To: (b) (6)
Christopher Broder; (b) (6)
Subject: Re: Nipah dynamics in P medius draft for PNAS

Dear co-authors,
Our paper has been submitted to PNAS! Thank you all for your thoughtful and helpful comments.
I think you've each received a link to the submission from PNAS, but if not, here's the version that was submitted.

I'll be in touch when I hear anything.

Cheers,
(b) (6)

On Wed, Dec 18, 2019 at 3:00 PM (b) (6) wrote:

> All,
> I hope this email finds you well. Attached is a draft of our manuscript entitled Nipah virus dynamics in bats and implications for spillover to humans. After multiple protracted periods of review in Science Advances, and ultimately a rejection, I've prepared this draft for submission to PNAS. I've revised the manuscript and updated figures and supplemental data in response to the last round of review (comments below, if you're interested).
>
> I have an editor at PNAS willing to send this for review, so please send me any input you may have by December 28th so that I can submit by the 31st. Unless I hear otherwise, I will assume you still consent to co-authorship.
>
> Thank you for your patience, and happy holidays!
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> Cheers,
> (b) (6)
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(b) (6)

(b) (6)

From: (b) (6)
To: (b) (6) Christopher Broder; (b) (6)
Subject: Re: Nipah dynamics in P medius draft for PNAS
Date: Friday, January 10, 2020 1:53:54 PM

Thanknyou so much for the hard work (b) (6) really appreciated mate (b) (6)

Science is Vital- http://scienceisvital.org.uk/

(b) (6)

Visit Sfam, the Society for Applied Microbiology website.

From: (b) (6)
Sent: 10 January 2020 17:35:43
To: (b) (6) Christopher Broder (b) (6)

(b) (6)

Subject: Re: Nipah dynamics in P medius draft for PNAS

Dear co-authors,
Our paper has been submitted to PNAS! Thank you all for your thoughtful and helpful comments.
I think you've each received a link to the submission from PNAS, but if not, here's the version that was submitted.

I'll be in touch when I hear anything.

Cheers,
(b) (6)

On Wed, Dec 18, 2019 at 3:00 PM (b) (6) wrote:

> All,
> I hope this email finds you well. Attached is a draft of our manuscript entitled Nipah virus dynamics in bats and implications for spillover to humans. After multiple protracted periods of review in Science Advances, and ultimately a rejection, I've prepared this draft for submission to PNAS. I've revised the manuscript and updated figures and supplemental data in response to the last round of review (comments below, if you're interested).
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> Thank you for your patience, and happy holidays!
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> Cheers,
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From: (b) (6)
To: (b) (6) Christopher Broder; (b) (6)
Subject: Re: Nipah dynamics in P medius draft for PNAS
Date: Friday, January 10, 2020 12:37:01 PM
Attachments: Nipah dynamics in bats (b) (6) et al 2020_complete.pdf

---

Dear co-authors,
Our paper has been submitted to PNAS! Thank you all for your thoughtful and helpful comments.
I think you've each received a link to the submission from PNAS, but if not, here's the version that was submitted.

I'll be in touch when I hear anything.

Cheers,
(b) (6)

On Wed, Dec 18, 2019 at 3:00 PM (b) (6) wrote:

> All,
> I hope this email finds you well. Attached is a draft of our manuscript entitled Nipah virus dynamics in bats and implications for spillover to humans. After multiple protracted periods of review in Science Advances, and ultimately a rejection, I've prepared this draft for submission to PNAS. I've revised the manuscript and updated figures and supplemental data in response to the last round of review (comments below, if you're interested).
>
> I have an editor at PNAS willing to send this for review, so please send me any input you may have by December 28th so that I can submit by the 31st. Unless I hear otherwise, I will assume you still consent to co-authorship.
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> Thank you for your patience, and happy holidays!
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> Cheers,
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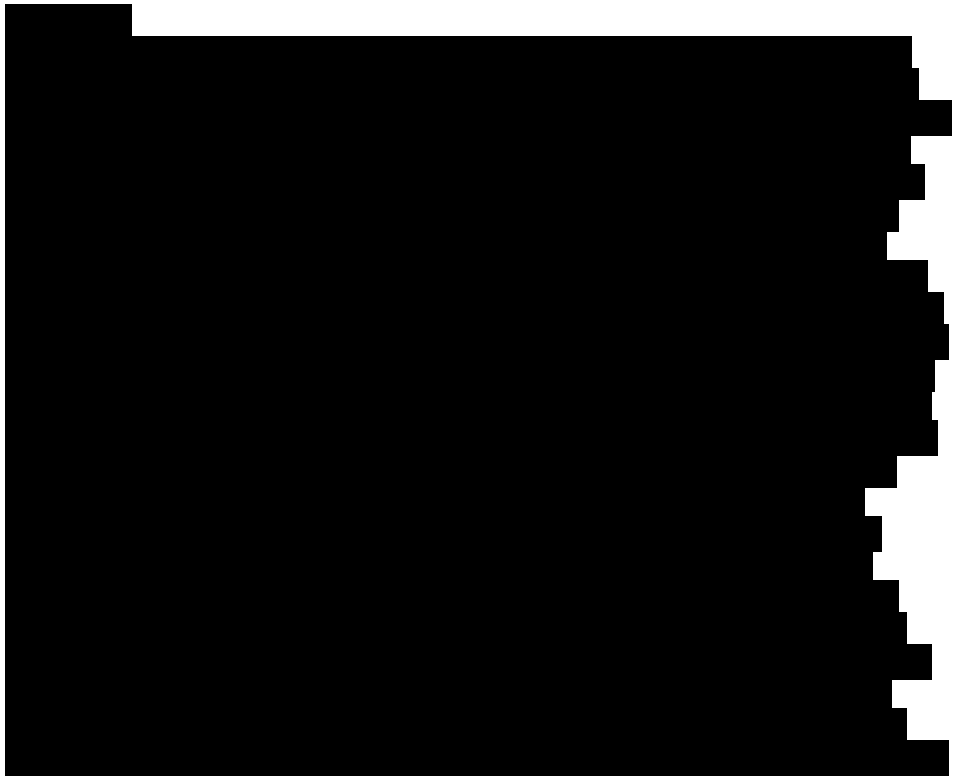

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From: (b) (6)
To: (b) (6)
Cc: (b) (6) ; Christopher Broder; (b) (6)
Subject: Re: Nipah dynamics in P medius draft for PNAS
Date: Saturday, December 21, 2019 4:08:43 PM
Attachments: Nipah dynamics in bats (b) (6) et al 2019 (b) (6) docx

---

Nice work (b) (6).

Attached are a few minor comments.

Good luck with submission.

(b) (6)

(b) (6) Friday, December 20, 2019 3:09 AM

Hi (b) (6),

It's reading pretty well! See my suggested revisions and comments on the main doc and supplemental material. Let me know if you have questions!

(b) (6)

On 12/18/2019 10:00 AM, (b) (6) wrote:

--

(b) (6)

(b) (6) Wednesday, December 18, 2019 2:00 PM

All,

I hope this email finds you well. Attached is a draft of our manuscript entitled Nipah virus dynamics in bats and implications for spillover to humans. After multiple protracted periods of review in Science Advances, and ultimately a rejection, I've prepared this draft for submission to PNAS. I've revised the manuscript and updated figures and supplemental data in response to the last round of review (comments below, if you're interested).

I have an editor at PNAS willing to send this for review, so please send me any input you may have by December 28th so that I can submit by the 31st. Unless I hear otherwise, I will assume you still consent to co-authorship.

Thank you for your patience, and happy holidays!

Cheers,

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From: (b) (6)
To: (b) (6) [redacted]; Christopher Broder; Gary
Subject: Re: Nipah dynamics in P medius draft for PNAS
Date: Friday, December 20, 2019 4:12:47 AM
Attachments: Supplemental Data_PNAS 2019 (b) (6) .docx
Nipah dynamics in bats (b) (6) et al 2019 (b) (6) .docx

Hi (b) (6),

It's reading pretty well! See my suggested revisions and comments on the main doc and supplemental material. Let me know if you have questions!

(b) (6)

On 12/18/2019 10:00 AM, (b) (6) wrote:

> All,
> I hope this email finds you well. Attached is a draft of our manuscript entitled Nipah virus dynamics in bats and implications for spillover to humans. After multiple protracted periods of review in Science Advances, and ultimately a rejection, I've prepared this draft for submission to PNAS. I've revised the manuscript and updated figures and supplemental data in response to the last round of review (comments below, if you're interested).
>
> I have an editor at PNAS willing to send this for review, so please send me any input you may have by December 28th so that I can submit by the 31st. Unless I hear otherwise, I will assume you still consent to co-authorship.
>
> Thank you for your patience, and happy holidays!
>
> Cheers,
> (b) (6)

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(b) (6)

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(b) (6)

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From: Broder, Christopher (b) (6)
To: (b) (6)
Subject: Re: Nipah dynamics in P medius draft for PNAS
Date: Thursday, December 19, 2019 3:03:45 PM

---

good luck (b) (6)

boy reviewer #2 was harsh....
wonder who that was?

On Wed, Dec 18, 2019 at 3:01 PM (b) (6) > wrote:

> All,
> I hope this email finds you well. Attached is a draft of our manuscript entitled Nipah virus dynamics in bats and implications for spillover to humans. After multiple protracted periods of review in Science Advances, and ultimately a rejection, I've prepared this draft for submission to PNAS. I've revised the manuscript and updated figures and supplemental data in response to the last round of review (comments below, if you're interested).
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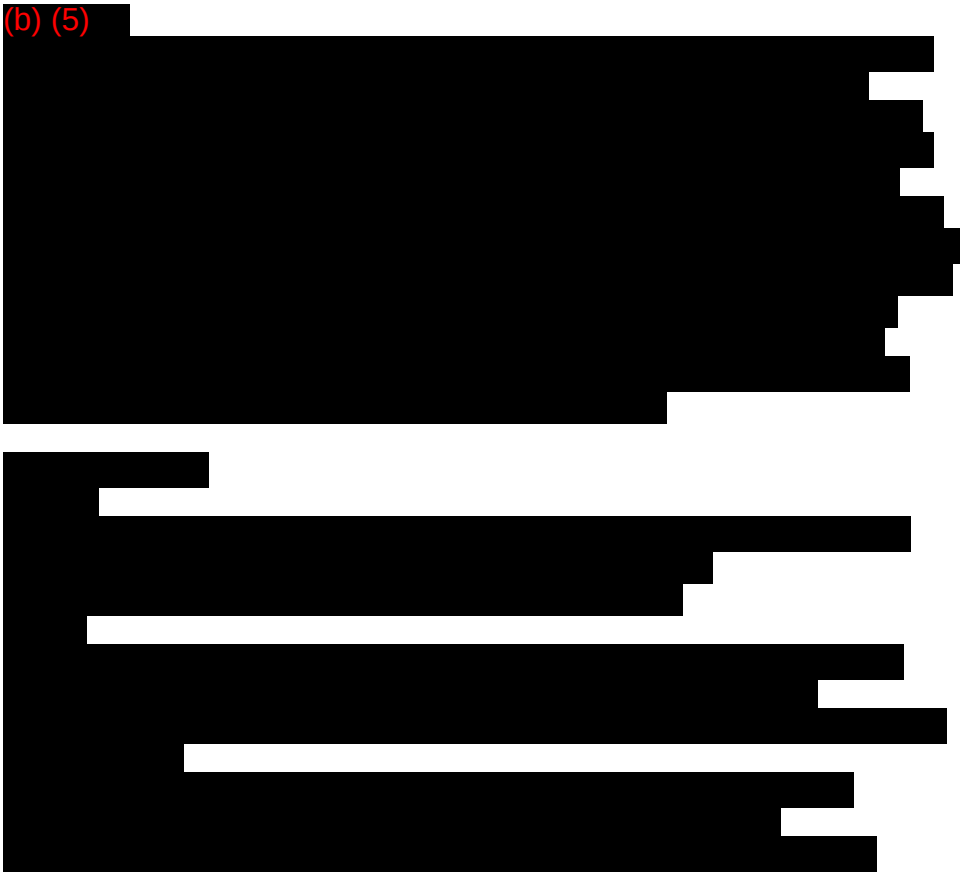
> (b) (6)
>
> (b) (5)

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(b) (6)

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(b) (6) [redacted]

[redacted]

From: (b) (6)
To: (b) (6)
Christopher Broder; (b) (6)
Subject: RE: Nipah dynamics in P medius draft for PNAS
Date: Wednesday, December 18, 2019 9:47:28 PM

Dear (b) (6)

I had a quick read and have nothing to add.

Fingers crossed!

Thanks

(b) (6)

[redacted]

From: (b) (6)
Sent: Thursday, 19 December 2019 4:01 AM
To: (b) (6)
[redacted]
Christopher Broder (b) (6)
[redacted]
Subject: Nipah dynamics in P medius draft for PNAS

- External Email -

All,
I hope this email finds you well. Attached is a draft of our manuscript entitled Nipah virus dynamics in bats and implications for spillover to humans. After multiple protracted periods of

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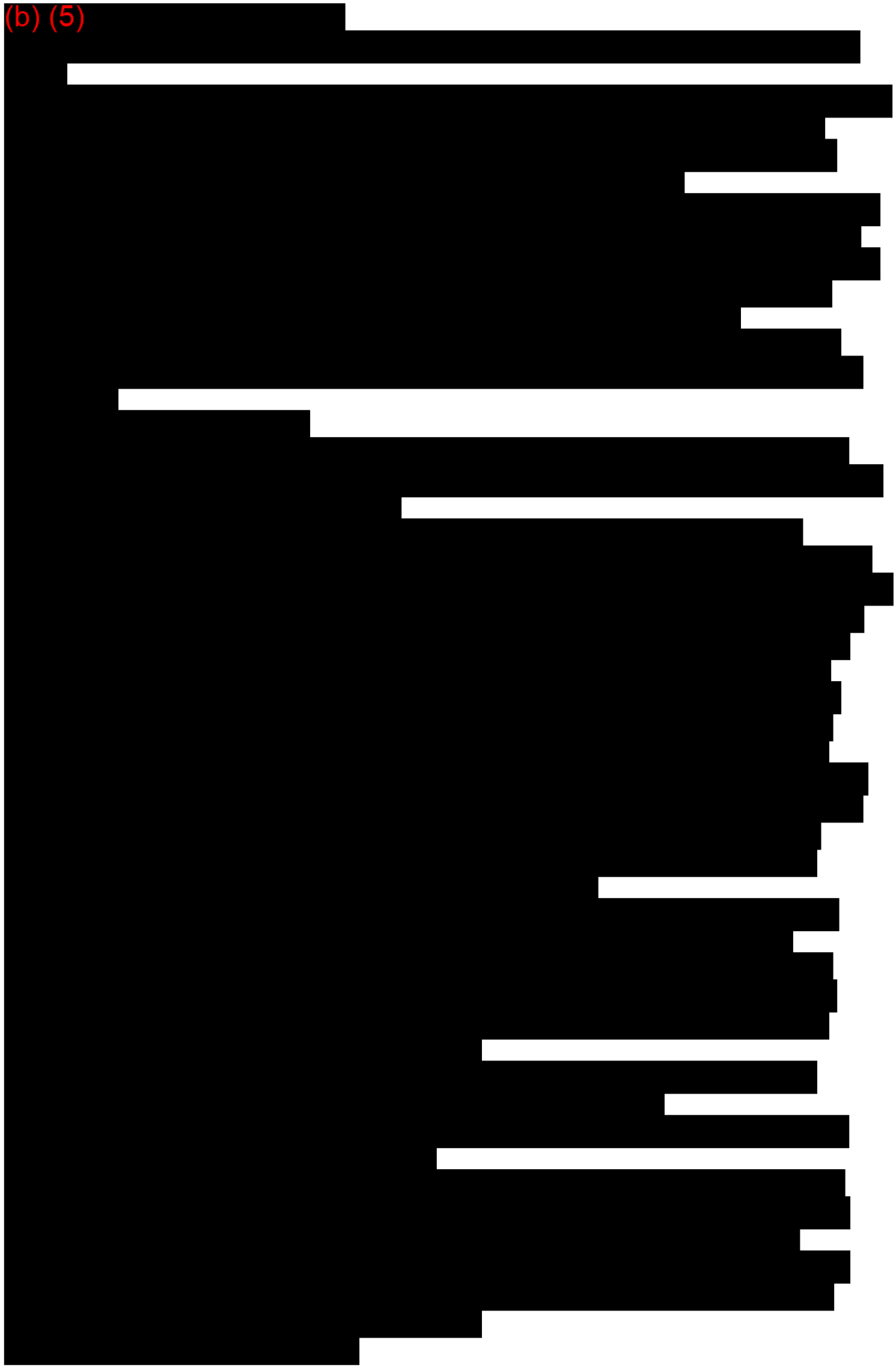
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From: (b) (6)
To: Laing, Eric; (b) (6)
Cc: (b) (6) ; Chris Broder; (b) (6)
Subject: RE: EIDRC Summary Statement from NIAID
Date: Friday, December 6, 2019 7:08:35 PM
Attachments: Summary Statement - EIDRC Grant.pdf
Importance: High

Dear All,

Attached is the summary statement from our EIDRC review. We didn't do badly at all – 3 reviewers, two of whom gave us 2's and 3's, and the second reviewer who loved it (mainly 1's).

The good news is that the summary statement starts with "in this excellent …proposal", which is a good sign, although there were some negatives of course.

In my opinion, we're touch and go on whether we'll get funded on this, so I quickly wanted to check in with who of you will be at the Singapore Nipah meeting over the next few days? I'll be there, as will (b) (6) (cc'd here so he can help). (b) (6) and (b) (6), obviously. (b) (6) – **will you be there in Singapore?**

The reason I'm asking is that (b) (6), and possibly others from NIH/NIAID/DMID will be there as well, and they're in a key position to decide whether to push this forwards for funding. The council meeting is on January 27th.

(b) (6) or (b) (6) – can you email me a list of who's registered for the meeting from NIAID or NIH please? (b) (6) is the Program Officer and (b) (6) will be part of the process also.

For those of you going – we can't lobby NIH directly, but it will be good for us to be seen as a cohesive team at that meeting, and talk very positively about our work together over the past few years, including on PREDICT and the samples that have been collected that could be also used in this project. Also, if you have chance, please re-read some of the proposal in case we do get chance to talk with them about it (attached in a previous email).

Fingers crossed on this one!

Cheers,

(b) (6)

[redacted]

(b) (6)

From: (b) (6)
Sent: Monday, November 18, 2019 9:24 PM
To: 'Laing, Eric'; (b) (6)
Cc: (b) (6) 'Chris Broder'; (b) (6)
Subject: EIDRC impact scores from NIH
Importance: High

Dear All,

Just to let you know that we've got the results back from NIH. EHA submitted two EIDRC proposals, and we were linked to a third one as a subcontract. Out of these, the only one to get a score was the Southeast Asia EIDRC (EID-SEARCH) which you are all collaborators on. I also know of another good group that wasn't scored, so just to get a score is actually a good result…

Our 'impact score' was (b) (5) which normally would put us right on the edge of being funded. I've already contacted the NIAID program officer (b) (6) to ask about the likelihood of being funded. She told me that NIH staff are currently working out the structure of the network and a funding plan. She felt our proposal was well-received and scored well enough to be considered, and she asked me to hold off for a week or two and then they'd know more.

My take on this is that they're now deciding what the geography and scope of the network should be, how many EIDRC centers they can afford to fund, and then they'll decide priorities for funding. With this being strongly driven by NIAID's internal concerns about their reach into different geographies, it's really going to be a decision driven by their own considerations of who else is in the mix and what strengths/weaknesses different proposals have. That means we're in with a chance, but we don't really know the factors driving a decision.

I'll contact NIAID again in a few days, but in the meantime, please do the following: 1) try not to share this information with colleagues because we don't want to have a situation where they scored better than us and we got funded, leading to a bad feeling (or worse, the other way round!); 2) if you meet people from NIAID, tell them how keen and enthusiastic you are about the proposal and the work we're conducting (I've attached the final text again as a reminder) – you never know how this might help. Key people at NIAID who will be making this decision are: (b) (6) (head of NIAID),

(b) (6) (Deputy Head, and in charge of this line of work), (b) (6) (Program Officer directly in charge of the EIDRCs), (b) (6) (also directly in charge of this work), (b) (6) (I think she's involved).

Finally – I've attached the Review Committee Roster for you to look at who the people are who either liked or disliked our proposal! I know a few of them, and I'm sure you know the others…

Will be in touch as soon as I hear more.

Cheers,

(b) (6)

[redacted]

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From: (b) (6)
Sent: Friday, June 28, 2019 8:59 PM
To: 'Laing, Eric'; (b) (6)
Cc: (b) (6) 'Chris Broder'; (b) (6)
Subject: EIDRC grant submitted
Importance: High

Dear All,

Just to let you all know that the grant was successfully submitted today with time to spare, and no errors. It's in the system, and it's all down to the reviewers now! Thanks to all of you for your help and support in getting this finalized and completed. Please pass on my personal thanks to the Co-Investigators, Key Personnel and Consultants who you also brought into the team.

I've attached a pdf of the final proposal text and will send the Word version in a few minutes. It's actually a good read, and I'm especially grateful to (b) (6) who generated all the cool graphics, based largely on (b) (6) previous NIH grant proposals.

As you read the text, please remember that the wording is very carefully targeted to a typical US-based NIH reviewer, and to the Program Officers, with the sole purpose of trying to win the grant. If I've exaggerated or made mistakes, or used language that isn't quite right, I apologize, but I did it for the key goal of getting funded.

In the meantime, please don't share this proposal beyond our group on this email chain, so we don't give our competitors an edge!

Good luck, and I'll be keeping my fingers crossed all summer in the hope that we win this...

Cheers,

(b) (6)

[redacted]

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From: (b) (6)
Sent: Wednesday, June 26, 2019 5:19 PM
To: 'Laing, Eric'; (b) (6)
Cc: (b) (6) ; Chris Broder; (b) (6)
Subject: EID-SEARCH Comments

Thanks everyone for sending the comments back. Working on v5 now, while others in the office are cranking out sample size calculations, new figures, human and vert. subjects and all the additional information needed after the research plan.

As I go through all your comments, I'll send the occasional email with questions to clarify specific points, so please be on standby for a quick turnaround.

Cheers,

(b) (6)

[redacted]

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From: Laing, Eric (b) (6)
Sent: Wednesday, June 26, 2019 12:59 PM
To: (b) (6)
Cc: (b) (6)
Chris Broder; (b) (6)
Subject: Re: EID-SEARCH v4

Hi (b) (6),

Built on top of (b) (6) suggestions. Included a prelimin data figure from Hughes et al, in prep. Double check that is ok with (b) (6)?

- (b) (6)

[redacted]

(b) (6)

On Wed, Jun 26, 2019 at 11:26 AM (b) (6) >
wrote:

> Hi (b) (6),
>
> I have added my edits and comments to (b) (6).
>
> Please let me know if you ave any questions or need more details.
>
> Thanks.
>
> (b) (6)

From: (b) (6)
Sent: 26 June 2019 2:51 PM
To: (b) (6)
Cc: (b) (6) ; Chris Broder; Eric Laing; (b) (6)
Subject: Re: EID-SEARCH v4

(b) (6), v4 with my edits, some revised figs, and additional comments.

Cheers,
(b) (6)

(b) (6)
[redacted]

On Jun 25, 2019, at 9:26 AM, (b) (6) [redacted] > wrote:

Dear All,

Here's version 4. Apologies for the delay. It's in better shape now, but there are still lots of areas that need your attention. Please put in time today, and tomorrow to edit and firm up the text. I've put your names in some of the comment boxes where I need specific help but I also need you to read this critically now and edit and tighten it up. We have 30 pages of space, and our research plan is now 21 pages, so we need to lose maybe 3 or 4 pages. There are also some weak points, specifically on Filoviruses, on the details of where we'll do our clinical work, and on what we'll do with the animal models for henipas and filos.

Please get comments back with me before Wednesday 26th 4pm New York time, so that I have Wendesday evening and all day Thursday to incorporate them. In the meantime, I'll be working with folks here on better figures, and all the extra sections that come after the research plan. I'll need you all to help with those on Thursday while I'm finishing off the draft.

Thanks to all of you in advance and look forward to the next round.

Cheers,

(b) (6)

[redacted]

[redacted]

[redacted]

(b) (6)

From: (b) (6)
Sent: Thursday, June 20, 2019 9:41 PM
To: (b) (6)
Christopher Broder
(b) (6) Eric Laing; (b) (6)
Cc: (b) (6)
Subject: EIDRC-SEA v.3
Importance: High

Dear all,

Thanks to everyone for comments and text for the earlier draft at the beginning of the week. (b) (6)s spent the last couple of days pulling all the comments together into one draft and inserting his changes, and I've now attached a v3 of the draft. It's still rough, and now too long, but it's getting there. I wanted you to look at this rough version because I'm stuck in Liberia in meetings Friday, then flying back home Saturday. I'll continue editing this draft on the plane and will be working solely on this grant from home on Sunday, Monday, Tues, Wed, Thurs.

Please work on this rapidly if possible. If you can all push your comments through, using track changes, and get them back to me **by Sunday evening New York Time**, I will turn round a polished draft by Tuesday, giving us time for significant last go-over on Wed, Thurs. It's tight, but unfortunately, it is what it is, so please commit the time if you can!! As well as general edits (using track changes, with references in comment boxes), please do the following…

(b) (6) – We especially need your input in section 1.1, 1.3, 1.4 and 2.5

(b) (6) - Please work your magic on this draft, inserting text, editing and reducing text, and making sure the technical details of the characterization are correct and fit the proposed work on CoVs, PMVs and Henipaviruses

(b) (6) – (b) (6) – congratulations on your (b) (6) and thanks for working on it at this time!
(b) (6) – please help and edit/finesse the language in the relevant sections for you

(b) (6) – Your edits crossed over with (b) (6) changes, so please insert all the bits that were new in the last version you sent to me, into this version. I think these are mainly the sections and comments from the other Malaysian colleagues, but please check and re-insert all key text. We especially need to be specific about which partner in Malaysia is doing what cohorts, and where, and about who's doing the testing

Thanks again all, and I'm looking forward to non-stop work on this as soon as I get on my flight in 24 hours…

Cheers,

(b) (6)

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<EIDRC Southeast Asia v4.docx>

MEETING ROSTER
National Institute of Allergy and Infectious Diseases Special Emphasis Panel
NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES
Emerging Infectious Diseases Research Centers (U01 Clinical Trial Not Allowed)
ZAI1 EC-M (J2)
11/04/2019 - 11/05/2019

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CHAIRPERSON(S)

(b) (6) W., PHD
PROFESSOR
DEPARTMENT OF MICROBIOLOGY AND IMMUNOLOGY
DREXEL COLLEGE OF MEDICINE
DREXEL UNIVERSITY
PHILADELPHIA, PA 19129

MEMBERS

(b) (6) ., PHD
PROFESSOR
DEPARTMENTS OF MEDICINE, EPIDEMIOLOGY
AND PUBLIC HEALTH
INSTITUTE OF HUMAN VIROLOGY NIGERIA &
UNIVERSITY OF MARYLAND SCHOOL OF MEDICINE
BALTIMORE, MD 21201

(b) (6) ., PHD
PROFESSOR
DEPARTMENT OF ENTOMOLOGY
TEXAS A&M UNIVERSITY
COLLEGE STATION, TX 77843

(b) (6), PHD
ASSOCIATE PROFESSOR
DEPARTMENT OF MICROBIOLOGY AND IMMUNOLOGY
COLLEGE OF VETERINARY MEDICINE
CORNELL UNIVERSITY
ITHACA, NY 14853

(b) (6), PHD
ASSISTANT PROFESSOR
DEPARTMENT OF VETERINARY AND BIOMEDICAL SCIENCES
UNIVERSITY OF MINNESOTA, TWIN CITIES
ST. PAUL, MN 55108

(b) (6), PHD
PROFESSOR
DEPARTMENT OF COMPUTATIONAL MEDICINE
CINCINNATI CHILDREN'S HOSPITAL MEDICAL CENTER
UNIVERSITY OF CINCINNATI
CINCINNATI, OH 45229

(b) (6), MD
PROFESSOR
DEPARTMENT OF BIOMEDICAL SCIENCES
OREGON STATE UNIVERSITY
CORVALLIS, OR 97331

(b) (6), PHD
ASSISTANT PROFESSOR
DEPARTMENT OF PATHOLOGY AND MICROBIOLOGY
UNIVERSITY OF NEBRASKA MEDICAL CENTER
OMAHA, NE 68198

(b) (6), PHD
PROFESSOR
DEPARTMENT OF MOLECULAR BIOLOGY, BIOCHEMISTRY,
MEDICINE, MICROBIOLOGY AND MOLECULAR GENETICS
DIVISION OF INFECTIOUS DISEASE
UNIVERSITY OF CALIFORNIA, IRVINE
IRVINE, CA 92697

(b) (6), PHD
PROFESSOR
CENTRE FOR TROPICAL MEDICINE & GLOBAL HEATLH
UNIVERSITY OF OXFORD
UNITED KINGDOM

(b) (6), MD
PROFESSOR
DEPARTMENT OF OBSTETRICS AND GYNECOLOGY
SCHOOL OF MEDICINE
UNIVERSITY OF CALIFORNIA SAN FRANCISCO
SAN FRANCISCO, CA 94105

(b) (6), MD
PROFESSOR
DEPARTMENT OF MOLECULAR GENETICS AND
MICROBIOLOGY
SCHOOL OF MEDICINE
STONY BROOK UNIVERSITY
STONY BROOK, NY 11794

(b) (6), BS, PHD
PROFESSOR
DEPARTMENT OF BIOLOGICAL SCIENCES
PURDUE UNIVERSITY
WEST LAFAYETTE, IN 47907

(b) (6), PHD
CLINICAL ASSISTANT PROFESSOR / SENIOR SCIENTIST
BC CENTRE FOR DISEASE CONTROL
VANCOUVER
CANADA

(b) (6), PHD
ASSOCIATE PROFESSOR
DEPARTMENT OF BIOLOGICAL SCIENCE
HEALTH PROMOTION RESEARCH INSTITUTE
CALIFORNIA STATE UNIVERSITY, FULLERTON
FULLERTON, CA 92831

MEETING ROSTER
National Institute of Allergy and Infectious Diseases Special Emphasis Panel
NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES
Emerging Infectious Diseases Research Centers (U01 Clinical Trial Not Allowed)
ZAI1 EC-M (J2)
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(b) (6), MD
ASSOCIATE PROFESSOR
DEPARTMENT OF MEDICINE
DIVISION OF INFECTIOUS DISEASES
UNIVERSITY OF NORTH CAROLINA CHAPEL HILL
CHAPEL HILL, NC 27599

(b) (6), PHD
PROFESSOR
DEPARTMENT OF BIOLOGICAL SCIENCES
THE UNIVERSITY OF SOUTHERN MISSISSIPPI
HATTIESBURG, MS 39406

(b) (6), DSC
BIOLOGIST
DIVISION OF VECTOR-BORNE DISEASES
CENTERS FOR DISEASE CONTROL AND PREVENTION
FORT COLLINS, CO 80521

(b) (6), PHD
SENIOR SCIENTIST
DEPARTMENT OF PATHOGEN INFORMATICS
VECNA TECHNOLOGIES, INC.
COLLEGE PARK, MD 20740

(b) (6), MD
ASSOCIATE PROFESSOR
DIVISION OF MALARIA RESEARCH,
CENTER FOR VACCINE DEVELOPMENT AND GLOBAL HEALTH
UNIVERSITY OF MARYLAND SCHOOL OF MEDICINE
BALTIMORE, MD 21201

(b) (6), PHD
ASSOCIATE PROFESSOR
DEPARTMENT OF EPIDEMIOLOGY
SCHOOL OF PUBLIC HEALTH
UNIVERSITY OF MICHIGAN
ANN ARBOR, MI 48109

(b) (6), PHD, DVM
DISTINGUISHED PROFESSOR OF VIROLOGY
DEPARTMENT OF COMPARATIVE PATHOBIOLOGY
COLLEGE OF VETERINARY MEDICINE
PURDUE UNIVERSITY
WEST LAFAYETTE, IN 47907

(b) (6), SCD
PROFESSOR
DEPARTMENT OF GLOBAL HEALTH
MILKEN INSTITUTE SCHOOL OF PUBLIC HEALTH
GEORGE WASHINGTON UNIVERSITY
WASHINGTON, DC 20052

(b) (6), MD
PROFESSOR
INSTITUTE FOR GLOBAL HEALTH
DEPARTMENT OF MEDICINE AND BIOMEDICAL ENGINEERING
NORTHWESTERN UNIVERSITY
CHICAGO, IL 60611

(b) (6), PHD
CHAIR AND PROFESSOR
DEPARTMENT OF MICROBIOLOGY
MOUNT SINAI SCHOOL OF MEDICINE
NEW YORK, NY 10029

(b) (6), PHD
PROFESSOR OF VIROLOGY
DEPARTMENT OF POPULATION HEALTH
COLLEGE OF VETERINARY MEDICINE
UNIVERSITY OF GEORGIA
ATHENS, GA 30602

(b) (6)., PHD
UNIVERSITY DISTINGUISHED PROFESSOR
BIODIVERSITY INSTITUTE
UNIVERSITY OF KANSAS
LAWRENCE, KS 66045

(b) (6) PHD
PROFESSOR
COORDINATING CENTERS FOR BIOMETRIC RESEARCH
DIVISION OF BIOSTATISTICS
UNIVERSITY OF MINNESOTA
MINNEAPOLIS, MN 55455

(b) (6), PHD
PROFESSOR
DEPARTMENT OF EPIDEMIOLOGY
SCHOOL OF PUBLIC HEALTH
UNIVERSITY OF CALIFORNIA LOS ANGELES
LOS ANGELES, CA 90095

(b) (6) PHD
PROFESSOR
DEPARTMENT OF BIOLOGY
UNIVERSITY OF VERMONT
BURLINGTON, VT 05405-0086

(b) (6), PHD
PROFESSOR OF VIROLOGY
INSTITUTE FOR VIROLOGY AND IMMUNOLOGY
UNIVERSITY OF BERN
BERN
SWITZERLAND

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(b) (6), MD, PHD
ASSISTANT PROFESSOR OF MEDICINE
INDIANA UNIVERSITY SCHOOL OF MEDICINE
INDIANA UNIVERSITY
NDIANAPOLIS, IN 46202

(b) (6), PHD
PROFESSOR
BIOSCIENCES DIVISION
UNIVERSITY OF ARKANSAS FOR MEDICAL SCIENCES
LITTLE ROCK, AR 72205

(b) (6), PHD
PROFESSOR
DEPARTMENT OF BIOLOGY
UNIVERSITY OF TEXAS AT SAN ANTONIO
SAN ANTONIO, TX 78249

SCIENTIFIC REVIEW OFFICER

(b) (6), PHD
SCIENTIFIC REVIEW OFFICER
SCIENTIFIC REVIEW PROGRAM
DIVISION OF EXTRAMURAL ACTIVITIES
NATIONAL INSTITUTES OF HEALTH/NIAID
BETHESDA, MD 20892

PROGRAM REPRESENTATIVE

(b) (6), PHD
PROGRAM OFFICER
VIROLOGY BRANCH
DIVISION OF MICROBIOLOGY AND INFECTIOUS DISEASES
NATIONAL INSTITUTES OF HEALTH
ROCKVILLE, MD 20892

GRANTS MANAGEMENT REPRESENTATIVE

(b) (6)
GRANTS MANAGEMENT SPECIALIST
GRANTS MANAGMENT OFFICE
DIVISION OF EXTRAMURAL ACTIVITIES
NATIONAL INSTITUTES OF HEALTH/NIAID
ROCKVILLE, MD 20892

OTHER REVIEW STAFF

(b) (6)., MS
CONTRACT PEER REVIEW SPECIALIST
SCIENTIFIC REVIEW PROGRAM
DIVISION OF EXTRAMURAL ACTIVITIES
NATIONAL INSTITUTES OF HEALTH, NIAID
ROCKVILLE, MD 20892

Consultants are required to absent themselves from the room during the review of any application if their presence would constitute or appear to constitute a conflict of interest.

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From: (b) (6)
To: Laing, Eric; (b) (6)
Cc: (b) (6) ; Chris Broder; (b) (6)
Subject: EIDRC impact scores from NIH
Date: Monday, November 18, 2019 9:26:16 PM
Attachments: Study section roster 1573258030732.pdf
EIDRC Southeast Asia v7 FINAL_FINAL.pdf
Importance: High

Dear All,

Just to let you know that we've got the results back from NIH. EHA submitted two EIDRC proposals, and we were linked to a third one as a subcontract. Out of these, the only one to get a score was the Southeast Asia EIDRC (EID-SEARCH) which you are all collaborators on. I also know of another good group that wasn't scored, so just to get a score is actually a good result…

Our 'impact score' was (b) (6), which normally would put us right on the edge of being funded. I've already contacted the NIAID program officer ((b) (6)) to ask about the likelihood of being funded. She told me that NIH staff are currently working out the structure of the network and a funding plan. She felt our proposal was well-received and scored well enough to be considered, and she asked me to hold off for a week or two and then they'd know more.

My take on this is that they're now deciding what the geography and scope of the network should be, how many EIDRC centers they can afford to fund, and then they'll decide priorities for funding. With this being strongly driven by NIAID's internal concerns about their reach into different geographies, it's really going to be a decision driven by their own considerations of who else is in the mix and what strengths/weaknesses different proposals have. That means we're in with a chance, but we don't really know the factors driving a decision.

I'll contact NIAID again in a few days, but in the meantime, please do the following: 1) try not to share this information with colleagues because we don't want to have a situation where they scored better than us and we got funded, leading to a bad feeling (or worse, the other way round!); 2) if you meet people from NIAID, tell them how keen and enthusiastic you are about the proposal and the work we're conducting (I've attached the final text again as a reminder) – you never know how this might help. Key people at NIAID who will be making this decision are: (b) (6) (head of NIAID), (b) (6) (Deputy Head, and in charge of this line of work), (b) (6) (Program Officer directly in charge of the EIDRCs), (b) (6) (also directly in charge of this work), (b) (6) (I think she's involved).

Finally – I've attached the Review Committee Roster for you to look at who the people are who either liked or disliked our proposal! I know a few of them, and I'm sure you know the others…

Will be in touch as soon as I hear more.

Cheers,

(b) (6)

[redacted]

[redacted]

[redacted]

[redacted]

From: (b) (6)
Sent: Friday, June 28, 2019 8:59 PM
To: 'Laing, Eric'; (b) (6)
Cc: (b) (6) 'Chris Broder'; (b) (6)
Subject: EIDRC grant submitted
Importance: High

Dear All,

Just to let you all know that the grant was successfully submitted today with time to spare, and no errors. It's in the system, and it's all down to the reviewers now! Thanks to all of you for your help and support in getting this finalized and completed. Please pass on my personal thanks to the Co-Investigators, Key Personnel and Consultants who you also brought into the team.

I've attached a pdf of the final proposal text and will send the Word version in a few minutes. It's actually a good read, and I'm especially grateful to (b) (6) who generated all the cool graphics, based largely on (b) (6)'s previous NIH grant proposals.

As you read the text, please remember that the wording is very carefully targeted to a typical US-based NIH reviewer, and to the Program Officers, with the sole purpose of trying to win the grant. If I've exaggerated or made mistakes, or used language that isn't quite right, I apologize, but I did it for the key goal of getting funded.

In the meantime, please don't share this proposal beyond our group on this email chain, so we don't give our competitors an edge!

Good luck, and I'll be keeping my fingers crossed all summer in the hope that we win this…

Cheers,

(b) (6)

[redacted]

[redacted]

[redacted]

[redacted]

From: (b) (6)
Sent: Wednesday, June 26, 2019 5:19 PM
To: 'Laing, Eric'; (b) (6)
Cc: (b) (6) ; Chris Broder; (b) (6)
Subject: EID-SEARCH Comments

Thanks everyone for sending the comments back. Working on v5 now, while others in the office are cranking out sample size calculations, new figures, human and vert. subjects and all the additional information needed after the research plan.

As I go through all your comments, I'll send the occasional email with questions to clarify specific points, so please be on standby for a quick turnaround.

Cheers,

(b) (6)

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[redacted]

From: Laing, Eric [redacted]
Sent: Wednesday, June 26, 2019 12:59 PM
To: [redacted]
Cc: [redacted]
Chris Broder; [redacted]
Subject: Re: EID-SEARCH v4

Hi [redacted],

Built on top of [redacted]' suggestions. Included a prelimin data figure from Hughes et al, in prep. Double check that is ok with [redacted]?

-[redacted]

[redacted]

[redacted]

On Wed, Jun 26, 2019 at 11:26 AM [redacted] > wrote:

> Hi [redacted],

I have added my edits and comments to (b) (6)s.

Please let me know if you ave any questions or need more details.

Thanks.

(b) (6)

From: (b) (6) >
Sent: 26 June 2019 2:51 PM
To: (b) (6)
Cc: (b) (6) ; Chris Broder; Eric Laing; (b) (6)
Subject: Re: EID-SEARCH v4

(b) (6), v4 with my edits, some revised figs, and additional comments.

Cheers,
(b) (6)

On Jun 25, 2019, at 9:26 AM, (b) (6) > wrote:

Dear All,

Here's version 4. Apologies for the delay. It's in better shape now, but there are still lots of areas that need your attention. Please put in time today, and tomorrow to edit and firm up the text.

I've put your names in some of the comment boxes where I need specific help but I also need you to read this critically now and edit and tighten it up. We have 30 pages of space, and our research plan is now 21 pages, so we need to lose maybe 3 or 4 pages. There are also some weak points, specifically on Filoviruses, on the details of where we'll do our clinical work, and on what we'll do with the animal models for henipas and filos.

Please get comments back with me before Wednesday 26th 4pm New York time, so that I have Wendesday evening and all day Thursday to incorporate them. In the meantime, I'll be working with folks here on better figures, and all the extra sections that come after the research plan. I'll need you all to help with those on Thursday while I'm finishing off the draft.

Thanks to all of you in advance and look forward to the next round.

Cheers,

(b) (6)

[redacted]

[redacted]

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[redacted]

From: (b) (6)
Sent: Thursday, June 20, 2019 9:41 PM
To: (b) (6) ; Christopher Broder
(b) (6)
Cc: (b) (6)
Subject: EIDRC-SEA v.3
Importance: High

Dear all,

Thanks to everyone for comments and text for the earlier draft at the beginning of the week. (b) (6)'s spent the last couple of days pulling all the comments together into one draft and inserting his changes, and I've now attached a v3 of the draft. It's still rough, and now too long, but it's getting there. I wanted you to look at this rough version because I'm stuck in Liberia in meetings Friday, then flying back home Saturday. I'll continue editing this draft on the plane and will be working solely on this grant from home on Sunday, Monday, Tues, Wed, Thurs.

Please work on this rapidly if possible. If you can all push your comments through, using track changes, and get them back to me **by Sunday evening New York Time**, I will turn round a polished draft by Tuesday, giving us time for significant last go-over on Wed, Thurs. It's tight, but unfortunately, it is what it is, so please commit the time if you can!! As well as general edits (using track changes, with references in comment boxes), please do the following…

(b) (6) – We especially need your input in section 1.1, 1.3, 1.4 and 2.5

(b) (6) v - Please work your magic on this draft, inserting text, editing and reducing text, and making sure the technical details of the characterization are correct and fit the proposed work on CoVs, PMVs and Henipaviruses

(b) (6) – congratulations on your (b) (6) and thanks for working on it at this time!
(b) (6) – please help and edit/finesse the language in the relevant sections for you

(b) (6) – Your edits crossed over with (b) (6)'s changes, so please insert all the bits that were new in the last version you sent to me, into this version. I think these are mainly the sections and comments from the other Malaysian colleagues, but please check and re-insert all key text. We especially need to be specific about which partner in Malaysia is doing what cohorts, and where, and about who's doing the testing

Thanks again all, and I'm looking forward to non-stop work on this as soon as I get on my flight in 24 hours…

Cheers,

(b) (6)

[redacted]

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[redacted] (b) (6) [redacted]

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<EIDRC Southeast Asia v4.docx>

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From: (b) (6)
To: Christopher Broder
Subject: Nipah R01
Date: Tuesday, September 24, 2019 6:01:09 PM
Attachments: Reviewer summary 1R01AI143978-01A1.pdf
Nipah virus R01 Research Strategy 2018_12p_R1_Dec 7_final_no refs.docx

---

(b) (6),

I'm going to resubmit our Nipah R01 on Oct 7th. I've had extensive discussions with (b) (6) about it - it got really close to being funded, but now it's got to go back in as a new submission.

Given your stellar track record with NIH, would you be willing to go through it and the reviewer comments with me and let me know what you think we could change to get it funded?

(b) (6)

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From: (b) (6)
To: Chris Broder; Eric Laing
Subject: Panel Review of our GHERI FY18 submission
Date: Tuesday, July 16, 2019 1:17:22 PM
Attachments: GHERI (b) (6) 2018_summarystatement.pdf
ATT00002.bin

(b) (6),

Attached is the panel summary statement from our GHERI proposal we submitted on CoV assay development, and MERS and bat borne CoV surveillance.

Disappointing we didn't get this, and unfortunately MERS isn't listed as a priority for FY19, so not sure this will be worth putting back in. Let me know your thoughts after reviewing the FY19 priorities.

In any case, Reviewer 1 was most critical, Reviewers 2 and 3 ranked it quite high overall. The main criticism were related to the assay development, validation, justifying the need for additional MERS/CoV serological assays, and our study design (i.e. not doing it in Saudi Arabia, and sampling unexposed people with an additional assay for validation).

Cheers,
(b) (6)

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From: (b) (6)
To: Laing, Eric; (b) (6)
Cc: (b) (6); Chris Broder; (b) (6)
Subject: EID-SEARCH Word version of proposal
Date: Friday, June 28, 2019 9:00:08 PM
Attachments: EIDRC Southeast Asia v7 FINAL_FINAL.docx
Importance: High

..and here's the word version so you can use text for other purposes...

Cheers,

(b) (6)

[redacted]

[redacted]

[redacted]

[redacted]

From: (b) (6)
Sent: Wednesday, June 26, 2019 5:19 PM
To: 'Laing, Eric'; (b) (6)
Cc: (b) (6); Chris Broder; (b) (6)
Subject: EID-SEARCH Comments

Thanks everyone for sending the comments back. Working on v5 now, while others in the office are cranking out sample size calculations, new figures, human and vert. subjects and all the additional information needed after the research plan.

As I go through all your comments, I'll send the occasional email with questions to clarify specific points, so please be on standby for a quick turnaround.

Cheers,

(b) (6)

[redacted]

[redacted]

[redacted]

[redacted]

From: Laing, Eric (b) (6)
Sent: Wednesday, June 26, 2019 12:59 PM
To: (b) (6)
Cc: (b) (6)
Chris Broder; (b) (6)
Subject: Re: EID-SEARCH v4

Hi (b) (6),

Built on top of (b) (6)' suggestions. Included a prelimin data figure from Hughes et al, in prep. Double check that is ok with Tom?

- (b) (6)

[redacted]

(b) (6)

On Wed, Jun 26, 2019 at 11:26 AM (b) (6) >
wrote:

> Hi (b) (6),
>
> I have added my edits and comments to Kevin's.
>
> Please let me know if you ave any questions or need more details.
>
> Thanks.
>
> (b) (6)

From: (b) (6) >
Sent: 26 June 2019 2:51 PM
To: (b) (6)
Cc: (b) (6) ; Chris Broder; Eric Laing; (b) (6)
Subject: Re: EID-SEARCH v4

(b) (6), v4 with my edits, some revised figs, and additional comments.

Cheers,
(b) (6)

(b) (6) [redacted]

On Jun 25, 2019, at 9:26 AM, (b) (6) [redacted] > wrote:

Dear All,

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Thanks to all of you in advance and look forward to the next round.

Cheers,

(b) (6)

[redacted]

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(b) (6)

From: (b) (6)
Sent: Thursday, June 20, 2019 9:41 PM
To: (b) (6) ; Christopher Broder (b) (6) ; Eric Laing; (b) (6)
Cc: (b) (6)
Subject: EIDRC-SEA v.3
Importance: High

Dear all,

Thanks to everyone for comments and text for the earlier draft at the beginning of the week. (b) (6)'s spent the last couple of days pulling all the comments together into one draft and inserting his changes, and I've now attached a v3 of the draft. It's still rough, and now too long, but it's getting there. I wanted you to look at this rough version because I'm stuck in Liberia in meetings Friday, then flying back home Saturday. I'll continue editing this draft on the plane and will be working solely on this grant from home on Sunday, Monday, Tues, Wed, Thurs.

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From: (b) (6)
To: Laing, Eric; (b) (6)
Cc: (b) (6) ; Chris Broder; (b) (6)
Subject: EIDRC grant submitted
Date: Friday, June 28, 2019 8:59:44 PM
Attachments: EIDRC Southeast Asia v7 FINAL_FINAL.pdf
Importance: High

Dear All,

Just to let you all know that the grant was successfully submitted today with time to spare, and no errors. It's in the system, and it's all down to the reviewers now! Thanks to all of you for your help and support in getting this finalized and completed. Please pass on my personal thanks to the Co-Investigators, Key Personnel and Consultants who you also brought into the team.

I've attached a pdf of the final proposal text and will send the Word version in a few minutes. It's actually a good read, and I'm especially grateful to (b) (6) who generated all the cool graphics, based largely on (b) (6)'s previous NIH grant proposals.

As you read the text, please remember that the wording is very carefully targeted to a typical US-based NIH reviewer, and to the Program Officers, with the sole purpose of trying to win the grant. If I've exaggerated or made mistakes, or used language that isn't quite right, I apologize, but I did it for the key goal of getting funded.

In the meantime, please don't share this proposal beyond our group on this email chain, so we don't give our competitors an edge!

Good luck, and I'll be keeping my fingers crossed all summer in the hope that we win this…

Cheers,

(b) (6)

[redacted]

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[redacted]

(b) (6)

[redacted]

From: (b) (6)
Sent: Wednesday, June 26, 2019 5:19 PM
To: 'Laing, Eric'; (b) (6)
Cc: (b) (6) Chris Broder; (b) (6)
Subject: EID-SEARCH Comments

Thanks everyone for sending the comments back. Working on v5 now, while others in the office are cranking out sample size calculations, new figures, human and vert. subjects and all the additional information needed after the research plan.

As I go through all your comments, I'll send the occasional email with questions to clarify specific points, so please be on standby for a quick turnaround.

Cheers,

(b) (6)

[redacted]

[redacted]

[redacted]

[redacted]

From: Laing, Eric (b) (6)
Sent: Wednesday, June 26, 2019 12:59 PM
To: (b) (6)
Cc: (b) (6) ;

Chris Broder; (b) (6)

Subject: Re: EID-SEARCH v4

Hi (b) (6),

Built on top of (b) (6)' suggestions. Included a prelimin data figure from Hughes et al, in prep. Double check that is ok with (b) (6)?

(b) (6)

On Wed, Jun 26, 2019 at 11:26 AM (b) (6) > wrote:

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>
> I have added my edits and comments to (b) (6)'s.
>
> Please let me know if you ave any questions or need more details.
>
> Thanks.
>
> (b) (6)

From: (b) (6)
Sent: 26 June 2019 2:51 PM
To: (b) (6)
Cc: (b) (6); Chris Broder; Eric Laing; (b) (6)
Subject: Re: EID-SEARCH v4

(b) (6), v4 with my edits, some revised figs, and additional comments.

Cheers,
(b) (6)

[redacted]

[redacted]

[redacted]

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From: (b) (6)
Sent: Thursday, June 20, 2019 9:41 PM
To: (b) (6) [redacted] hristopher Broder
(b) (6) ; Eric Laing; (b) (6) '
Cc: (b) (6) [redacted]
Subject: EIDRC-SEA v.3
Importance: High

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From: Laing, Eric (b) (6)
To: (b) (6)
Cc: (b) (6) ; Chris Broder; (b) (6)
Subject: Re: EID-SEARCH v4
Date: Wednesday, June 26, 2019 12:59:39 PM
Attachments: EIDRC Southeast Asia v4-(b) (6) .docx

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Subject: Re: EID-SEARCH v4

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From: (b) (6)
To: (b) (6)
Cc: (b) (6) Chris Broder; Eric Laing; (b) (6)
Subject: RE: EID-SEARCH v4
Date: Wednesday, June 26, 2019 4:51:04 AM
Attachments: EIDRC Southeast Asia v4(b) (6) .docx

Hi (b) (6),

Here is the rest of my edits. I have to send this through now and go back to the other required documents to send back to (b) (6).

From: (b) (6)]
Sent: Wednesday, 26 June, 2019 2:51 PM
To: (b) (6)
Cc: (b) (6) Chris Broder (b) (6) >; Eric Laing (b) (6)
Subject: Re: EID-SEARCH v4
Importance: High

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Sent: Thursday, June 20, 2019 9:41 PM
To: (b) (6) [redacted] Christopher Broder (b) (6) [redacted] ; Eric Laing; (b) (6) [redacted]
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Cc: (b) (6) ; Chris Broder; Eric Laing (b) (6)
Subject: RE: EID-SEARCH v4
Date: Wednesday, June 26, 2019 2:58:21 AM
Attachments: EIDRC Southeast Asia v4 (b) (6) .docx

I was working on this at the same time as (b) (6), so I will stop here on this version.
I will continue from where I left off using (b) (6) new version

---

From: (b) (6)]
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Cheers,
(b) (6)

On Jun 25, 2019, at 9:26 AM, (b) (6) wrote:

Dear All,

Here's version 4. Apologies for the delay. It's in better shape now, but there are still lots of areas that need your attention. Please put in time today, and tomorrow to edit and firm up the text. I've put your names in some of the comment boxes where I need specific help but I also need you to read this critically now and edit and tighten it up. We have 30 pages of space, and our research plan is now 21 pages, so we need to lose maybe 3 or 4 pages. There are also some weak points, specifically on Filoviruses, on the details of where we'll do our clinical work, and on what we'll do with the animal models for henipas and filos.

Please get comments back with me before Wednesday 26th 4pm New York time, so that I have Wendesday evening and all day Thursday to incorporate them. In the meantime, I'll be working with folks here on better figures, and all the extra sections that come after the research plan. I'll need you all to help with those on Thursday while I'm finishing off the draft.

Thanks to all of you in advance and look forward to the next round.

Cheers,

(b) (6)

[redacted]

From: (b) (6)
Sent: Thursday, June 20, 2019 9:41 PM
To: (b) (6) Christopher Broder (b) (6)
Cc: (b) (6)
Subject: EIDRC-SEA v.3
Importance: High

Dear all,

Thanks to everyone for comments and text for the earlier draft at the beginning of the week. (b) (6)'s spent the last couple of days pulling all the comments together into one draft and inserting his changes, and I've now attached a v3 of the draft. It's still rough, and now too long, but it's getting there. I wanted you to look at this rough version because I'm stuck in Liberia in meetings Friday, then flying back home Saturday. I'll continue editing this draft on the plane and will be working solely on this grant from home on Sunday, Monday, Tues, Wed, Thurs.

Please work on this rapidly if possible. If you can all push your comments through, using track changes, and get them back to me **by Sunday evening New York Time**, I will turn round a polished draft by Tuesday, giving us time for significant last go-over on Wed, Thurs. It's tight, but unfortunately, it is what it is, so please commit the time if you can!! As well as general edits (using track changes, with references in comment boxes), please do the following…

(b) (6) – We especially need your input in section 1.1, 1.3, 1.4 and 2.5

(b) (6) - Please work your magic on this draft, inserting text, editing and reducing text, and making sure the technical details of the characterization are correct and fit the proposed work on CoVs, PMVs and Henipaviruses

(b) (6) – congratulations on your (b) (6) and thanks for working on it at this time! (b) (6) – please help and edit/finesse the language in the relevant sections for you

(b) (6) – Your edits crossed over with (b) (6)'s changes, so please insert all the bits that were new in the last version you sent to me, into this version. I think these are mainly the sections and comments from the other Malaysian colleagues, but please check and re-insert all key text. We especially need to be specific about which partner in Malaysia is doing what cohorts, and where, and about who's doing the testing

Thanks again all, and I'm looking forward to non-stop work on this as soon as I get on my flight in 24 hours…

Cheers,

(b) (6)

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<EIDRC Southeast Asia v4.docx>

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From: (b) (6)
To: (b) (6) ; Christopher Broder (b) (6)]; Eric Laing; (b) (6) s
Cc: (b) (6)
Subject: Re: CORRECTION - please ignore last email!!! re. EIDRC-SEA v.3
Date: Sunday, June 23, 2019 6:45:45 AM
Attachments: EIDRC Southeast Asia v3 (b) (6)).docx

Hi (b) (6) (and all),

Here are mine and (b) (6) 's edits. I will have to add more after some of the CoV stuff has been removed. At the moment it is too CoV specific (I know we took a lot of text from our other grant proposal), but can some of this be removed before we add in the final filo/paramyxo info- see my comments.

In the next version, can you delete the tracked comments if they have been answered or corrected in the text? I didn't delete any comments.

I should have time on Monday afternoon (SG time) to look at this again.

Cheers,
(b) (6)

From: (b) (6)
Sent: 21 June 2019 10:45 AM
To: (b) (6)
Christopher Broder (b) (6)); Eric Laing; (b) (6)
Cc: (b) (6)
Subject: CORRECTION - please ignore last email!!! re. EIDRC-SEA v.3

Many apologies – I sent the wrong attachment – here is the correct version!!!

Dear all,

Thanks to everyone for comments and text for the earlier draft at the beginning of the week. (b) (6) 's spent the last couple of days pulling all the comments together into one draft and inserting his changes, and I've now attached a v3 of the draft. It's still rough, and now too long, but it's getting there. I wanted you to look at this rough version because I'm stuck in Liberia in meetings Friday, then flying back home Saturday. I'll continue editing this draft on the plane and will be working solely on this grant from home on Sunday, Monday, Tues, Wed, Thurs.

Please work on this rapidly if possible. If you can all push your comments through, using track changes, and get them back to me **by Sunday evening New York Time**, I will turn round a polished draft by Tuesday, giving us time for significant last go-over on Wed, Thurs. It's tight, but unfortunately, it is what it is, so please commit the time if you can!! As well as general edits (using track changes, with references in comment boxes), please do the following…

(b) (6) – We especially need your input in section 1.1, 1.3, 1.4 and 2.5

(b) (6) - Please work your magic on this draft, inserting text, editing and reducing text, and making sure the technical details of the characterization are correct and fit the proposed work on CoVs, PMVs and Henipaviruses

(b) (6) – congratulations on your (b) (6) and thanks for working on it at this time!
(b) (6) – please help and edit/finesse the language in the relevant sections for you

(b) (6) – Your edits crossed over with (b) (6)'s changes, so please insert all the bits that were new in the last version you sent to me, into this version. I think these are mainly the sections and comments from the other Malaysian colleagues, but please check and re-insert all key text. We especially need to be specific about which partner in Malaysia is doing what cohorts, and where, and about who's doing the testing

Thanks again all, and I'm looking forward to non-stop work on this as soon as I get on my flight in 24 hours…

Cheers,

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From: (b) (6)
To: (b) (6) Christopher Broder (b) (6) ; Eric Laing; (b) (6)
Cc: (b) (6)
Subject: EIDRC-SEA v.3
Date: Thursday, June 20, 2019 9:40:59 PM
Attachments: NEIDL EIDRC EHA proposed work v3.docx
Importance: High

Dear all,

Thanks to everyone for comments and text for the earlier draft at the beginning of the week. (b) (6)'s spent the last couple of days pulling all the comments together into one draft and inserting his changes, and I've now attached a v3 of the draft. It's still rough, and now too long, but it's getting there. I wanted you to look at this rough version because I'm stuck in Liberia in meetings Friday, then flying back home Saturday. I'll continue editing this draft on the plane and will be working solely on this grant from home on Sunday, Monday, Tues, Wed, Thurs.

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(b) (6) – congratulations on your (b) (6) and thanks for working on it at this time (b) (6) – please help and edit/finesse the language in the relevant sections for you

(b) (6) – Your edits crossed over with (b) (6)'s changes, so please insert all the bits that were new in the last version you sent to me, into this version. I think these are mainly the sections and comments from the other Malaysian colleagues, but please check and re-insert all key text. We especially need to be specific about which partner in Malaysia is doing what cohorts, and where, and about who's doing the testing

Thanks again all, and I'm looking forward to non-stop work on this as soon as I get on my flight in 24 hours…

Cheers,

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From: Laing, Eric (b) (6)
To: (b) (6)
Cc: Broder, Christopher
Subject: Re: (b) (6) collab at DukeNUS
Date: Tuesday, June 18, 2019 8:44:21 PM
Attachments: EIDRC Southeast Asia v2(b)(6).docx

Hey (b) (6)

Have a look at some of the proposal suggestions. My (b) (6). I'm running on fumes right now and having difficulty finding time to incorporate suggestions, apologies if the word-dump is disconnected. If these are on point I'll send over some accompanying data figures you can embed.

(b) (6)

[redacted]

On Sun, Jun 16, 2019 at 2:59 PM (b) (6) > wrote:

> Thanks (b) (6),
>
> As you go through the text of the grant making revisions, can you add in a bit on preliminary data at the appropriate point re. prior collab with DukeNUS with (b) (6). For example, if this work is on developing the Luminex strategy, put it in that section in Aim 1 under preliminary data (section 1.1) and say something like:
>
> "In a collaboration between USUHS and Duke-NUS, Co-Is Laing and Broder have refined and tested the Luminex serology platform on 500 samples of bats and humans with Duke-NUS ((b) (6)). The results suggest that we're all doomed....etc..."
>
> I'll be talking with (b) (6) tonight and will check in with them on how/if to formally include (b) (6) in the proposal.

Cheers,

(b) (6)

[redacted]

From: Laing, Eric (b) (6)
Sent: Monday, June 10, 2019 12:00 PM
To: (b) (6)
Cc: Broder, Christopher
Subject: Re: EIDRC SE Asia V. Rough first draft for edits

Hi (b) (6),

I wanted to pass along the names our two collaborators at Duke-NUS: Drs. (b) (6) and (b) (6). (b) (6) is a Professor in the EID Programme that (b) (6) is chair of and (b) (6) is a Principal Research Scientist in (b) (6)'s lab. (b) (6) and I have worked with (b) (6) and (b) (6) on 2 wildlife/human interface surveillance projects in SE/South Asia and (b) (6) have a large footprint in Cambodia (bats/rats/collaborators). (b) (6) and I also have a small GEIS project thru NAMRU-2 Phnom Penh, Their surveillance projects are independent of (b) (6)'s research at Duke-NUS. I'll leave the decision to fold them into this proposal yours to make. Here are there contact addresses: (b) (6)

Best regards,

(b) (6)

(b) (6)

On Mon, Jun 10, 2019 at 3:59 AM (b) (6) > wrote:

> Dear all – here's the first rough draft.
>
> Please start editing as fast and furious as you all can. I've based this on our previous conversations, so there are lots of bits for you to expand on, insert references etc. Also, you'll see that lots of the text in the Aims is from our recent CoV proposal and will need extending for Henipas, CoVs and Filos, and then editing back on the SARSr-CoV text. Please get stuck into that.
>
> Don't worry about all editing separately – I can collate this, although it is great if some of you go rapidly and others build on theirs.
>
> Main thing – please draft as much as you can, please use TRACK CHANGES, and please don't mess up the ENDNOTE! Prob best for references that you just insert them into comment boxes. It's a bit of a pain, but it's less work than if the whole library decomposes…
>
> Anyway – thanks for being great collaborators, and I look forward to what you send back
>
> NB (b) (6) – please share with your collaborators, and cc me, (b) (6) and (b) (6) so I have their email addresses.
>
> Cheers,

(b) (6)

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(b) (5)

From: (b) (6)
To: (b) (6)); Broder, Christopher; Eric Laing; (b) (6)
Cc: (b) (6)
Subject: RE: Introduction. (b) (6). Regards to Ecohealth NIAID submission.
Date: Friday, June 14, 2019 10:41:38 AM
Attachments: EIDRC SE Asia Specific aims v3.docx

Great – here are the high level aims (still a draft, of course!).

Much appreciate you moving ahead on this.

Cheers,

(b) (6)

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From: (b) (6)
Sent: Wednesday, June 12, 2019 11:41 PM
To: (b) (6) ; Broder, Christopher; Eric Laing; (b) (6)
Cc: (b) (6)
Subject: RE: Introduction. (b) (6). Regards to Ecohealth NIAID submission.

Sounds great. I'm working on another grant with a Friday deadline - so I will put together some language for you this weekend after I get that out (b) (6) is currently traveling, though he is aware of our conversation. He will be back in Thailand next week and I will discuss the potential LOS with him ASAP. Do you have a high level summary and Aims that I can share with (b) (6) for our discussion? I would also use this to draft the LOS.

(b) (6)

From: (b) (6)
Sent: Thursday, June 13, 2019 8:44 AM
To: (b) (6) Broder, Christopher (b) (6) ; Eric Laing (b) (6)
Cc: (b) (6)
Subject: RE: Introduction. (b) (6) . Regards to Ecohealth NIAID submission.
Importance: High

Thanks very much for the rapid response (b) (6), and a pleasure to virtually meet you (b) (6).

I really appreciate your willingness to provide a letter of support and I will absolutely respect your caveats below, of course. I think this is a great opportunity because NIAID are looking for leverage and partnerships, and although there's always that issue about one federal agency not being allowed to fund another, there are plenty of ways we can use this to help build up your capacity through cross training and joint research projects.

I've answered some of your questions in the body of your email below, and again thank you for your openness for collaboration.

Cheers,

(b) (6)

(b) (6)

From: (b) (6)
Sent: Wednesday, June 12, 2019 12:26 AM
To: (b) (6) ; Broder, Christopher; Eric Laing; (b) (6)
Cc: (b) (6)
Subject: RE: Introduction. (b) (6). Regards to Ecohealth NIAID submission.

Hi (b) (6). Thanks for the email. I cannot provide a letter of support individually, but we can consider this as the CDC country office in Thailand.

(b) (6) That's great, because NIAID is looking for us to leverage partnerships

I'm adding Dr. (b) (6) who is filling multiple roles currently, including Director- CDC Thailand/SE Asia regional office, HHS Country Rep for Thailand, and Director for the CDC-Thailand Div. of Global Health Protection.

I am happy to share information to help support your grant, but request this be used for the grant only. The data potentially could be published eventually and I don't want to jeopardize that possibility. What kind of information/figures do you need? What is your deadline for this LOS and this information?

(b) (6) We'll definitely keep this solely for this grant and will maintain confidentiality. Given the space constraints, ideally if you could send a few sentences with some explanation of the site, subjects, number of individuals/other data with a reference, and a figure and I'll insert that or reduce a bit. The deadline is June 28th, but we're trying to get a final draft by the 19th, so if you can get me something by then that would be ideal. (b) (6) will send a sample of the sort of info for the Letter of Support, but it's always best if it's written in your own words…

(b) (6) may have mentioned, our CDC Director recently visited the laboratory and expressed his interest in our office starting some research on bat-borne viruses with the MOPH.

(b) (6) That's very interesting and great news also.

My goal is to make sure the CDC office is positioned to work well with the groups already performing this research in Thailand and not duplicating efforts. I reached out to (b) (6) already and we plan to talk in July when he comes to Thailand for the bat meeting. Are you coming for the meeting as well? If so, it would be great to connect with you while you are here.

(b) (6) That's perfect re. CDC office collaborating on bat research. We've been working with Supaporn for many years, but there is so much to do there's little chance of duplication and tons of opportunity for collaboration. We are definitely interested in supporting your efforts any way we can. I won't be able to be there in July – but I'll check in with others in the office here to see if any of our staff will be there and can meet with you. I'll be in Malaysia in September and maybe that's a chance to meet also.

Again – thanks for your help and I look forward to future collaboration!

(b) (6)

From: (b) (6)
Sent: Saturday, June 8, 2019 3:26 AM
To: Broder, Christopher (b) (6)
Eric Laing (b) (6)
Cc: (b) (6)
Subject: RE: Introduction. (b) (6). Regards to Ecohealth NIAID submission.
Importance: High

Hi (b) (6) and thanks for the introduction (b) (6).

We're pulling together an EIDRC proposal with the goal of funding work to identify evidence of hidden, misdiagnosed, or under-reported spillover events in SE Asia. We're working with (b) (6) in Thailand, (b) (6) in Malaysia and (b) (6) in Singapore. In the USA, we'll be collaborating with (b) (6) and the NEIDL lab (just for sample submission).

As (b) (6) mentioned, I heard about the interesting findings from (b) (6), but wasn't sure what the details are – (b) (6) filled me in. It's particularly interesting to me because our whole premis for this proposal is that viruses like henipaviruses, CoVs and filoviruses are found in wildlife hosts in SE Asia, and likely spillover regularly but the cases are either clinically inconsequential, don't get reported, or get misdiagnosed (e.g. like the original Nipah virus outbreaks in India which were called 'aberrant measles', or like the outbreaks in Bangladesh which we know how occur annually, but prior to 1999 must have been undiagnosed).

We don't have spare funds right now, given that we're pretty close to the deadline and have a full set of partners with their budgets already fixed. But, if you are interested in drafting a letter of support, maybe we could mention the preliminary findings, and make the case that these samples would benefit from further work with this collaborative group. I'm not sure of all the details, but (b) (6) has a good idea of how we could move forwards, and given that we'll be collaborating with him, NEIDL, (b) (6), I'm sure that there would be availability of resources to run the next step of the diagnostic path to move this to a validated finding. (b) (6) – please let me know if that's correct, or if this is a case of running SNTs, which we could do through NEIDL.

If you were able to sign on, we would certainly appreciate your involvement, and make sure that there would be opportunity for co-authorship of some of the work we're planning, as well as trying to mobilize the findings that you've already made..

Cheers,

(b) (6)

(b) (6)

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From: Broder, Christopher (b) (6)
Sent: Thursday, June 6, 2019 3:35 PM
To: (b) (6) ; Eric Laing
Subject: Introduction. (b) (6). Regards to Ecohealth NIAID submission.

Hey (b) (6),

I know you are only just getting back to Bangkok.
(b) (6) and I just had another TelCon with (b) (6) (cc'd here)

(b) (6) is going to submit this RFA:

Letter of Intent: RFA-AI-19-028 Emerging Infectious Diseases Research Centers
This letter of intent indicates that we plan to submit a proposal in response to the FOA RFA-AI-19-028 "Emerging Infectious Diseases Research Centers". Below are the details requested in the RFA:

Descriptive Title of Proposed Activity
Understanding risk of zoonotic virus emergence in EID hotspots of Southeast Asia

(b) (6) is a part of this network. (b) (6) and I will be as well but with only a small budget.
In his chat with (b) (6) she mentioned preliminary data of NiV spillover evidence in children in Thailand.
This is your data, with (b) (6). I briefly explained that and how it was left unpublished and told him
you and I chatted again about that just yesterday when discussing the CDC NiV ELISA vs Bioplex in
her bat samples (perhaps we should by some of those ELISAs)

I suggested to (b) (6) that he include you and your folks in this proposal. and also mention that DTRA wants to fund you and get you started as well.

(b) (6) can share with you the LOI and other documents and perhaps have a chat with you.

cheers

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From: (b) (6)
To: (b) (6) Christopher Broder (b) (6) ; Eric Laing (b) (6)
Cc: (b) (6)
Subject: EIDRC SE Asia V. Rough first draft for edits
Date: Monday, June 10, 2019 3:59:31 AM
Attachments: EIDRC Southeast Asia v2.docx
Importance: High

Dear all – here's the first rough draft.

Please start editing as fast and furious as you all can. I've based this on our previous conversations, so there are lots of bits for you to expand on, insert references etc. Also, you'll see that lots of the text in the Aims is from our recent CoV proposal and will need extending for Henipas, CoVs and Filos, and then editing back on the SARSr-CoV text. Please get stuck into that.

Don't worry about all editing separately – I can collate this, although it is great if some of you go rapidly and others build on theirs.

Main thing – please draft as much as you can, please use TRACK CHANGES, and please don't mess up the ENDNOTE! Prob best for references that you just insert them into comment boxes. It's a bit of a pain, but it's less work than if the whole library decomposes…

Anyway – thanks for being great collaborators, and I look forward to what you send back

NB (b) (6) – please share with your collaborators, and cc me, (b) (6) so I have their email addresses.

Cheers,

(b) (6)

(b) (6) [redacted]

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From: (b) (6)
To: (b) (6)
Christopher Broder; (b) (6)
Subject: Nipah dynamics manuscript re-submitted to Science Advances
Date: Wednesday, February 13, 2019 2:22:55 AM
Attachments: Nipah virus dynamics in bats_manuscript + suppl_02132019.docx

---

All,
I wanted to update you that our manuscript on Nipah virus dynamics in bats is being re-submitted to *Science Advances*. Although it was originally rejected, the reviews were positive enough that the Deputy Editor has agreed to receive the revised manuscript as a new submission and send it out again for review. Thank you all for your input into this manuscript in its various stages of development. I'll follow up once we receive reviewers' comments.

Attached is the submitted version.

Cheers,
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From: [redacted]
To: [redacted]
Cc: Christopher Broder; Laing, Eric
Subject: Sub-Award Documents on behalf of Drs Laing and Broder
Date: Wednesday, December 19, 2018 3:46:04 PM
Attachments: Budget Justification_[redacted]_CGHE_Fina[redacted].docx
Research and Related Budget[redacted]_CGHE.pdf
Performance Site[redacted].docx
CC[redacted].Biosketch-NIH-EcoH-GHERI-120718.docx
CC[redacted]-USU-EcoH-LOC-Dec2018.pdf
SOW_[redacted]Lab-USU_VP-18-011 (GHERI FY18 FOA) v2.docx
[redacted]_Biosketch_GHERI v2.docx

Hello all,

Please accept the following documents for Drs [redacted]' contribution to the CGHE GHERI award.

Once we hear back from USU-VPR with their review, we will follow up.

If you have any questions, please let me know.

Thanks much .... [redacted]

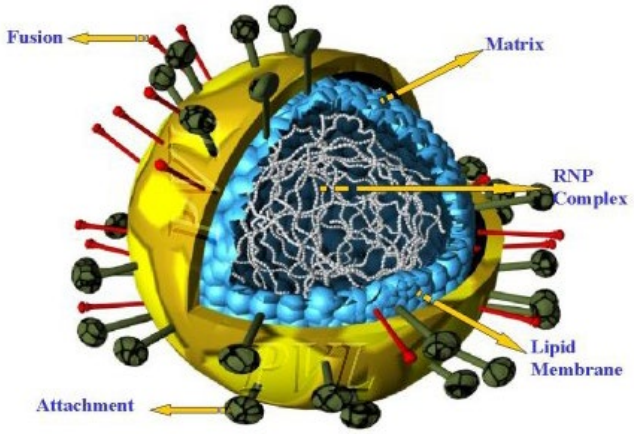
[redacted]

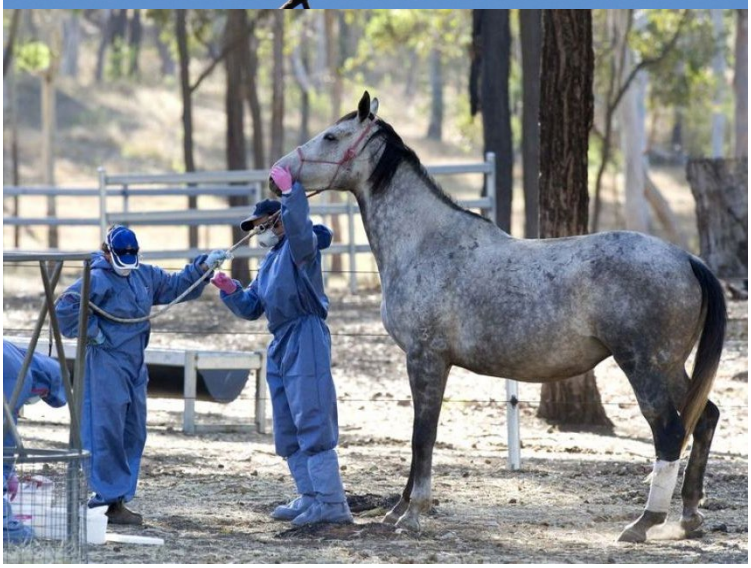
United States Department of Agriculture

Agricultural Research Service

November 2018

Henipavirus Gap Analysis
Workshop Report





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EXECUTIVE SUMMARY

Henipavirus is the taxonomic genus for a group of viruses in the family Paramyxoviridae that includes *Hendra virus* (HeV) and *Nipah virus* (NiV). These viruses are zoonotic agents that are highly pathogenic in humans with case fatality rates of 40% to 70%. As such, these viruses are classified as Biosafety Level 4 (BSL-4) agents, requiring the highest level of laboratory biocontainment. Importantly, they have many of the physical attributes to serve as potential agents of bioterrorism, and are also considered emerging zoonotic pathogens with increasing geographical distribution in Australia, New Caledonia, Southeast Asia, and Madagascar.

Hendra virus first emerged in 1994 in Australia spilling over from bats to horses to humans, causing several disease outbreaks since with significant fatality rates. Nipah virus emerged in Malaysia in 1999, resulting in nearly 300 human cases with over 100 deaths.

The Nipah virus outbreak in Malaysia was especially concerning, causing widespread panic and fear because of the high mortality rate in people and the inability to control the disease initially. There were also considerable social disruptions and tremendous economic loss to an important pig-rearing industry. This highly virulent virus, believed to be introduced into pig farms by fruit bats, spread easily and silently among pigs and was transmitted to humans who came into close contact with infected animals. A NiV outbreak in Bangladesh in 2001 resulted from direct bat to human transmission via contaminated date palm juice with further spread within the human population. From 2001 to 2012, the World Health Organization (WHO) reported a total of 209 cases, with 161 deaths due to of NiV infections. In 2014, the WHO reported a NiV outbreak in fourteen districts of Bangladesh, resulting in 24 cases and 21 deaths. In 2015, three fruit bats tested positive for NiV in New Caledonia at the Noumea National Park, including three bats at the Noumea Zoo.

This gap analysis report focuses primarily on NiV and its potential impact on agricultural swine production. However, information is also provided on the threat henipaviruses pose to public health, both as emerging zoonotic agents and as potential agents of bioterrorism. Included in this report is scientific information on *Henipavirus* virology, epidemiology, pathogenesis, immunology, and an assessment of the available veterinary medical countermeasures to detect, prevent, and control disease outbreaks. Importantly, gaps are provided to inform research needs and priorities. Some of the major gaps and obstacles for disease control can be summarized as follows:

Diagnostics

The availability of safe laboratory diagnostic tests are limited. Virus isolation and serum neutralization assays require live NiV; thus, BSL-4 containment laboratories are required. Nucleic acid-based assays, such as RT-PCR are available, but genetic variation amongst henipaviruses are reported to impact sensitivity and real time RT-PCR may not be able to detect all divergent and novel henipavirus strains. Serological assays are limited in their ability to differentiate between known and unknown henipaviruses, as cross-reactivity to one or more known viruses is possible. Commercial diagnostic test kits are not available. International standards for NiV assay validation are needed. Gaps include a lack of positive experimental and field samples for test validation (or even evaluation) and there are restrictions on material transfer (e.g., obtaining animal samples that could be used to validate tests) due to biosecurity concerns. Low biosafety level reference sera

against various isolates are not yet available. There is a need for high throughput antibody assays for disease outbreaks, recovery and surveillance purposes. There is also a need to develop operator-safe diagnostics tests and reagents that can be produced in low biocontainment facilities.

Vaccines

There is currently a commercial vaccine available for horses, but there are no vaccines for swine or humans. There are several experimental vaccine candidates that may be safe and effective in swine and other domestic animals. However, all these vaccine candidates will require further research to establish their efficacy, and they will need to be fully developed to be licensed and stockpiled for rapid use in an emergency disease outbreak in swine.

Surveillance

Surveillance is the first line of defense against a disease outbreak. Rapid and accurate detection affects the time when control measures can be implemented and affects the extent of the disease outbreak. Because of limitations with laboratory diagnosis, surveillance programs are dependent on the reporting of clinical signs in populations at risk. Diagnosis of NiV infections based on clinical presentation has a low positive predictive value as there are numerous etiologies for encephalitis in humans, and clinical signs in pigs are difficult to differentiate from many common endemic infectious diseases.

Depopulation

Depopulation is the primary countermeasure to reduce virus shedding and stop the spread of NiV in livestock. Disease outbreaks have shown that the control of NiV in pig populations through stamping out is complex due to the zoonotic nature of the agent. In addition, depopulation may be logistically difficult and may be impossible in a rapidly spreading outbreak in countries where there are pig dense regions with millions of pigs, such as the states of Iowa, North Carolina, and Minnesota in the United States, or South East China.

GROUP PICTURES

Henipavirus Gap Analysis Working Group, Winnipeg, Canada
November 14-17, 2017

The Nipah Virus Countermeasures Working Group, Geelong, Australia
March 17-19, 2009

GLOSSARY

APHIS: Animal and Plant Health Inspection Service, USDA, United States of America

ARS: Agricultural Research Service

AAHL: Australian Animal Health Laboratory

BSL-4: Biosafety Level 4

CDC: U.S. Centers for Disease Control and Prevention, HHS, United States of America

CFIA: Canadian Food Inspection Agency

DIVA: Differentiating between infected and vaccinated animals

ELISA: Enzyme-linked immunosorbent assay

FADDL: U.S Foreign Animal Disease Laboratory, Plum Island Animal Disease Center

FLI: Friedrich Loeffler Institute

GMP: good manufacturing practice

HeV: Hendra virus

HHS: Department of Human Health Services, United States of America

HSPD-9: Homeland Security Presidential Directive Nine

ICAR: Indian Council of Agricultural Research

Ig: Immunoglobulin

IEDCR: Institute of Epidemiology, Disease Control and Research in Bangladesh

MLV: Modified live virus vaccine

NAHLN: National Animal Health Laboratory Network, USA

NIHSAD: National Institute of High Security Animal Diseases, ICAR, India

NCFAD: National Center for Foreign Animal Disease, CFIA, Canada

NiV: Nipah virus

NiV-B: Nipah virus Bangladesh

NiV-M: Nipah virus Malaysia

NiV N: Nipah virus nucleoprotein

NVCWG: Nipah Virus Countermeasures Working Group

NVS: National Veterinary Stockpile

OIE: World Organisation for Animal Health

PCR: Polymerase Chain Reaction.

PPE: Personal Protective Equipment

RT-PCR: Reverse transcription-polymerase chain reaction

rRT-PCR: Real-time reverse transcription-polymerase chain reaction

sHeV G: recombinant soluble Hendra virus G protein

sNiV G: recombinant soluble Nipah virus G protein

USDA: United States Department of Agriculture, United States of America

INTRODUCTION

Nipah virus (NiV) is an emerging zoonotic virus. First isolated in pigs and people from an outbreak in Malaysia in 1998 (Ang *et al.* 2018), this emerging virus causes severe disease in humans. The source of transmission was determined to be from bats to pigs to humans, through close contact with infected animals. The virus is named after the location where it was first detected in Sungai Nipah, a village in the Malaysian Peninsula where exposed pig farmers became severely ill with encephalitis.

Nipah virus is closely related to another zoonotic virus called Hendra virus (HeV), formerly called Equine *Morbillivirus*, and named after the town where it first appeared in Australia. Hendra virus infection was first recognized in 1994, when it caused an outbreak of acute, fatal respiratory disease that killed 14 horses. Three human cases, leading to two deaths were recorded during the outbreak. The precise mode of virus transmission to the three Australian patients is not fully understood. All three individuals appear to have acquired their infection as a result of close contact with horses, which were ill and later died.

Although members of this group of viruses have only caused a few focal outbreaks, their ability to infect a wide range of animal hosts and to produce a high mortality rate in humans has made this emerging zoonotic viral disease a significant public health threat.

Certain species of bats of the genus *Pteropus* (fruit bats, also called flying foxes) are the principal natural reservoir hosts for NiV and HeV – see Table I. Bats are susceptible to infection with these viruses but do not develop disease. Fruit bats are distributed across an area encompassing Australia, Southeast Asia; including Indonesia, Malaysia, the Philippines and some of the Pacific Islands, the Indian subcontinent, and Madagascar (See Fig. 1). There is also growing evidence that viruses related to NiV and HeV circulate in non-pteropid fruit bats across the globe (Clayton, 2017).

The exact mode of transmission of henipaviruses is uncertain, but appears to require close contact with contaminated tissue or body fluids from infected animals. The role of domestic species other than pigs in transmitting NiV infection to other animals has not yet been determined. In 2014, an outbreak was reported in the Philippines involving the consumption of meat from NiV-infected horses, further expanding the potential routes of transmission for henipaviruses.

Despite frequent contact between fruit bats and humans there is no serological evidence of human infection among persons that are in contact with bats. Pigs were the apparent source of infection among most human cases in the Malaysian outbreak of NiV in 1998-1999. Nipah virus has continued to spillover over from animals with at least six outbreaks resulting in human fatalities in Bangladesh in 2013, one in India in 2014, and two in Bangladesh in 2015. The World Health Organization (WHO) had not reported any NIV cases 2016-2017, but in 2018 twenty three new cases and 21 deaths were reported in Kerala, India - See Table II.

The spread of henipaviruses to new geographical areas is a concern. In 2014, the Philippines reported an outbreak with a zoonotic paramyxovirus in horses and people. There is further evidence for broader distribution of NiV in pteropid fruit bats species. There is also growing evidence that viruses related to NiV and HeV also circulate in non-pteropid fruit bats worldwide.

BACKGROUND

Organization of the Gap Analysis Working Groups on Nipah Virus (2009 and 2017)

The United States Department of Agriculture (USDA) organized the first Nipah virus gap analysis workshop in Australia in 2009 with the support of the Australian Animal Health Laboratory (AAHL). The working group was charged by the USDA National Veterinary Stockpile Steering Committee with making recommendations on specific materials, commercially available and in the pipeline, which will ensure the United States has an arsenal of highly efficacious countermeasures to control and mitigate the impact of an outbreak of Nipah virus. Nipah virus experts representing laboratories in South East Asia, Australia, Canada, and the United States were invited to participate and contributed to this report. The second workshop was organized in 2017 by the Special Pathogens Unit, National Centre for Foreign Animal Disease, Canadian Food Inspection Agency (CFIA), in collaboration with BSL4ZNet and DISCONTOOLS (http://www.discontools.eu/). The participants were charged with assessing available veterinary medical countermeasures to control and respond to a Nipah virus disease outbreak. In addition, the workshop participants agreed to update the gap analysis conducted at the AAHL in Geelong, Australia, in 2009.

Report Updates

This report will be updated periodically with new scientific information, research breakthroughs, and/or the successful development of veterinary medical countermeasures. This report was last updated with the support of Henipavirus experts November 2018.

Reference Material

The following reports and websites are recommended:

OIE – World Organisation for Animal Health - Nipah in Animals
http://www.oie.int/en/animal-health-in-the-world/animal-diseases/Nipah-Virus/
Accessed July 22, 2018

FAO – Food and Agriculture Organization
Manual on the diagnosis of Nipah virus infection in animals
www.fao.org/DOCREP/005/AC449E/AC449E00.htm
Accessed July 22, 2018

CDC – Center for Disease Control and Prevention - Special Pathogens Branch
https://www.cdc.gov/vhf/nipah/index.html
Accessed July 22, 2018

WHO - World Health Organization
http://www.who.int/news-room/fact-sheets/detail/nipah-virus
Accessed July 22, 2018

Guidelines for Veterinarians Handling potential Hendra Virus infection in Horses (QDPI)
https://www.daf.qld.gov.au/__data/assets/pdf_file/0005/126770/2913_-Guidelines-for-veterinarians-handling-potential-Hendra-virus-infection-in-horses-V5.1.pdf

Accessed July 22, 2018

CFSPH – Center for Food Security and Public Health
Nipah Virus Infection
http://www.cfsph.iastate.edu/Factsheets/pdfs/nipah.pdf
Accessed July22, 2018

DEFINITION OF THE THREAT

The threat for a natural introduction of henipaviruses in the United States is low, but there is significant concern that henipaviruses could be used for nefarious purposes to harm agriculture and people. Both Hendra virus and Nipah virus are on the HHS and USDA list of overlap Select Agents and Toxins. Henipaviruses are listed as APHIS Tier 3 high-consequence foreign animal diseases and pests. Henipaviruses are promiscuous in their ability to cause severe morbidity in several animal species, including people, and human infections result in a very high mortality rate. The mortality rate in pigs is actually reported as about 2.5% in adult pigs – high morbidity, but low mortality. Mortality rates in humans range from 40% (Malaysia) to 75% (up to 100%) in Bangladesh. The animal reservoir includes several species of bats, and henipaviruses may thus be readily available in these wildlife reservoirs.

INFECTION IN PEOPLE

Between September 1998 and June 1999, a NiV outbreak in Malaysia resulted in severe viral encephalitis in 105 patients (Goh *et al.,* 2000; Epstein *et al.,* 2006). Ninety-three percent had had direct contact with pigs, usually within two weeks prior to the onset of illness, suggesting that there was direct viral transmission from pigs to humans and a short incubation period. The main presenting features were fever, headache, dizziness, and vomiting. Fifty-two patients (55%) had a reduced level of consciousness and prominent brain-stem dysfunction. Distinctive clinical signs included segmental myoclonus, areflexia and hypotonia, hypertension, and tachycardia. The initial cerebrospinal fluid findings were abnormal in 75% of patients. Antibodies against Hendra virus were detected in serum or cerebrospinal fluid in 76 percent of 83 patients tested. Thirty patients (32%) died after rapid deterioration in their condition. An abnormal doll's-eye reflex and tachycardia were factors associated with a poor prognosis. Death was probably due to severe brain-stem involvement. Neurologic relapse occurred after initially mild disease in three patients. Fifty patients (53%) recovered fully, and 14 (15%) had persistent neurologic deficits.

Unlike Malaysia, the NiV outbreaks in Bangladesh were strictly confined to human populations with significantly higher mortality rate (Hossain *et al.,* 2008). NiV outbreaks in Bangladesh have continued annually since 2008 resulting in a total of 207 reported cases, 152 of which were fatal resulting in a 70% mortality rate (Clayton,. 2017). In 2018, NiV infection was confirmed in Kerala, India, where 23 confirmed cases were reported and case fatality rates were 90% (Arunkumar *et al.,* 2018).

INFECTION IN PIGS

The NiV outbreak in Maylasia in 1999 was facilitated by the rapid spread of the virus in pig populations. Although some pigs demonstrated a febrile respiratory illness with epistaxis, dyspnoea, and cough, few animals exhibit neurological signs, and the majority of pigs had subclinical infections. There are no clinical signs in pigs that are specific for NiV infection. Both, apparently healthy pigs and pigs showing clinical signs shed significant amount of virus.

ECONOMIC IMPACT

The NiV outbreak in Malaysia in 1999 destroyed the main market for Malaysian hogs in Singapore. The Malaysia outbreak resulted in an 80% drop in pork consumption in the domestic market. Over half the standing pig population in the country was culled to halt the outbreak. Half the pig farms

went out of business. The cumulative economic losses based on government figures was estimated to be approximately $217 million USD.

BIOTERRORISM

NiV has many of the physical attributes needed for a biological weapon, including easy access to virus resulting from its wide distribution in nature and laboratories, easy to produce, easy to disseminate, and the potential for high morbidity and mortality in people.

GAP ANALYSIS

The following section summarizes what we know about henipaviruses, gaps in our knowledge, and the threat of bioterrorism.

VIROLOGY

The following summarizes our current knowledge of viral strains, taxonomy, reservoir, genome, morphology, determinants of virulence, host range, and tissue tropism.

Virus species

Nipah virus (NiV) was first isolated in 1999 from samples collected during an outbreak of encephalitis and respiratory illness among pig farmers. The name Nipah originated from Sungai Nipah, a village in the Malaysian Peninsula where pig farmers became sick. There are currently two genotypes identified: NiV-Malaysia and NiV-Bangladesh. Different strains/genotypes of NiV have emerged: Malaysia, Bangladesh, and Cambodia. NiV Malaysia resulted in the culling of a million pigs and 250 human cases (106 fatal). NiV Bangladesh is associated with outbreaks in people (Clayton, 2017).

Hendra virus (HeV) was first isolated in 1994 from specimens obtained during an outbreak of respiratory and neurologic disease in horses and humans in Hendra, a suburb of Brisbane, Australia.

Cedar virus (CedV) is a novel *Henipavirus* isolated from Australian bats, which appears to be non-pathogenic in lab animal experiments (Marsh et al. 2012).

Ghanaian bat henipavirus (GhV) is a species of henipaviruses assembled from sequences collected from *Eidolon helvum*, a bat species in the family Pteropodidae (Drexler *et al.* 2009; Drexler *et al*. 2012). No isolates have been reported, and both pathogenicity and the cross-species transmission remain unknown. Partial sequences of 19 phylogenetically novel African henipaviruses have also been discovered, suggestive of a further diversity of African henipaviruses.

Mòjiāng henipavirus (MojV) was discovered during retrospective surveillance for the etiologic agent responsible for cases of fatal respiratory illness in cave-miners, China. A full-genome was assembled from sequences detected from a cave-dwelling rodent species (Wu Z. *et al.* 2014). MojV is circumstantially associated with the fatal respiratory illness, however, pathogenicity studies have not been completed.

Taxonomy

NiV and HeV are members of the family Paramyxoviridae, order *Mononegavirales*. Comparison of nucleic acid and deduced amino acid sequences with other members of the family confirms that NiV and HeV are members of the family Paramyxoviridae, but with limited homology with members of the *Morbillivirus*, *Rubulavirus* and *Respirovirus* genera (See Fig. 2). The name *henipavirus* was recommended for the genus of both HeV and NiV (Wang *et al.,* 2000). HeV appear to be less diverse that NiV but molecular epidemiology studies are needed to identify new isolates that may bridge the gap between HeV and NiV.

Reservoir

The natural reservoir, or primary animal host, of the henipaviruses are fruit bats mainly from the genus *Pteropus* (flying foxes). Nucleic acid and antibody signatures of exposure to NiV or NiV-like viruses has been documented in a diversity of bat species across the globe (Table 1).

Genome

The complete genomes of both HeV and NiV have been sequenced (Wang *et al.,* 2001). Henipaviruses have a large non-segmented genome comprised of single-stranded negative-sense RNA. Their genomes are 18.2 kb in size and contain six genes corresponding to six structural proteins. All genes are of similar size to homologues in the respirovirus and morbillivirus genera, with the exception of P which is 100-200 amino acids longer (See Fig. 3). Most of the increase in genome length is due to longer untranslated regions between genes, mainly at the 3' end of each gene. The role of these long untranslated regions are not understood. Henipaviruses employ an unusual process called RNA editing to generate multiple proteins from a single gene. The process involves the insertion of extra guanosine residues into the P gene mRNA prior to translation. The number of residues added determines whether the P, V or W proteins are synthesized. The C protein is made via an alternative translational initiation mechanism. The functions of the V, W, and C proteins are unknown, but they may be involved in disrupting host antiviral mechanisms (see Immunology below). The function of the G protein is to attach the virus to the surface of a host cell via the major receptors ephrin-B2 and ephrin-B3 ligands, highly conserved proteins present in many mammals. G glycoprotein is the major neutralizing antigen and the target protein for vaccine development. X-ray crystal structure for NiV G complex with ephrin-B3 has been determined. This interaction is highly conserved between NiV and HeV. This interaction is a prime candidate for developing henipavirus specific therapeutics. The F protein fuses the viral membrane with the host cell membrane, releasing the virion contents into the cell. It also causes infected cells to fuse with neighboring cells to form large multinucleated syncytia.

The genome size and organization of CedPV is very similar to that of HeV and NiV. The nucleocapsid protein displays antigenic cross-reactivity with henipaviruses and CedPV uses the same receptor molecule (ephrin-B2) for entry during infection. Clinical studies with CedPV in *Henipavirus* susceptible laboratory animals confirmed virus replication and production of neutralizing antibodies although clinical disease was not observed. In this context, it is interesting to note that the major genetic difference between CedPV and HeV or NiV lies within the coding strategy of the P gene, which is known to play an important role in evading the host innate immune system. Unlike NiV and HeV, and almost all known paramyxoviruses, the CedPV P gene lacks both RNA editing and also the coding capacity for the highly conserved, interferon pathway antagonists, V or W proteins (Marsh *et al.* 2012).

Although, GhV and MojV have not yet been isolated from hosts, sequence constructed genomes are similar in size, organization, and coding capacity to HeV, NiV, and CedV (Wu Z *et al.* 2014, Drexler *et al*. 2012). Like HeV and NiV, both GhV and MojV are predicted to possess the RNA editing site in the P gene and presumably coding capacity for V and W proteins. Receptor-usage studies with recombinant GhV G glycoprotein demonstrated that like CedV, GhV G was capable of binding to

ephrin-B2, but not ephirn-B3 (Lee B *et al.* 2015). A receptor remains undiscovered for MojV; however, ephrin-B2, -B3 appear to be unlikely candidates (Rissanen I *et al.* 2017).

Morphology

Henipaviruses are pleomorphic ranging in size from 40 to 600 nm in diameter. They possess a lipid membrane overlying a shell of viral matrix protein. At the core is a single helical strand of genomic RNA tightly bound to the nucleocapsid (N) protein and associated with the large (L) and phosphoprotein (P) proteins, which provide RNA polymerase activity during replication. Embedded within the lipid membrane are spikes of fusion (F) protein trimers and attachment (G) protein tetramers.

Determinants of virulence, host range, and tissue tropism

Molecular determinants of virulence, host range and cell tropism have been extensively studied and are well understood for many paramyxoviruses. Infectivity is determined by the cell-attachment and fusion glycoproteins and the presence of appropriate P gene products modulate virulence by antagonizing the cellular interferon response.

Henipaviruses have a large host range, unlike other members of the Paramyxoviridae, which generally have a very narrow host range. The cell attachment protein, unlike many other members for the paramyxovirus subfamily, does not have haemagglutinating activity and as a consequence does not bind sialic acid on the surface of cells.

The receptor for henipavirus is present on many different cultured cell types from many different species. The receptors for HeV and NiV are the same and have been identified as ephrin-B2 and ephrin-B3. Ephrin-B2 or -B3 are highly conserved across vertebrate species and are members of a family of receptor tyrosine kinase ligands. Ephrin-B2 is highly expressed on neurons, smooth muscle, arterial endothelial cells and capillaries, which closely parallels the known tissue tropism of HeV and NiV *in vivo*. Ephrin-B3 is also widely expressed but particularly in specific regions of the central nervous system and may facilitate pathogenesis in certain neural subsets.

Virology Research Priorities

- Molecular epidemiology and determinants of strain variation
- Need sequencing of henipaviruses from bats, especially Bangladesh
- Determine molecular basis for virulence

PATHOGENESIS

The following summarizes our current knowledge of viral pathogenesis, including routes of infection, tissue tropism, pathogenesis, clinical signs, and clinical pathology reported in naturally acquired infections. It should be noted that experimental infection in other animal models have been developed. NiV and HeV (henipaviruses) are distinguished from all other paramyxoviruses particularly by their broad species tropism and ability to cause fatal disease in multiple vertebrate hosts including humans, monkeys, pigs, horses, cats, dogs, ferrets, hamsters and guinea pigs, spanning 6 mammalian Orders (Broder CC *et al.,* 2012; Geisbert TW *et al.,* 2012).

NiV infections in humans and pigs are linked to contact with bats. Clinical signs in human cases indicate primarily involvement of the central nervous system with 40% of the patients in the Malaysian outbreak having also respiratory syndromes, while in pigs the respiratory system is considered the primary virus target, with only rare involvement of the central nervous system.

Humans

The main histopathological findings include a systemic vasculitis with extensive thrombosis and parenchymal necrosis, particularly in the central nervous system (Wong *et al.,* 2002). Endothelial cell damage, necrosis, and syncytial giant cell formation are seen in affected vessels. Characteristic viral inclusions are seen by light and electron microscopy. Immunohistochemistry (IHC) analysis shows the widespread presence of NiV antigens in endothelial and smooth muscle cells of blood vessels (Hooper *et al*., 2001). Abundant viral antigens are also seen in various parenchymal cells, particularly in neurons. The brain appears to be invaded via the hematogenous route and virus has been isolated from the cerebrospinal fluid of patients with NiV encephalitis (Wong *et al*., 2002). Infection of endothelial cells and neurons as well as vasculitis and thrombosis seem to be critical to the pathogenesis of this new human disease.

NiV infection can rarely cause a late-onset encephalitis up to a couple of years following a non-encephalitic or asymptomatic infection, or a relapsed encephalitis in patients who had previously recovered from acute encephalitis (Wong *et al*., 2001; Goh *et al*., 2000; Tan *et al*., 2002).

The most recent NiV outbreak, and first reported in South India, resulted in 23 human cases with a case-fatality rate of 91% (Arunkumar *et al*., 2018). The clinical manifestations and high fatality rate among people were similar to those of earlier NiV outbreaks in India and Bangladesh, and the NiV isolate from this outbreak showed a 97% genetic similarity to the NiV-B lineage. All human cases, following the index case, were due to nosocomial transmission in three different hospitals. Although it was not possible to establish the exact NiV transmission event to the identified index case, the most likely zoonotic route was from *P.* giganteus (Indian flying fox). It was noted that in Kerala, date palms are not used for obtaining sap, and the narrow-mouthed vessels used to collect sap from coconut and Asian Palmyra palm do not allow access by bats. The human-to-human transmission rate was very high in this recent outbreak, and the index case transmitted NiV to 19 contacts (primary cases), while three cases were reported as secondary (Arunkumar *et al*., 2018). These nosocomial transmissions to the primary cases were concomitant with the index case presenting with a persistent cough and near the terminal stage of NiV illness. Of the 23 cases, 20 (87%) had respiratory symptoms presumably increasing the possibility of human-to-human transmission by droplet, and it was reported that only those with direct exposure to the patient's coughing appeared to have acquired NiV infection.

Pigs

Experimental challenge studies in piglets conducted at the National Centre for Foreign Animal Diseases, Winnipeg, Canada, demonstrated neurological signs in several inoculated pigs (Weingartl *et al.,* 2005; Berhane et al., 2008; Weingartl, H.M., personal communication of unpublished data). The rest of the pigs remained clinically healthy. NiV was detected in the respiratory system (turbinates, nasopharynx, trachea, bronchus, and lung), the lymphoreticular system (endothelial cells of blood and lymphatic vessels), submandibular and bronchiolar lymph nodes, tonsil, and spleen, with observed necrosis or lymphocyte depletion in lymphoid tissues, most importantly in lymph nodes (Hooper et al.,

2001, Weingartl et al., 2006; Berhane et al., 2008). NiV presence was confirmed in the nervous system of both sick and apparently healthy animals (cranial nerves, trigeminal ganglion, brain, and cerebrospinal fluid). No virus was detected urine, although NiV antigen was found in kidneys of field swine cases (Tanimura et al., 2004). This study suggests NiV invaded the porcine host central nervous system via cranial nerves after initial virus replication in the upper respiratory tract, and later in the infection also by crossing the blood-brain barrier as a result of viremia. Additional information on NiV and HeV pathogenesis in pigs are summarized in Middleton and Weingartl, 2012.

Cats

Cats were recognized as a naturally susceptible host for NiV during the 1998-99 Malaysian outbreak (Hooper *et al.*, 2001). Experimental infections of cats revealed they are highly susceptible to productive infection by both HeV and NiV and disease is severe. HeV infected cats develop fever and elevated respiratory rates, and there is rapid progression to severe illness and death within 24 hours of the onset of clinical signs (Westbury *et al.,* 1996). HeV disease in cats is similar to that observed in horses, with wide-spread vasculitis and parenchymal lesions in a wide range of organ systems particularly the lungs (Hooper *et al.,* 2001; Hooper *et al.,* 1997). Experimental NiV infection in the cat is essentially identical in outcome as compared to HeV infection and closely resembles most of the pathogenic processes seen in cases of henipavirus infection of people (Broder *et al.,* 2012).

Dogs

Middleton *et al*., 2017, conducted experimental infections with HeV in dogs and determined that the virus is not highly pathogenic in dogs but their oral secretions pose a potential transmission risk to people. The time window for potential oral transmission corresponded to the period of acute infection.

Horses

The pathology caused by HeV or NiV in horses (natural or experimental infection with HeV or natural infection with NiV) is more severe than that caused by either virus in pigs. Naturally acquired HeV infection in horses is often associated with severe disease, and experimental infections are essentially uniformly fatal. Animals initially become anorexic and depressed with general uneasiness and ataxia, with a developing fever with sweating. Respiration becomes rapid, shallow and labored with pulmonary edema and congestion with nasal discharge being a common terminal feature 1 to 3 days following the onset of clinical signs. Neurologic disease is also present but less frequent and noted in both terminally ill horses and in those that recovered from respiratory infection (Rogers *et al.,* 1996; Williamson *et al.,* 1998). Infection is wide-spread with an endothelial cell tropism with syncytia (Hooper *et al.,* 2001; Hooper *et al.,* 1997; Marsh *et al.,* 2011; Murray *et al.,* 1995; Williamson *et al.,* 1998). Experimental infection of horses with NiV has not been carried out, but the brain and spinal cord of one naturally infected horse was confirmed and revealed non-suppurative meningitis (Hooper *et al.,* 2001).

Bats

Fruit bats in the *Pteropus* genus have been identified as the reservoir hosts for HeV, NiV, and CedV. Henipaviruses have been isolated to date in *Pteropus* spp. from Australia (HeV, CedV) and Malaysia/Bangladesh/Cambodia/Thailand (NiV). Serological evidence of NiV or NiV-like exposure was detected in bats sampled in Madagascar and Ghana (Iehle C., *et al.,* 2007, Hayman *et al.,* 2008). Subsequently, 19 novel henipavirus sequences and one full-length genome of an African henipavirus,

GhV, were identified from related pteropodid bats, *Eidolon helvum*, sampled in Ghana (Drexler *et al.*, 2009; Drexler *et al.*, 2012). Nucleic acid and antibody signatures of henipaviruses have been detected serologically and by PCR in non-*Pteropus*, but related pteropodid bats in Central and West Africa, China, and Southeast Asia (Table 1); however the role that these non-*Pteropus* spp. play in the maintenance and transmission of henipaviruses remains unclear. The genome of MojV was constructed from sequences collected from a rodent, *Rattus flavipectus*, but comprehensive surveys have not been performed to rule out whether bats also host MojV.

There is no significant pathology in bats, and the frequency of viral shedding from wild bats is rare, with prevalence ranging from (1-3%) with temporal variation of infection and viral shedding observed among different bat populations (Gurley *et al.,* 2017 and Wacharaplusadee *et al.* 2010, 2016). Henipavirus isolation from bat excreta is challenging, potentially due to low viral load.

Pathogenesis Research Priorities

- Identify determinants of virulence in pigs
- Develop experimental infection models in bats to study shedding
- Comparative genomic studies of contemporaneous NiV strains collected from bats and humans during outbreaks.
- Expand knowledge of spectrum of henipaviruses in bat hosts in NiV hotspots (e.g. western Bangladesh & West Bengal India)
- Determine whether the innate immune system in bats is responsible for limiting viral replication
- Determine how the net reproductive value of henipaviruses are sustained in bats
- Determine how transmission effected within bats, and between bats and other species

IMMUNOLOGY

The following summarizes our current knowledge of NIV immunology, including innate and adaptive immune responses to wild-type virus, immune evasion mechanisms, and protective immunity.

Innate and adaptive immune responses to wild-type NiV

Viral RNA can be detected by both cytoplasmic and endosomal pattern recognition receptors (PRRs), resulting in innate immune Type I IFN induction/ and signaling pathways:

- Retinoic Acid-inducible Gene I (RIG- I)- recognizes 5' triphosphorylated RNA
- Melanoma Differentiation Antigen 5 (Mda-5)-recognizes cytosolic dsRNA
- RNA-dependent Protein Kinase (PKR)- recognizes cytosolic dsRNA
- Toll-like Receptor (TLR) 3- recognizes endosomal dsRNA
- TLR 7-8- recognizes endosomal ssRNA

Immune evasion mechanisms

The NiV uses unusual processes called RNA editing and internal translational initiation to generate multiple proteins from the phosphoprotein (P) gene, resulting in 4 proteins (P, C, V, and W) that function in inhibiting Type I interferon pathways:

- NiV P, V, and W have individually been shown to bind STAT1 and STAT2, effectively preventing STAT1 phosphorylation in type I IFN-stimulated cells.
- The V protein localizes to the cytoplasm, while the W protein localizes to the nucleus.

- The C protein can partially rescue replication of an IFN-sensitive virus, but the mechanism is unknown.
- Nuclear localization of W enables it to inhibit both dsRNA and TLR 3 (IRF-3 dependent) IFN-β induction pathways.
- A single point mutation in the V protein abrogates its ability to inhibit of IFN signaling.
- The V proteins of paramyxoviruses interact with the intracellular helicase Mda-5, and inhibits its IFN-β induction, but not with RIG-I.
- NiV V, W, and P bind polo-like kinase (PLK) via the STAT1 binding domain (Ludlow *et al.,* 2008).
- The P, V, and W proteins of NiV Malaysia and NiV Bangladesh inhibit IFN-stimulated response element (ISRE), which have a role in inducing transcription of IFN-stimulated genes (ISGs). Some of these ISGs include IRF-7, 2'5' Oligoadenylate Synthetase (OAS), RnaseL, p56, and double-stranded RNA-induced protein kinase (PKR). These ISGs all contribute to the generation of an 'antiviral state' in the cell.

Protective immunity

The G and F protein induce neutralizing antibodies that protect against challenge. Recent evidence from vaccination challenge studies indicates that both serum neutralizing antibody and T cell-mediated immunity are needed for protection from NiV infection in pigs (Pickering *et al.,* 2016).

Research needs

- Innate immunity and immunosuppression
  - Need studies in NiV infected cells and animal models
  - Need to study infection in various cell types, including cells of the immune system and bat cells
  - Use infectious clone to study virulence determinants
  - Identify targets for antiviral agents
  - Cytokine response to infection in human and bat cell lines
  - Need to study the potential for type 1 interferon or other cytokines to provide early protection from Nipah virus infection, transmission and/or clinical signs.

- Protective Immunity
  - Need to better define correlates of protection
  - Study T lymphocyte subset responses and cellular targets (e.g., N)

EPIDEMIOLOGY

Certain species of fruit bats of the genus *Pteropus* are the principal natural reservoir hosts for NiV and HeV. Bats are susceptible to infection with these viruses but do not develop disease. Other zoonotic viruses like Ebola, Marburg, and SARS virus, have also been identified in various bats (Leroy *et al*. 2005; Towner *et al*. 2009; Li W *et al*. 2005). Fruit bats are distributed across an area encompassing Australia, Southeast Asia; including Indonesia, Malaysia, the Philippines and some of the Pacific Islands, the Indian subcontinent, and Madagascar (See Fig. 1). There is further evidence for broader

distribution of NiV in pteropid fruit bats species across their range (Wacharapluesadee S. and Hemachudha T., 2007). There is also growing evidence that viruses related to NiV and HeV also circulate in non-pteropid fruit bats worldwide.

Hendra Virus

Hendra virus infection was first recognized in 1994 in Australia, when it caused an outbreak of acute, fatal respiratory disease that killed 14 horses. Three human cases, leading to two deaths were recorded during the outbreak. In 1995, a horse was infected with associated human cases. The precise mode of virus transmission to the three Australian patients is not fully understood. All three individuals appear to have acquired their infection as a result of close contact with horses, which were ill and later died.

There have been several recognized outbreaks in Australia since 1994. Hendra virus reemerged in 1999, 2004, and 2006-2010. All known HeV cases have occurred in Queensland or northern New South Wales. From 1994 to 2010, HeV was confirmed on 11 premises in Queensland and one premise in northern New South Wales. In Australia, GlobalincidentMap.com reported: 21 cases in 2011; 12 cases in 2012; 10 cases in 2013; four cases in 2014; three cases in 2015; one case in 2016; and four cases in 2017. Al cases have involved horses as an intermediate host along with some additional human infections, resulting in several fatalities. The Australian Veterinary Association's national president, Dr. Ben Gardiner, was quoted as stating "no state or territory was immune from the virus."

The natural reservoirs for HeV are flying foxes found in Australia. Bats are susceptible to infection with these viruses but do not develop disease.

Hendra virus infection has also been detected in two dogs that were in close contact with infected horses. Both dogs remained clinically normal with no history of related illness.

Updated statistics on HeV outbreaks, including locations, dates and confirmed human and animal cases may be found on the Australian Veterinary Association website (Assessed July 22, 2018).

Nipah Virus

Nipah virus is a recently-recognized, zoonotic paramyxovirus that causes severe disease and high fatality rates in people. Outbreaks have occurred in Malaysia, Singapore, India and Bangladesh, and a putative Nipah virus was also recently associated with human disease in the Philippines (Clayton, 2017). The following summarizes our current knowledge of NiV epidemiology taking into account disease outbreaks in Malaysia and Bangladesh.

Malaysia

Nipah virus was first described in 1999 in Malaysia. The outbreak in Malaysia resulted in over a million pigs culled, 800 pig farms demolished, 36,000 jobs lost, $120+ million exports lost, and over 300 human cases (106 fatal, ~35% mortality) in pig farmers (Chinese) and Singapore abattoir workers (Field *et al.,* 2001). The NiV outbreak in pigs was described as highly infectious, frequently asymptomatic, low mortality rate (~5%), with respiratory and neurological syndromes. The pig farm pattern of disease included 30% morbidity and 5% mortality with sows first affected, followed by weaners, growers and finishers. The duration of clinical disease on a farm lasted ~ 2 weeks with a sero-prevalence approaching 100% in some cases. The outbreak in Malaysian pigs was associated

with an increased incidence of human viral encephalitis cases, strongly associated with pig farm workers, with temporal and spatial links to disease in pigs.

During the outbreak, evidence of NiV infection was found in domestic animals such as goats and cats, but especially dogs (Field *et al.,* 2001). After pig populations were destroyed, but before residents were allowed to return to their homes, studies were undertaken in the epidemic area to determine whether domestic animal populations maintained active infection in the absence of infected pigs (Mills *et al.,* 2009). Dogs were especially suspected because they live commensally with both pigs and humans. However, serologic screening showed that in the absence of infected pigs, dogs were not a secondary reservoir for NiV.

Although human-to-human transmission of NiV during the 1998-1999 outbreak in Malaysia was not reported, a small number of infected people had no history of contact with pigs, suggesting human-to-human transmission occurred in these cases (Clayton, 2017).

The reservoir and natural host of NiV was determined to be fruit bats. Fruit bats have a wide geographic distribution, high antibody prevalence (17-30%), but no apparent clinical disease. A NiV neutralizing antibody study (Yob *et al.,* 2001) from 237 wild-caught bats surveyed on Peninsular Malaysia April 1–May 7, 1999, found four different species of fruit bats, and one species of insectivorous bats, tested positive for NiV (see Table I).

The routes of NiV excretion in bats include urine, saliva, and foetal tissues and fluids but the exact modes of transmission have yet to be determined.

The drivers of the emergence of NiV in Malaysia were determined to be large piggery (30,000+) adjacent to primary forest/fruit bat habitat and a network of other large farms close by. The stages of emergence associated with the outbreak included a spillover from flying foxes to domestic pigs near Ipoh (see Fig. 4), where farming practices and high pig densities facilitated the dissemination of the infection. Transportation of pigs for commerce led to the southern spread of the outbreak with the amplifying pig host facilitating the transmission of the virus to humans.

The epidemic enhancement of the outbreak included the initial introduction of infection in a naive pig population resulting in a rapid epidemic peak, followed by burn-out and localized human infections. Subsequent introduction(s) into partially immune pig populations resulted in a lower epidemic peak but prolonged duration and increased total number of infectious pigs, increasing the chances of spread to surrounding farms.

Bangladesh

Bangladesh experienced its first reported NiV outbreak in Siliguri and Naogaon in 2001 (Fig. 5). Unlike Malaysia, outbreaks in Bangladesh appeared to be strictly confined to human populations and significantly higher mortality rate. From 2001 to 2018, the WHO reported a total of 261 cases, with 198 deaths (76% mortality) due to NiV infection (see Table II).

The transmission of NiV to humans in Bangladesh was determined to be associated with drinking date palm juice, considered a delicacy in this region of the world. In the Tangail outbreak of 2005, it was

estimated that persons drinking raw date palm sap had a 7.0 odds ratio of developing a NiV infection when compared to controls (95% confidence level, 1.6).

NiV cases in Bangladesh have been seasonal, with human cases reported between the months of January and April. This coincides with the season for collecting date palm sap, late November through April. However, there is significant heterogeneity in the number of spillovers detected by district and year that remains unexplained. Cortes et al., in 2018 analyzed data from all 57 spillovers occurring during 2007–2013 and found that temperature differences explained 36% of the year-to-year variation in the total number of spillovers each winter, and that distance to surveillance hospitals explained 45% of spatial heterogeneity. January, when 40% of the spillover events occurred, was the month with the lowest mean temperature during every year of the study.

Bats are recognized as a nuisance and frequently drink the juice, defecate into juice, and occasionally drown in the palm sap collecting pot. Measures have been put in place to prevent bats access to the sap collecting pot, which has been very effective in reducing the spread of NiV from bats to humans in Bangladesh.

India

In 2001, an outbreak occurred within a hospital in Siliguri, West Bengal. Nosocomial transmission likely occurred, though it is unknown how primary cases were infected. Another outbreak in 2007 was reported in Nadia, West Bengal. Consumption of date palm sap was identified as the likely route of infection of primary cases there. In May of 2018, another outbreak was reported in Kerala. A total of 85 cases were reported in these three outbreaks in 2001, 2007, and 2018, with 62 deaths (73% mortality) due to NiV infection (see Table II).

In 2012, Yadav et al. surveyed the Indian states of Maharashtra and West Bengal to evaluate the presence of viral RNA and IgG against NiV in different bat populations belonging to the species Pteropus giganteus, Cynopterus sphinx and Megaderma lyra. The authors found NiV RNA in Pteropus bat thus suggesting it may be a reservoir for NiV in India.

In 2018, an outbreak of 23 cases of NiV disease was reported in Kerala, India. This was the first spillover in NiV in South India. 18 cases were lab-confirmed and the case fatality rate during this outbreak was 91% (Arunkumar G *et al*. 2018).

Philippines

In 2014, the Philippines reported an outbreak with a zoonotic paramyxovirus in horses and people that is very closely related to NiV based on sequence analysis. Virus isolation was unsuccessful so it was impossible to confirm that there was transmission from presumably bats to horses, from horses to people, and also human to human (Ching P.K., *et al.*, 2015; Clayton, 2017).

New Caledonia

In 2015, three fruit bats tested positive for NiV in New Caledonia at the Noumea National Park, including three bats at the Noumea Zoo.

Research needs

- Improved understanding of infection dynamics in flying foxes: modes of transmission, immune response, evidence of disease, and the implications of co-infection with NiV and other henipaviruses
- Better understanding of co-circulation of different strains / species of henipaviruses within Pteropus populations and the effect of waning herd immunity on outbreaks.
- Other animals such as infected dogs and cats need to be further studied to determine their potential role in the transmission of NiV.
- Improved understanding of infection dynamics in humans: modes of transmission, implications of genetic diversity of the virus for infection, transmission & pathogenicity
- Research into bat populations: additional research regarding bat distributions & ecological impacts
- Research aimed at improving the capacity to diagnose henipavirus infections and improve human health outcomes
- Research into infection and clinical signs in pigs in Bangladesh and potential for pig to human and human to pig transmission.

BIOTERRORISM

The following summarizes the rationale for considering NiV as a potential agent of bioterrorism.

NiV is classified by CDC as a Category C pathogen – emerging pathogens that could be engineered for mass dissemination in the future. Category C include pathogens are readily available, easy to produce, easy to disseminate, and have the potential for high morbidity and mortality with major health impact.

NiV has many of the physical attributes to serve as a potential agent of bioterrorism. The outbreak in Malaysia caused widespread panic and fear because of its high mortality and the inability to control the disease initially. There were considerable social disruptions and tremendous economic loss to an important pig-rearing industry. This highly virulent virus, believed to be introduced into pig farms by fruit bats, spread easily among pigs and was transmitted to humans who came into close contact with infected animals. From pigs, the virus was also transmitted to other animals such as dogs, cats, and horses.

Nipah Virus Bioterrorism Threat Assessment

Virology

- Reverse genetic methods are available for negative strand RNA viruses, including Nipah, and all genomic sequence data is available.
- Many laboratories are actively engaged in research programs on the cell biological properties of the henipaviruses.

- Virus can be amplified to reasonably high unconcentrated titers (>10^7). Several cell culture lines can be used, Vero cell use most often reported, and wild-type virus can be grown and harvested from cell cultures.
- A major constraint in handling Nipah is the requirement for BSL4 facilities; , however, potential terrorists may not respect this need.
- Inactivation of virus can be achieved with a variety of agents typically used for envelope viruses; but extensive environmental stability testing not reported.
- Vaccines and passively-delivered countermeasures are under development both for human and veterinary use. A commercial Hendra virus vaccine is available for horses, and the soluble G protein based vaccine has shown experimental efficacy against Nipah virus in nonhuman primates.
- Bats are sold (often live) in markets throughout their range, providing a potential source of virus; and serological tests are available for identifying henipaviruses

Economic Impact
- Destroyed the main market for Malaysian hogs in Singapore
- ~80% drop in pork consumption in the domestic market.
- Over half the standing pig population in the country was culled to halt the outbreak.
- Half the pig farms went out of business.
- During the outbreak cumulative economic losses based on government figures >$217 million USD.
- Cumulative government costs in operations and lost revenues >$298 million USD.
- Other countries in South East Asia often have a higher pig density than Malaysia. China, with approximately half of the pigs in the world, is especially vulnerable to an economic and public health disaster if NiV were to emerge and be rapidly transmitted between pigs and from pigs to people.

Epidemiology and Clinical Disease
- In outbreaks to date henipaviruses do not appear to be highly infectious. Infection requires close contact with secretions of diseased animals. Many infections can be mild to asymptomatic.
- In the initial 1998-99 outbreak the virus was *initially misdiagnosed* as Japanese Encephalitis; amplification occurred from veterinary reuse of needles in immunization programs to control JE, increasing outbreak severity and extent.
- Time from exposure to signs of infection averages ~2 weeks for humans and seroconversion occurs within a month of onset (dose / route dependent).
- Transmission directly to the vascular system could occur through bites from infected animals or broken skin exposed to secretions of infected animals.
- It is quite likely that an outbreak in animals would result in transmissions to humans.
- An outbreak of Nipah pneumonia or ARDs-like disease with human-to-human transmission as demonstrated in the Bangladesh outbreak could cause significant mortality. Nipah could cause more severe or different disease presentations in older or sick populations.

Viral Transmission
- Deliberate release of virus in some manner is possible.

- Aerosol delivery might transmit the disease effectively to domestic animals, but the environmental requirements for maintaining virus stability are not well known.
- Transmission to humans through consumption of contaminated food has been documented.
- The veterinary reuse of needles in the Japanese Encephalitis immunization campaign and in artificial insemination may have been a factor in the near 100% infection level of Nipah in pigs observed on affected farms.
- Deliberate contamination of veterinary needles could initiate an outbreak in susceptible domestic animals.
- Human-to-human transmission through travel has not been documented, but is possible.
- Transport of infected pigs on trucks was a transmission route in the Malaysian outbreak. Generalizing-- transportation of infected humans on crowded airplanes, buses or trains could also transmit the disease. Human cases have been transported to highly populous cities (e.g. Dhaka) where risk of exposure and spread among the public is increased.

Summary

- Nipah (henipaviruses) can be isolated from animal hosts.
- Several species of fruit bats, including *Pteropus spp.* widely distributed throughout Southeast Asia. The live animals are sold in food markets.
- A Nipah outbreak in swine producing areas can cause an economic crisis, even if human cases do not occur.
- Nipah virus can be amplified in permissive cell cultures (e.g., Vero cells) providing adequate laboratory facilities are available (Biosafety Level 4), although a bioterrorist group would not be restricted from growing the virus because of the lack of BSL-4 facilities.
- Effective aerosol delivery is likely possible but unpredictable on the basis of publicly available information. General unknowns are-- titers necessary for infection, virion stability in vitro, and how infectious the virus would be with this delivery.
- Effective surveillance programs, particularly in pig farming areas, are the best defense for early detection and containment of infection, whatever the source.

SUMMARY OF OBSTACLES TO PREVENTION AND CONTROL

The 2017 gap analysis working group determined that the following countermeasures were important but several weaknesses were identified.

DIAGNOSIS

NiV and HeV are zoonotic paramyxoviruses capable of causing severe disease in humans and animals. These viruses require biosafety level 4 (BSL-4) containment. The availability of safe laboratory diagnostic tests is limited. Sequence variation effects molecular diagnostics; both Clifton Beach (2007) and Redlands (2008) reported that Hendra virus strains failed in AAHL Hendra virus specific real-time PCR. Most published diagnostic PCRs only detect HeV or NiV, but not both. There is a need for a more general PCR to detect divergent and novel strains. Pan-paramyxovirus PCR assays exist and are in use to detect henipaviruses, but limitations in sensitivity limit diagnostic value. The USAID PREDICT program previously used its pan-paramyxovirus PCR assay for surveillance in more than 20 countries in Africa and Asia. Virus isolation and serum neutralization assays require live NiV. There is a need for diagnostics that can be used safely in the laboratory. There is a need for rapid nucleic acid-based assays that can detect all henipaviruses. There is also a critical need for improved antibody-based assays for disease outbreaks and disease surveillance. Importantly, there is a need to develop operator-safe diagnostic tests for which reagents can be produced without requiring high containment facilities.

Currently there are no expectations that validated tests will become available for livestock (or other species) in the near future. Nothing has been done in terms of test harmonization since 2009; however, a number of technology transfers have occurred: from AAHL to laboratories in Asia (Malaysia mainly); limited transfer from NCFAD to India (Bhopal High Containment Animal Health Laboratory); limited transfer from AAHL to the FLI and bilateral transfers between NCFAD and FLI.

VACCINATION

There is currently a commercially available vaccine for horses but no vaccines for swine or human vaccines. The goal for a HeV vaccine for horses is to vaccinate horses in areas at risk for transmission from bats to horses in order to prevent bat to horse transmission and subsequent horse to human transmission. The goal for a NiV vaccine for swine is to have a large stockpile of vaccine available for rapid use in an outbreak situation to prevent swine to swine, swine to human, and perhaps human to swine transmission to control the outbreak. A large stockpile of NiV vaccine, or vaccine antigen concentrate, for rapid emergency use in swine to control a potential outbreak that spreads too quickly to be stamped out in swine dense areas is needed. The vaccine should be licensed in the U.S., E.U or Australia for stockpiling as well as in the countries where NiV is endemic in bats. The stockpile should be available for use internationally where ever it may be needed.

SURVEILLANCE

Passive surveillance is the primary and most economical method used. Passive surveillance in commercial swine herds based on clinical signs has many weaknesses due to the difficulty of differentiating NiV from many common endemic infectious diseases of pigs; e.g., classical swine

fever, porcine reproductive and respiratory syndrome, pseudorabies, swine enzootic pneumoniae, and porcine pleuropneumonia.

In the case of infections in swine where recognition of Nipah symptoms is less likely, surveillance activities must be based on diagnostic testing to supplement surveillance based on clinical signs.

Active surveillance programs are expensive and would have to rely on direct diagnostic tests such as viral isolation and nucleic acid-based assays but available tests have significant weaknesses and have not been validated.

Rapid confirmation of cases is essential. Knowledge on serological cross-reactions with other henipaviruses and/or morbilliviruses in bats is improving. There is an urgent need to establish diagnostic capacity for Nipah virus in countries that are most likely to experience spillovers from the bat reservoirs.

DEPOPULATION

Depopulation is the primary countermeasure to reduce virus shedding and stop the spread of Nipah virus in swine. Recent outbreaks have shown that the control of Nipah virus in pig populations through stamping out is complex due to the zoonotic nature of the agent and may be very expensive, particularly in areas with high pig densities. Because Nipah virus spreads rapidly and silently in pigs, a large number of animals would need to be pre-emptively culled if an outbreak occurred in the U.S, or in other swine dense countries in order to minimize the virus spread in the vicinity of infected herds. Thus, this method of control would have significant financial implications due to the culling of thousands or millions of animals.

COUNTERMEASURES ASSESSMENT

ASSUMPTIONS

The following captures assumptions made by the gap analysis working group to assess potential countermeasures to enhance our ability to contain and eradicate an outbreak of NiV.

Situation
Countermeasures assessed for worst case scenario: A coordinated intentional distribution of NiV-contaminated material in a high density highly populated pig region of the United States.

Target Population
Countermeasures assessed for target pig production segments in priority order:

1. Backyard pigs
2. Comprehensive commercial swine operations (farrowing, nursery, and finishing)
3. Commercial indoor farrowing operations
4. Large intensive indoor pig farms
5. Valuable commercial genetic swine stock

Scope of Outbreak
Countermeasures assessed for multiple outbreaks occurring simultaneously in backyard pigs, three farrowing commercial operations, a finishing pig commercial operation, a sow replacement operation, and evidence of infection in feral swine.

DECISION MODEL

The quantitative Kemper-Trego (KT) decision model was used to assess available vaccines and diagnostics. For the criteria and weights used to assess NiV vaccines and diagnostics (See Appendices II, III).

Criteria
The following critical criteria were selected to enable the comparison of countermeasures using a pertinent and valid analysis, as follows:

<u>Vaccines</u>

- Efficacy
- Safety
- One dose
- Manufacturing safety
- DIVA compatible
- Manufacturing yield
- Rapid production
- Reasonable cost
- Short withdrawal period
- Long shelf life

Diagnostics

- Sensitivity
- Specificity
- DIVA detection
- Multispecies
- Validation to purpose
- Speed of scale-up
- Throughput
- Pen-side test
- Rapid result
- No need for a confirmatory test
- Easy to perform
- Safe to operate
- Availability
- Storage/Distribution
- Low cost to implement
- Perform at BSL-2
- Does not require use of live virus to prepare reagents

Weight

Each criterion was weighted to allow a quantitative comparison of the impact of the selected interventions (See Appendices II and III).

Product profile

To ensure a consistent and meaningful assessment, the desired product profile (i.e., the benchmark) was identified for each countermeasure:

Desired Vaccine Profile

1. Highly efficacious: prevent transmission; efficacy in all age animal target hosts, including maternal antibody override; cross protection across all henipavirus strains; quick onset of immunity; multiple animal target hosts; one year duration of immunity
2. Safe in all age animal target hosts; no reversion to virulence for live vaccines
3. One dose
4. Safe vaccine strain for manufacturing
4. DIVA compatible
5. Manufacturing method yields high number of doses
6. Rapid speed of production and scale-up
7. Reasonable cost
8. Short withdrawal period for food consumption
9. Long shelf life

Desired Diagnostic Test Profile
1. Detect all henipavirus
2. Identify Nipah virus specific strains
3. Direct tests for control and eradication
4. Indirect tests for post-control monitoring
5. Rapid test
6. >95% specificity
7. >95% sensitivity
8. Pen-side test
9. DIVA Compatible
10. Field validated
11. Easy to perform/easily train NAHLN's personnel
12. Scalable
13. Reasonable cost
14. Operator safe
15. Reagents can be produced in low containment

Values
The values assigned for each of the interventions reflect the collective best judgment of members of the gap analysis working groups (See Appendices I and II)

VACCINES
The human infections in the 1999 outbreak in Malaysia were linked to transmission of NiV from pigs. Accordingly, a swine vaccine able to prevent virus transmission would be an important tool to safeguard commercial swine operations and people at risk. In addition, since henipaviruses have a very broad host range, a vaccine that is efficacious in multiple susceptible animal species would be desirable. Although the 2017 gap analysis working group determined that there are still no NiV commercial vaccines available, there are several vaccine candidates that may be safe and effective in swine and other domestic animals that were recently reviewed in: (Weingartl H.M., 2015; Broder, C.C., *et al, 2016*; and Satterfield, B.A., *et al.,* 2016). After these reviews were published, a manuscript was published demonstrating the efficacy of a virus-like-particle (VLP) Nipah virus vaccine in hamsters for inducing virus neutralizing antibodies and protection from challenge (Walpita P., *et al.,* 2017). Another manuscript was published that concluded that an adjuvanted Hendra soluble G vaccine in pigs induced neutralizing antibody titers considered to be protective against Nipah virus without detectable T cell-mediated immunity to Nipah, which did not protect from challenge with Nipah virus. However, pigs that had been previously challenged with a low dose of NiV developed neutralizing antibodies and cell-mediated immune memory and were protected from a high challenge dose of NiV. The conclusion of this manuscript was that both virus neutralizing antibodies and cell-mediated immunity were necessary for protection from NiV challenge (Pickering B.S., *et al.,* 2016). Subsequent unpublished research demonstrated that a different adjuvant used with the soluble Hendra G vaccine caused the induction of both high tittered virus neutralizing antibody and detectable T cell-mediated immunity in pigs to NiV. Challenge studies were not conducted (J.A. Roth, personal communication). All of these vaccine candidates would need further research and development to be licensed, and would need to be made available as a stockpile for rapid use in an emergency if an outbreak in swine were to occur that could not be effectively stamped out. A swine vaccine would

also be needed if the Nipah virus were to mutate to be efficiently transmitted between people and between people and pigs.

Summary

- Vaccination against NiV has been successfully demonstrated
- Experimental henipavirus vaccines can prevent clinical disease
- Experimental henipavirus vaccines elicit systemic and mucosal immunity
- Experimental henipavirus vaccines prevent viral replication in target tissues
- HeV commercial vaccine Equivac® HeV does not cross protect against NiV infection in swine
- Henipavirus vaccines appear to be effective in several mammalian animal species

Assessment of Commercial Vaccines

A commercial vaccine (Equivac® HeV) against Hendra virus approved for use in horses (Middleton D.J. *et al.,* 2014) was registered by Zoetis in Australia in 2015. A six month booster dose is required for full protection, followed by annual vaccination. The vaccine is also approved for pregnant mares. There is currently no NiV vaccine approved for swine. Likewise, there is no vaccine against HeV or NiV approved for human use.

Assessment of Experimental Vaccines

The working group felt that limited information was available to assess and contrast experimental vaccines that have been reported in the literature. Experimental animal vaccines under investigation are summarized in Table I. Experimental vaccines for humans are summarized in Table II. Several of the working group members have directly or indirectly been involved in the research associated with these vaccines so that an assessment could be made (See Appendix I). The following describes some of the most promising experimental vaccine technologies.

1) Canarypox-vectored NiV Vaccines

The ALVAC canarypox virus-based recombinant vaccine vector (Taylor *et al.,* 1994) was used to construct two experimental NiV vaccines (Weingartl *et al.,* 2006). These experimental vaccines were engineered by Merial.

The first construct carries the gene for NiV attachment glycoprotein G (ALVAC-G). The second construct carries the NiV fusion protein F (ALVAC-F).

The efficacy of both the ALVAC-G and ALVAC-F were tested in pigs either as monovalent vaccine or in combination (ALVAC-G/F). The vaccine dose used was 10(8) PFU. The vaccine regimen was two doses administered 14 days apart. Both non-vaccinated controls and vaccinated pigs were challenged with 2.5 x 10(5) PFU of NiV two weeks later.

The combined ALVAC-F/G vaccine induced the highest levels of neutralization antibodies. Despite the low neutralizing antibody levels induced by ALVAC-F all vaccinated animals were protected against challenge. Virus was not isolated from the tissues of any of the vaccinated pigs post-challenge, and a real-time reverse transcription (RT)-PCR assay detected only small amounts of viral

RNA in several samples. In challenge control pigs, virus was isolated from a number of tissues or detected by real-time RT-PCR. Vaccination of pigs with the ALVAC-F/G stimulated both type 1 and type 2 cytokine responses. No virus shedding was detected in vaccinated animals, in contrast to challenge control pigs, from which virus was isolated from the throat and nose.

Based on the data generated in this one study, both the ALVAC-G or the combined ALVAC-F/G vaccine appears to be a very promising vaccine candidate for swine.

2) Soluble G Henipavirus Vaccine

HeV and NiV infect cells by a pH-independent membrane fusion event mediated by their attachment (G) and fusion (F) glycoproteins. Scientists at the Uniformed Services University of the Health Sciences in Bethesda, Maryland, in collaboration with the Australian Animal Health Laboratory characterized HeV- and NiV-mediated fusion activities and detailed their host-cell tropism characteristics. These studies suggested that a common cell surface receptor was utilized by both viruses. To further characterize the G glycoprotein and its unknown receptor, soluble forms of HeV G (sG) were constructed by replacing its cytoplasmic tail and transmembrane domains with an immunoglobulin kappa leader sequence coupled with an S-peptide tag (sG) to facilitate purification and detection. Expression of sG was verified in cell lysates and culture supernatants by specific affinity precipitation. Analysis of sG by size exclusion chromatography and sucrose gradient centrifugation demonstrated tetrameric, dimeric, and monomeric species, with the majority of the sG released as a disulfide-linked dimer. Immunofluorescence staining revealed that sG specifically bound to HeV and NiV infection-permissive cells. The scientists further reported that administration of sG to rabbits can elicit a potent cross-reactive neutralizing antibody response against infectious HeV and NiV (Bossart *et al.* 2005). The HeV sG subunit vaccine has been the most extensively studied NiV/HeV vaccine platform because of its ability to elicit a potent cross-protective immune response to NiV and has been shown to induce potent cross-reactive neutralizing antibody responses in a variety of animals including mice, rabbits, cats, ferrets, monkeys and horses.

Experimental subunit vaccine formulations containing either HeV sG or NiV sG were first evaluated as potential NiV vaccines in the cat model. Two cats were immunized with HeV sG and two cats were immunized with NiV sG. Immunized animals and two additional naïve controls were then challenged subcutaneously with 500 TCID50 of NiV. Naive animals developed clinical disease 6 to 13 days post-infection, whereas none of the immunized animals showed any sign of disease (Mungall *et al.,* 2006).

In a subsequent experiment, an experimental subunit formulation containing HeV sG and CpG adjuvant was evaluated as a potential NiV vaccine in the cat model. Vaccinated animals demonstrated varying levels of NiV-specific Ig systemically and importantly, all vaccinated cats possessed antigen-specific IgA on the mucosa. Upon oronasal challenge with NiV (50,000 TCID50), all vaccinated animals were protected from disease although virus was detected on day 21 post-challenge in one animal. (McEachern *et al.,* 2008).

Additional studies with the HeV-sG vaccine in the ferret model formulated in CpG and Allhydrogel™ and could provide complete protection from a 5,000 TCID50 dose of HeV (100 times the minimal lethal dose) with no disease or evidence of virus or viral genome in any tissues or body fluids and only a low level of HeV genome detected in the nasal washes from 1 of 4 animals in a low-dose vaccine

group, and no infectious HeV could be recovered from any immunized ferrets (Pallister J, et al. A recombinant Hendra virus G glycoprotein-based subunit vaccine protects ferrets from lethal Hendra virus challenge. Vaccine. 2011;29:5623-30) In a similar study with NiV-B, vaccinated ferrets remained disease free, and virus or viral genome was undetectable in all tissues and fluids with no observed pathology in examined tissues. The study also revealed good durable immunity with other ferrets challenged 434 days post-vaccination, with 5 of 5 animals were disease free following challenge and viral genome was detected only from the nasal secretions of one ferret and the bronchial lymph nodes of another ferret that were given an intermediate vaccine dose (Pallister JA, et al. Vaccination of ferrets with a recombinant G glycoprotein subunit vaccine provides protection against Nipah virus disease for over 12 months. Virol J. 2013;10:237).

The HeV-sG subunit vaccine has also been evaluated in the African green monkey (AGM), which is the only nonhuman primate model that has uniformly recapitulated human disease for both NiV and HeV infection (Rockx B, et al. A novel model of lethal Hendra virus infection in African green monkeys and the effectiveness of ribavirin treatment. J Virol. 2010;84:9831-9; Geisbert TW, et al. Development of an acute and highly pathogenic nonhuman primate model of Nipah virus infection. PLoS One. 2010;5:e10690). HeV-sG was initially tested by formulation in Allhydrogel^{TM} and CpG and animals were challenged by intratracheal administration with a 10-fold lethal dose of NiV (1×10^5 TCID50). Complete protection was observed in all vaccinated animals with no evidence of clinical disease, virus replication, or pathology in any vaccinated subject with some having pre-challenge NiV neutralizing titers as low as 1:28. A second study demonstrated HeV-sG vaccination and protection from a HeV in the AGM model and also showed that HeV-sG in Allhydrogel^{TM} alone was suffient to confer complete protection from infection and disease (Mire CE, et al. A recombinant Hendra virus G glycoprotein subunit vaccine protects nonhuman primates against Hendra virus challenge. J Virol. 2014;88:4624-31). The HeV-sG subunit vaccine is now being evaluated as a NiV/HeV vaccine for human use with support from the Coalition for Epidemic Preparedness Innovations (CEPI) (Hum Vaccin Immunother. 2017 Dec 2;13(12):2755-2762. doi: 10.1080/21645515.2017.1306615. Vaccines for epidemic infections and the role of CEPI. Plotkin SA)

A recent publication demonstrated that an adjuvanted HeV-sG vaccine in pigs induced SN antibody titers considered to be protective against NiV without detectable T cell-mediated immunity to NiV which did not protect from challenge with NiV. Pigs which had been previously challenged with a low dose of NiV developed SN antibodies and cell-mediated immune memory and were protected from a high challenge dose of NiV. The conclusion of this manuscript was that both SN antibodies and cell-mediated immunity were necessary for protection from NiV challenge (Protection against henipaviruses in swine requires both, cell-mediated and humoral immune response, B.S. Pickering, J.M. Hardham, G. Smith, E.T. Weingartl, P.J. Dominowski, D.L. Foss, D. Mwangi, C.C. Broder, J.A. Roth, H.M. Weingartl, Vaccine 34(40): 4777-4786, 2016). Subsequent unpublished research demonstrated that a different adjuvant used with the soluble HeV-sG vaccine caused the induction of both high tittered SN antibody and detectable T cell-mediated immunity in pigs to NiV. Challenge studies were not conducted (J.A. Roth, personal communication).

3) Vaccinia-vectored NiV Vaccine

The NYVAC vaccinia virus-based recombinant vaccine vector (Tartaglia *et al.,* 1992) was used to construct an experimental NiV vaccine where the vaccinia virus expresses both the NiV glycoproteins G and F (Guillaume *et al.,* 2004). This experimental vaccine was engineered by the Pasteur Institute.

Scientists at the Pasteur Institute in collaboration with University of Malaysia scientists showed that both of the NiV glycoproteins G and F when expressed as vaccinia virus recombinants induced an immune response in hamsters that protected against a lethal challenge with NiV. Furthermore, this team of scientists demonstrated passive transfer of antibody induced by either of the glycoproteins protected the animals.

DIAGNOSTICS

The gap analysis working group determined that the availability of validated diagnostic tests for surveillance, early detection, and recovery during a NiV outbreak were critical to minimize the spread of disease and reduce the economic and public impact.

Currently the diagnosis of NiV infection is by virus isolation, detection of viral RNA, or demonstration of viral antigen in tissue collected at necropsy. Specific antibody detection can also be useful, particularly in pigs where NiV infection may go unnoticed. Demonstration of specific antibody to NiV in either animals or humans is of diagnostic significance because of the rarity of infection and the serious zoonotic implication of NiV transmission.

Summary

- Antibody responses to NiV take at least 14 days and therefore early diagnosis based on serology will be less reliable than antigen or molecular tests
- Recombinant N-ELISA will likely not pick up all infected pigs
- The concept of a pen-side test is attractive, but the development and regulation of such a test will be extremely challenging

Assessment of Laboratory Diagnostic Tests (See Appendix II)

Details in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2015, Chapter 2.1.14 Hendra and Nipah Virus Diseases, provides recommendations for the following tests.

Identification of the agent

1. Virus isolation and characterization
    1.1. sampling and submission of specimens
    1.2. isolation in cultured cells
    1.3. Identification: immunostaining and Immuno EM
2. Viral identification: differentiation of HeV and NiV
    2.1 comparative immunostaining
    2.2. immunofluorescence
    2.3. microtiter neutralization

3. Molecular methods
    3.1. real-time RT-PCR
    3.2. Conventional RT-PCR and Sanger sequencing

4. Immunohistochemistry

Serological tests
1. Virus neutralization tests
2. Enzyme-linked immunosorbent assay
3. Bead-based assays

Histopathology
1. Veterinary diagnostic labs might use histopathology to make the first diagnosis
2. NiV does not produce pathognomonic lesions, but a generalized vasculitis with fibrinoid necrosis in several tissues (e.g. lung and kidneys) is characteristic; NiV might be considered in the initial differential diagnosis by experienced veterinary pathologists.

Assessment of Available Diagnostic Tests
Australia, Canada, and Germany have diagnostic capability for henipaviruses in livestock; India (e.g. NIHSAD) is building its veterinary diagnostic capability; U.S. veterinary diagnostic laboratories do not have diagnostic capability to detect NiV in livestock, although the Center for Disease Control (CDC) in Atlanta, Georgia, is an OIE collaborating center for NiV.

Currently, there are no expectations of validated tests for livestock (or other species). Nothing has been done in terms of test harmonization for serological, antigen, or nucleic acid detection assays; however, successful technology transfers have taken place, as follows: from AAHL to laboratories in Asia (Malaysia mainly); limited transfer from NCFAD to India (Bhopal High Containment Animal Health Laboratory); limited transfer from AAHL to FLI and bilateral transfers between NCFAD and FLI.

Serologic testing plays an important role in the diagnosis and detection of NiV infections. Serologic tests are the most straightforward and practical means to confirm acute cases of disease and serologic evidence of infection is used in screening programs for reservoir hosts and domestic animals. However, serological assays are limited in their ability to differentiate between known and unknown henipaviruses, as cross-reactivity to one or more known viruses is possible. Both serum neutralization and Luminex assays have shown positive reactivity to both NiV and HeV in bats where the presence of a yet-to-be characterized henipavirus could not be ruled out.

Several standard and new experimental technologies that are currently being used or considered for the detection of NiV in the laboratory or as pen-side tests for field use. Shedding of NiV in oral fluids starts early post-infection and rope sampling could prove convenient for collecting samples that could be used to test larger numbers (i.e., pen tests) of pigs. Suitability of oral fluid samples for various test platforms should be investigated. There is a need to develop a formalized worldwide structure for test validation and ring trials (i.e., inter-laboratory comparisons).

The following describes some of the most promising diagnostic platforms with potential application for NiV detection.

1) *Quantitative (q) real-time PCR*

Real-time PCR is a sensitive and useful approach to the detection of henipavirus genome in specimens. Due to its nature, rRT-PCR may not be able to detect all divergent and novel henapivirus strains, although adaptation of molecular tests to new virus variants could be rapid. Test methods and primers used depend on the technology platform and associated chemistry being used in individual laboratories. Test procedures have been described by different laboratories (Mungall *et al.,* 2006; Wacharapluesadee and Hemachudha, 2007; Guillaume *et al*., 2004; Chang *et al*., 2006; Feldman *et al*., 2009).

The AAHL has developed a quantitative real-time PCR to detect NiV or HeV RNA synthesis. The most commonly targeted amplification regions are directed against the N gene (Feldman et al., 2009).

RT-PCR targeting the N gene of NiV will detect both, NiV-M and NiV-B, with somewhat lower sensitivity for NiV-B. Confirmatory RT-PCR targeting the F gene specific only for NiV-B has therefore been developed (publication in preparation; H.M. Weingartl, personal communication).

2) *Conventional PCR*

Classical RT-PCR followed by sequencing may be more successful in detecting novel henipavirus strains. Combination of both approaches may need to be considered. Genomic RNA detection can be performed on blood or serum samples collected from live animals as well as tissues from dead animals. RNA is extracted using an RNA extraction kit [e.g., RNeasy Mini Kit (Qiagen)]. Extracted total cellular RNA is first subjected to first-stand cDNA synthesis using a reverse transcriptase kit [e.g., SensiScript (Qiagen)] and a reverse transcriptase primer. The resulting cDNA is amplified using a Master Mix PCR kit (Qiagen) and primers that are designed to target HeV and NiV positive-sense mRNA from the N, M and G genes and negative-sense genomic viral RNA (vRNA) at the N/P, M/F and F/G gene junctions.

3) *Field PCR*

Not available. Isothermal real-time RT-PCR is promising as a field deployable assay.

While this will be costly and not be practical to have in large numbers, it is worth considering having the capabilities to establish in several strategically located regions across the nation to response rapidly in an emergency situation. Technically it will not be difficult to achieve if there is the will and financial support.

4) *Virus isolation (VI)*

Virus isolation in permissive cell culture is considered the "gold standard" for isolating all strains of henipaviruses. Virus isolation greatly facilitates identification procedures and definitive diagnosis should be undertaken where operator safety can be guaranteed. Isolation is especially relevant in any new case or outbreak, particularly in countries or geographical areas where infection by NiV or HeV has not been previously documented. Implication of wildlife species as natural hosts of the viruses requires positive serology, PCR or virus isolation from wild-caught animals (Daniels *et al.,* 2007). The range of tissues yielding virus in natural and experimental cases include the brain, lung, kidney and spleen (Crameri G., *et al.* 2002).

Henipaviruses grow rapidly to high titers in a large number of cell lines. African green monkey kidney (Vero) and rabbit kidney (RK-13) cells have been found to be particularly susceptible. A CPE usually develops within 3 days, but two 5-day passages are recommended before judging the attempt unsuccessful. After low multiplicity of infection, the CPE is characterised by formation of syncytia that may, after 24–48 hours, contain over 60 or more nuclei. Syncytia formed by NiV in Vero cell monolayers are significantly larger than those created by HeV in the same time period. Although the distribution of nuclei in NiV-induced syncytia early in infection resembles that induced by HeV, with nuclei aggregated in the middle of the syncytia, nuclei in mature NiV-induced syncytia are distributed around the outside of the giant cell (Hyatt *et al.*, 2001).

Very low virus load in bats makes isolation very difficult. Linfa Wang and colleagues at the AAHL have increased sensitivity of cell lines by "rational engineering," consisting of a single point mutation in ephrinB2 resulting in enhanced affinity for NiV.

5) *Pen-side test*

Not yet developed.

While the concept is attractive, it is a huge challenge technically and in regulatory sense, especially considering how presumable false positive results would be handled.

6) *N and G ELISA*

Indirect recombinant N- ELISA and G-ELISA have been developed, and are now in the stage of diagnostic evaluation (Fisher K., *et al.*, 2018). The N-ELISA protocol was transferred to HSADDL (India) and validated and used for surveillance (Kulkarni *et al.,* 2016).

Problems with specificity (i.e., false positives) could arise. For example, swine sero-surveillance in West Bengal, India, appears to be negative; however, 8/328 samples tested positive (i.e., presumably false positive) using the anti-N antigen ELISA antibody detection test. Evaluation of the indirect IgG ELISA based on the recombinant NiV-N antigen using swine samples from Canada yielded similar results, including an indirect IgG ELISA based on the G glycoprotein. In Canadian context, the problem is the diagnostic specificity, with 5% false positives, resulting in the decision to complement with the G-ELISA. Only sera positive on both tests are considered

positive. Confirmatory testing may be required, if this was to be the first case reported in non-endemic area.

A diagnostic test for differentiating infected from vaccinated animals (DIVA) would have to most likely target the N antigen, or alternatively P gene coded products depending on the level of expression and antigenicity in animals, and the number of reactors in non-endemic areas.

The N ELISA assay could fulfill DIVA requirements if the canarypox vectored NiV-G-NiV-F vaccine is used because antibodies to N would only occur after NiV infection.

7) *IgM ELISA*

The U.S Center for Disease Control and Prevention (CDC) developed an IgM ELISA for human serology. Detection of IgM was used to confirm recent infection with NiV in both Malaysia and Bangladesh. NiV-infected cells that have been inactivated by gamma irradiation are used as antigens.

In theory the same can be done for different animal species as long as we have the right anti-species antibodies. For bats, that is still a challenge.

8) *Virus neutralization test (VNT)*

VNT serves as the traditional gold standard of serological investigations. The VNT requires live virus and thus BSL-4 containment facilities are required (Crameri *et al.,* 2002). It has proven to be a very valuable specific and sensitive tool in the diagnosis of NiV.

VNT rely on quantification methods. Three different procedures are available to titer HeV and NiV. In the traditional plaque and microtiter assay procedures, the titer is calculated as plaque forming units (PFU) or the tissue culture infectious dose capable of causing CPE in 50% of replicate wells ($TCID_{50}$), respectively.

In an alternative procedure, the viruses are titrated on Vero cell monolayers in 96-well plates and after 18–24 hours, foci of infection are detected immunologically in acetone-fixed cells using anti-viral antiserum (Crameri G., *et al.* 2002). The virus titre is expressed as focus-forming units (FFU)/ml.

Neutralisation assays using these three methods are described in the OIE Manuel of Diagnostic Tests and Vaccines for Terrestrial Animals.

Virus quantification procedures should be conducted at BSL4. A new version of the differential neutralisation test has been recently described, which avoids the use of infectious virus by the use of ephrin-B2-bound biospheres (Bossart *et al.,* 2007). Although the test has yet to be formally validated, it appears to have the potential to be a screening tool for use in countries without BSL4 facilities.

9) Pseudotype virus plaque reduction neutralization test (PRNT)

The standard plaque reduction neutralization assay (PRNT) used to detect NiV and HeV must be performed in BSL-4 containment and takes several days to complete. The CDC and the AAHL have modified the PRNT by using recombinant Vesicular Stomatitis Virus (VSV) derived from the cDNA of VSV Indiana to construct pseudotype particles expressing the F and G proteins of NiV (pVSV-NiV-F/G) as target antigens (Chang *et al.,* 2006; Tamin *et al.,* 2009; Kaku *et al*., 2009). This rapid assay can be performed at BSL-2. The PRNT was evaluated using serum samples from outbreak investigations and more than 300 serum samples from an experimental NiV vaccination study in swine. The results of the neutralization assays with pVSV-NiV-F/G as antigen showed a good correlation with those of standard PRNT. The PRNT titers give an indication of protective immunity. Therefore, this new method has the potential to be a rapid and cost-effective diagnostic method, especially in locations that lack high containment facilities, and will provide a valuable tool for basic research and vaccine development. A similar assay has been developed by the Japanese-Australian group (Kaku et al., 2009), which proved to be as specific as the VNT and much more sensitive than VNT.

10) Serological Binding Assay

Currently, a Luminex®-based (e.g. Bio-Rad Bio-Plex) multiplex microsphere immunoassay for the detection of antibodies specific to HeV and NiV G glycoproteins is used for bat surveillance at the AAHL, and by other research investigators. This multiplex microsphere immunoassay detects antibodies to recombinant soluble G (sG) proteins from NiV and HeV in a multiplexed assay. In contrast to traditional ELISAs, these Luminex-based platforms are more sensitive and require less sample sera to generate results with multiple analytes. The sG proteins retain their ability to bind the cellular receptor molecule, indicating their native conformation is maintained, which is important for the detection of neutralizing antibodies. Since the G specific antibody response to both NiV and HeV can be measured simultaneously, this assay can differentiate between the serologic responses to NiV and HeV. A variety of statistical models have been developed to determine thresholds to determine the cutoff value between negative and positives median fluorescence intensities (MFI). Instances when negative control sera is available, a MFI value three standard deviations above the z score can be used to interpret the cutoff for positive valuves.

11) Luminex® multiplexed nucleic acid detection assay

Foord et al, 2012, reported microsphere suspension array systems enable the simultaneous fluorescent identification of multiple separate nucleotide targets in a single reaction using commercially available oligo-tagged microspheres (Luminex® MagPlex-TAG) to construct and evaluate multiplexed assays for the detection and differentiation of HeV and NiV. Assays were developed to target multiple sites within the nucleoprotein (N) and phosphoprotein (P) encoding genes. The relative specificities and sensitivities of the assays were determined using reference isolates of each virus type, samples from experimentally infected horses, and archival veterinary diagnostic submissions. Results were assessed in direct comparison with an established qPCR. Foord reported the microsphere array assays achieved unequivocal differentiation of HeV and NiV

and the sensitivity of HeV detection was comparable to qPCR, indicating high analytical and diagnostic specificity and sensitivity.

12) Blocking Luminex® Assay

This is an extension of the Binding Luminex Assay, developed as a surrogate VNT in the sense that it measures antibodies that block the binding of the soluble henipavirus G protein to the ephrin-B2 receptor molecule. It is highly specific, but needs further validation with field samples.

DEPOPULATION

Preemptive culling of herds in the neighborhood of an infected herd is an effective and even indispensable measure in the control of a NiV epidemic in areas with high pig densities. The purpose of this measure is to prevent infection of new herds, which would generate massive infectious virus production, and thus to reduce the virus infection load in an area. This reduced infection load subsequently results in a reduction of the between-herd virus transmission. However, recent outbreaks have shown that the control of Nipah virus in pig populations through stamping out is complex due to the zoonotic nature of the agent. In addition, depopulation may be logistically difficult and very expensive in swine dense area, and would not be effective if the Nipah virus mutates to become easily transmitted between people and from people to pigs. Depopulation will not be possible in situation like those that occurred in Bangladesh in which NiV was transmitted from bats to humans without an amplifying host. Depopulation of swine may be impossible in a rapidly spreading outbreak in a pig dense region with hundreds of millions of swine, such as in southeast China (Vergne T. *et. al*. 2017).

SURVEILLANCE

The initial expression of NiV in U.S swine would be variable and unpredictable due to the myriad of host factors and the broad diversity of virulence among strains of henipaviruses. Different surveillance strategies will be required to detect the different clinical manifestations.

For acute infection, surveillance activities can be based on clinical signs, but signs are unlikely to be noticed by producers and practitioners. It would be prudent to develop surveillance activities based on diagnostic testing to supplement surveillance based on clinical signs.

The following surveillance programs are in place to meet the objective of rapid detection of henipaviruses in Malaysia and Australia:

1. Population-based passive reporting of suspicious NiV cases. Efforts to enhance reporting will be focused on high risk areas.
2. Laboratory-based surveillance of serum and tissue submitted from sick pigs.

There is no diagnostic capability for henipaviruses in United States veterinary diagnostic laboratories due to the lack of BSL-4 laboratory space. The only diagnostic capability for henipaviruses in the U.S is the Center for Disease Control and Prevention (CDC). There are no active or passive surveillance

programs. Henipavirus suspect samples would be sent to the CDC, the OIE reference laboratory at the Australian Animal Health Laboratory, or the National Canadian Foreign Animal Disease Center, in Winnipeg, Canada.

DRUGS

There are no licensed anti-viral drugs available to treat people or animals against Henipaviruses.

DISINFECTANTS

People: Soaps and detergents.

Fomite disinfection: Sodium hypochlorite to supply 10,000 ppm chlorine or Virkon.

PERSONAL PROTECTIVE EQUIPMENT (PPE)

PPE should be suitable to prevent farm-to-farm virus spread by diagnostic or vaccination teams.

RECOMMENDATIONS

RESEARCH

The 2017 gap analysis working group recommended the implementation of the following research priorities.

Viral Pathogenesis

- Determine early events of NiV infection, immune evasion and identify determinants for virulence and host susceptibility

Immunology

- Characterize the antibody and cell-mediated immune response to NiV infection and vaccination
- Develop the basic knowledge of the mechanisms NiV uses to evade the innate immune response
- Characterize the ability of interferons to inhibit virus replication and shedding early in infection.

Vaccine Discovery and Development Research.

- Implement comprehensive vaccine research program to deliver next generation NiV vaccines (e.g., DIVA [differentiate infected from vaccinated animals] capable), and specifically design strategies for control in priority susceptible hosts
- Investment in Nipah vaccine development needs to include conducting studies to demonstrate safety and efficacy necessary for licensure by authorities in countries that may have an emergency need for vaccine in swine.

Diagnostics

- Develop a panel of reference standards for both molecular and serologic tests that can be made available to all of the laboratories performing diagnostic tests for henipaviruses. This panel should also include monoclonal antibodies and recombinant antigens that would be readily available as low biosecurity BSL-2 reagents.
- Develop a formalized structured worldwide network for reference panel development and assay validation and harmonization.
- Develop and validate broadly reactive PCR assays targeting highly conserved genetic targets within the henipaviruses. Evaluate the relative sensitivity and specificity of the currently used PCR assays.
- Develop and validate field tests (both protein- and nucleic acid-based) to detect henipaviruses.
- Explore new antigen detection assays, including antigen capture, Loop Mediated Isothermal Amplification Protocol (LAMP) suitable for resource limited situations, and nanotechnology.
- Develop species specific reagents to improve the quality of serologic assays.
- Evaluate the relative sensitivity and specificity of molecular and serologic tests, especially new serologic tests that could replace serum neutralization titers (SNT) and meet DIVA requirements.
- Explore the use of serological assays based on recombinant antigens that could be produced at BSL-2. Classical serological tests using low biosecurity (recombinant) reagents produced at BSL-2 facilities could be developed reasonably quickly and at a reasonable cost.
- Develop species independent serologic assays using recombinant antigens.

Epidemiology

- The epidemiology of NiV in disease outbreaks needs to be assessed and modeled on the level of the individual pig, the herd, and the demographics of the region.
- Epidemiological investigations should be performed on the implementation of emergency vaccination and the use of 'DIVA' and other diagnostic tests to detect infected pigs in vaccinated populations
- Risk assessments need to be performed with regard to control or spread of henipaviruses
- The epidemiological evaluation of wildlife needs to be carried out in order to improve the risk estimates of outbreaks in domestic animal and human populations

PREPAREDNESS

Many of the countermeasures discussed in this report will require preparation and integration in a coordinated disease control program and funding for a stockpile for use in an emergency response plan for an outbreak of NiV infection. The Henipavirus gap analysis working group recommends investing in the implementation of the following preparedness plan to ensure the effective use of the countermeasures in the NVS:

- See the Ausvetplan: https://www.animalhealthaustralia.com.au/our-publications/ausvetplan-manuals-and-documents/ Assessed July 22, 2018
- See Guidelines for Veterinarians Handling potential Hendra Virus infection in Horses (QDPI): https://www.daf.qld.gov.au/__data/assets/pdf_file/0005/126770/2913_-Guidelines-for-veterinarians-handling-potential-Hendra-virus-infection-in-horses-V5.1.pdf Accessed July 22, 2018

Surveillance

Routine surveillance for NiV is now limited to serologic screening of pigs in several Southeast Asian countries.

- Develop a regional surveillance strategy, including laboratory, to detect spillovers of NiV into domestic and agricultural animals.
- Determine the optimal surveillance strategy to detect circulation of NiV in the bats reservoirs and other wild life.
- Improve surveillance capacity to detect henipaviruses in high risk countries.
- Establish a formal laboratory network for henipavirus surveillance that includes standardized specimen collection, laboratory testing scheme, quality control, specimen referral and accreditation.

Biosecurity

Design NiV-specific on-farm biosecurity programs to implement in a disease outbreak situation.

Personal Protective Equipment and Decontamination

- See Australian procedures https://www.dpi.nsw.gov.au/__data/assets/pdf_file/0003/494202/Hendra-virus-ppe-procedures.pdf Assessed July 22, 2018

- FAO (Food and Agriculture Organization) – Manual on the Diagnosis of Nipah Virus in Animals: Chapter 2: Working safely with Nipah Virus
  http://www.fao.org/docrep/005/AC449E/ac449e05.htm#bm05
  Assessed July 22, 2018

Depopulation and Disposal

Develop plans for handling disposal of animals infected with a zoonotic agent, including an emergency plan to dispose of infected swine and decontaminate facilities and equipment determined to be infected.

- FAO (Food and Agriculture Organization) – Manual on the Diagnosis of Nipah Virus in Animals: Chapter 5: Control and eradication
  http://www.fao.org/docrep/005/AC449E/ac449e08.htm#bm08

CONCLUSION

The threat of an outbreak with a henipavirus in the United States due to a natural transmission from a reservoir host is very low since the reservoirs are known to be bats in South East Asia, South Asia, and Asia. However, an outbreak that is not controlled in swine or in people in Asia could result in infection being introduced accidentally into North America or Europe. There is considerable concern that henipaviruses could be used as a weapon of mass destruction (WMD) because they have many of the characteristics of the ideal biological weapon, including causing one of the highest mortality rate in people known for an infectious disease. The possibility of an intentional criminal spread at least in short clusters of terrorist attacks is a distinct possibility, for example by aerosolization in confined public spaces, or through infection of pigs. Surveillance brings challenges and weaknesses of diagnostic methods may impede the early detection of an outbreak in the United States. There are no commercially available diagnostic tests and although laboratory tests are available they have not been field validated. Depopulation is the primary method to eradicate NiV but present very high risks since henipaviruses are BSL-4 zoonotic agents. There are commercially available vaccines for horses, but none for swine and people. Accordingly, the gap analysis working group recommends investing in the research and development of countermeasures and ensure their use and integration in planning for preparedness and future control campaigns. Priority should be given to funding research to improve surveillance, diagnostics, and vaccines. Specific goals include 1) improving diagnostic tests to rapidly identify new disease outbreaks; 2) epidemiological research to better understand virus transmission in wildlife and maintain a passive surveillance program in high risk commercial livestock operations; and 3) develop safe and effective vaccines specifically designed for control and eradication. The United States should stockpile NiV vaccines when they become available for use in contact herds to create a buffer zone as an additional control measure to prevent the spread of henipaviruses should an outbreak ever occur.

FIGURES

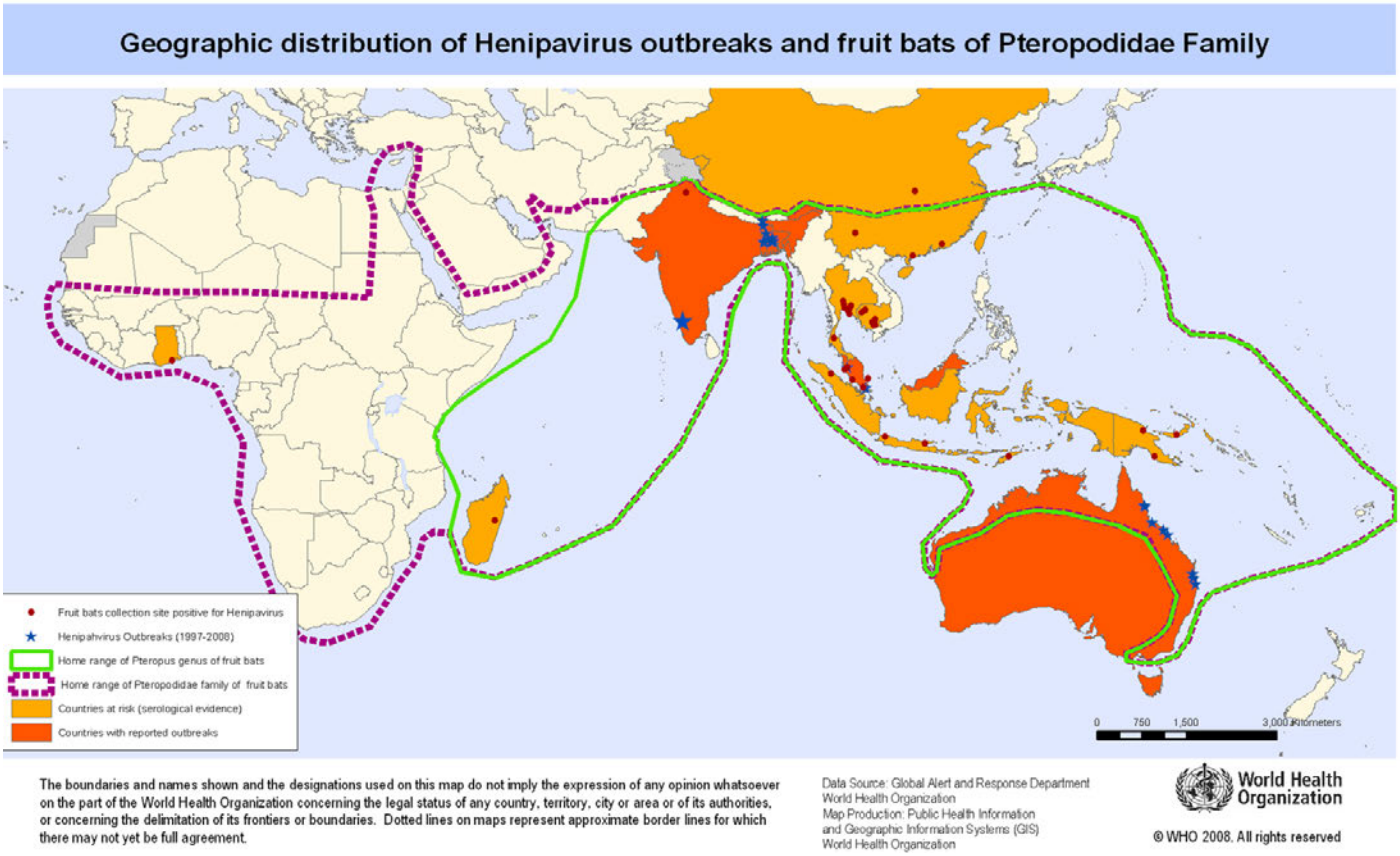

Figure 1: Geographic distribution of fruit bats of the Pteropodidae family. WHO: Nipah virus infections: http://www.who.int/csr/disease/nipah/en/ (Assessed and modified November 26, 2018).

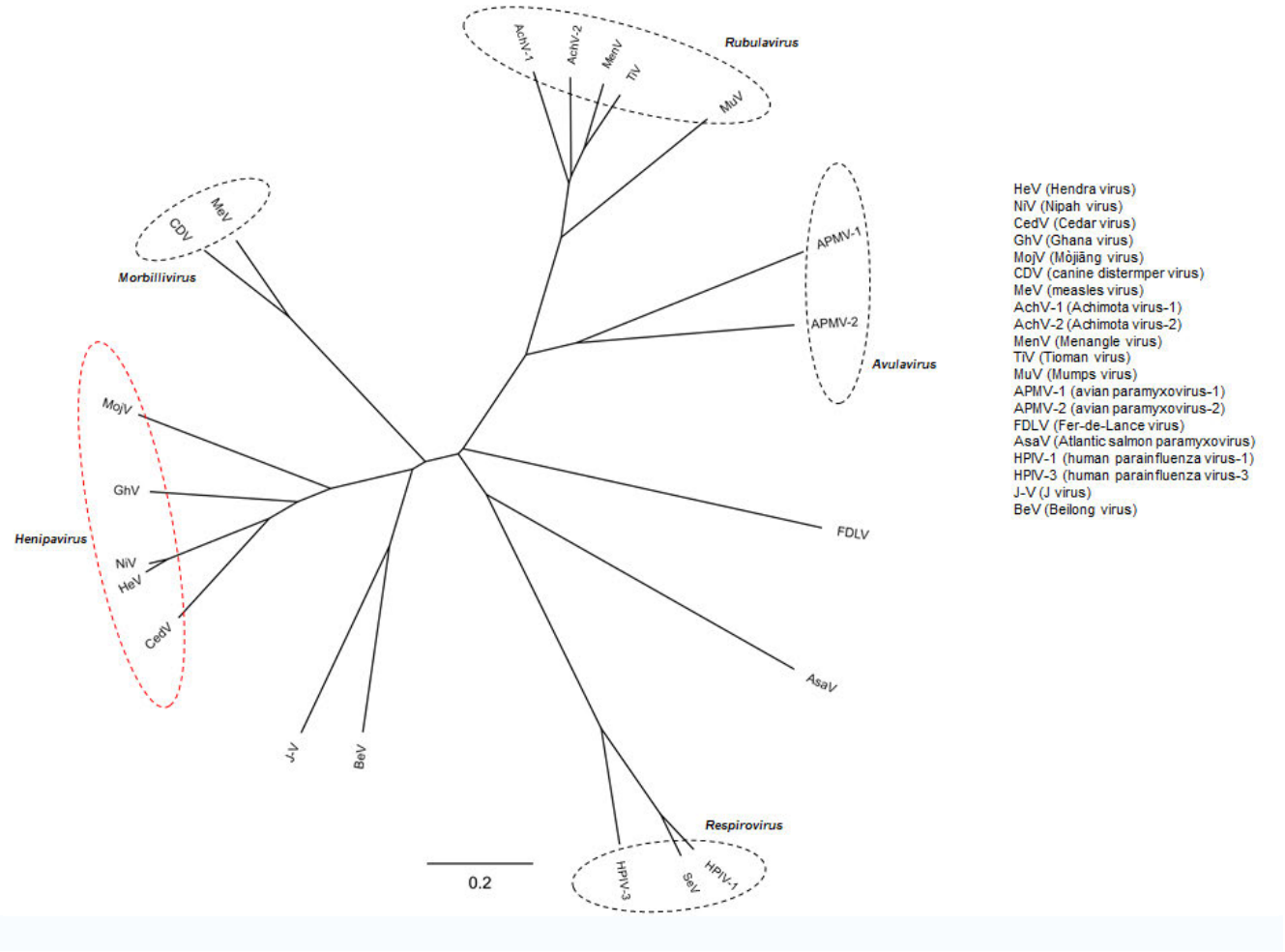

Figure 2: Phylogenetic tree based on alignment of amino acid sequence of the N-gene of selected *Paramyxovirinae* subfamily members.

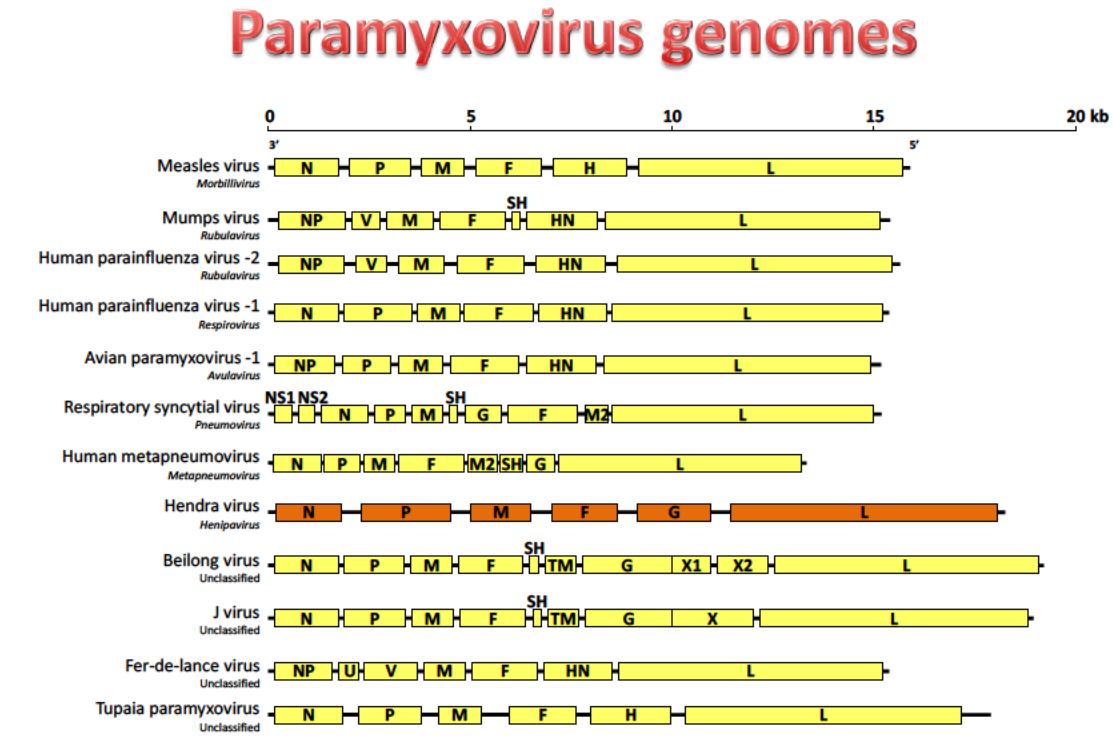

Figure 3: Comparison of *Paramyxoviridae* viruses genomes (Provided by Glen Marsh, AAHL)

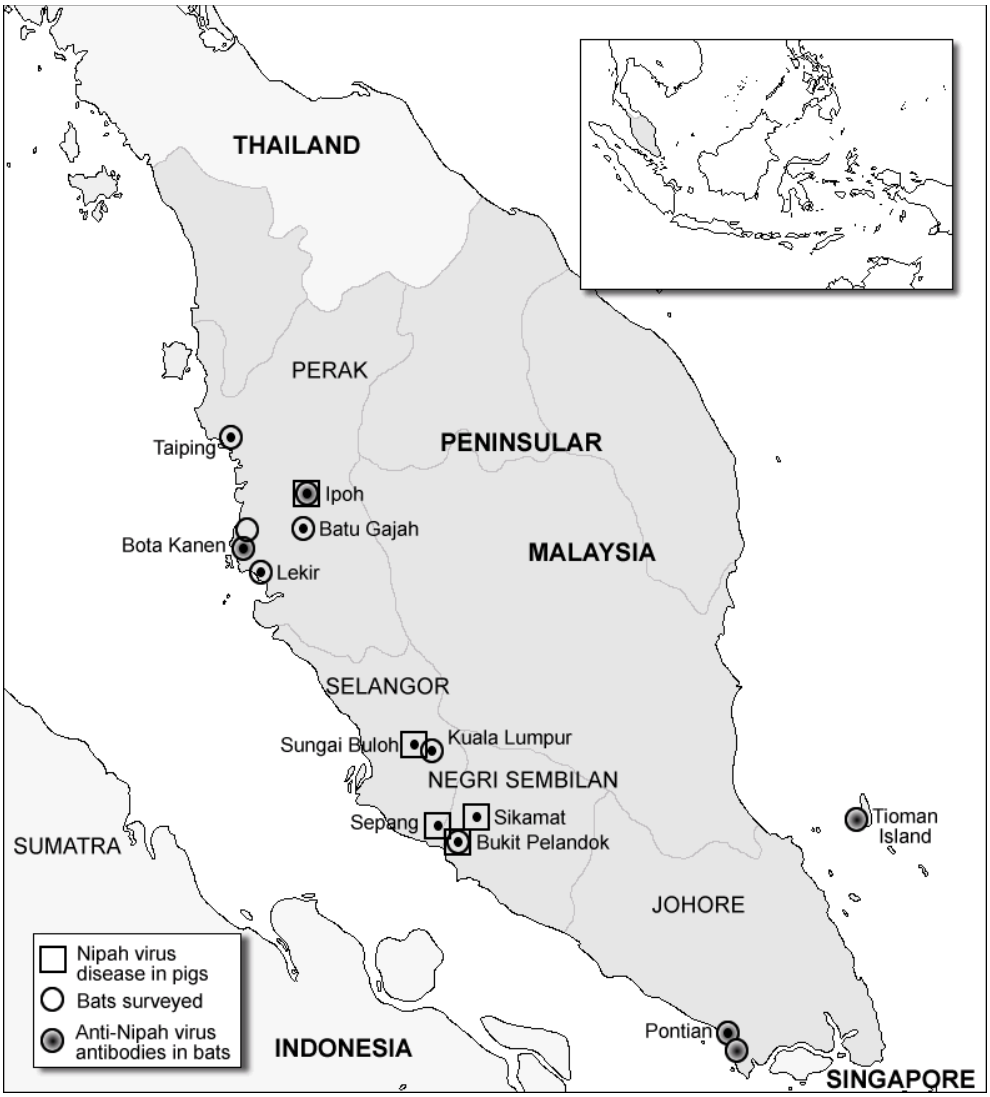

Figure 4: Descriptive map of NiV in Malaysia (Yob *et al.,* 2001)

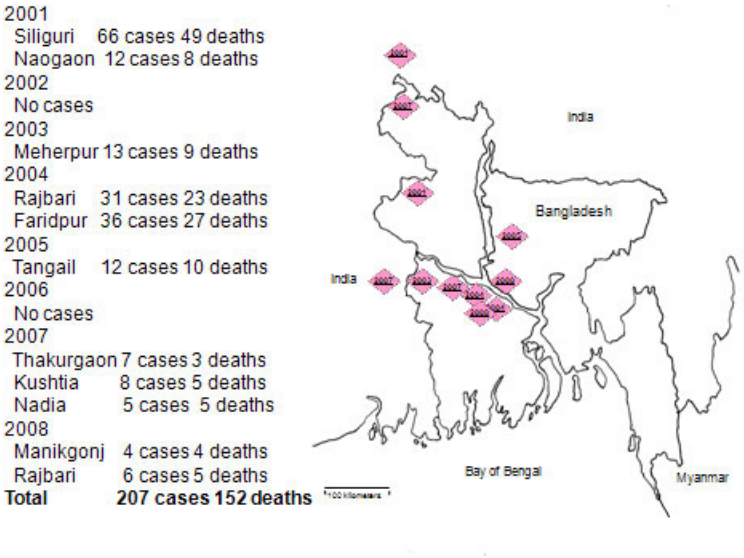

Figure 5: Epidemiology of Nipah Virus Infections in Bangladesh (Source: Steve Luby, icddr,b)

TABLE I: NIPAH VIRUS INFECTION IN BATS

Suborder	Species	No. of bats	No. Positive
Pteropodiformes	*Balionycterus macul*	4[*]	0
	Cynopterus brachyotis	56[*], 1[†]	2[*], 0[†]
	C. horsfieldi	24[*]	0
	C. sphinx	2[ǂ], 34[§], 68[†]	0[ǂ], 0[§], 0[†]
	Eidolon dupreanum	73[#]	14
	E. helvum	59[¶], 215[Δ]	23[¶], 3[Δ]
	Eonycteris spelaea	38[*], 64[§]	2[*], 0[§]
	Epomophorus gambianus	89[¶]	1
	Epomops buettikoferi	7[¶]	0
	E. franqueti	29[¶]	0
	Hipposideros armiger	63[*], 88[§], 1[†]	2[*], 0[§], 0[†]
	H. bicolor	1[*]	0
	H. larvatus	21[ǂ], 95[§], 81[†]	0[ǂ], 2[§], 0[†]
	H. pomona	60[*], 2[†]	1[*], 0[†]
	Hypsignathus monstrosus	18[¶]	1
	Macroglossus sobrinus	4[*], 1[†]	0, 0[†]
	Megaderma lyra	1[ǂ]	0
	Megaderma spasma	13[§]	0
	Megaerops ecaudatus	1[*]	0
	Nanonycteris veldkampii	4[¶]	0
	Rhinolophus acuminatus	2[†]	0
	R. affinis	6[*], 94[ǂ]	0[*], 1[ǂ]
	R. ferrumequinum	3[ǂ]	0
	R. luctus	11[ǂ], 1[†]	0[ǂ], 0[†]
	R. macrotis	3[ǂ]	0
	R. pearsoni	35[ǂ]	0
	R. pusillus	35[ǂ]	0
	R. refulgens	1[*]	0
	R. rex	1[ǂ]	0
	R. sinicus	51[*]	1
	Rousettus leschenaulti	52[*], 11[§], 15[†]	5[*], 0[§], 0[†]
	R. madagascariensis	5[#]	0
	Pteropus hypomelanus	35[*], 36[§]	11[*], 4[§]
	P. lylei	857[§], 408[†]	83[§], 50[†]
	P. medius	2790[«]	100
	P. rufus	349[#]	6
	P. vampyrus	29[*] 39[§]	5[*], 1[§]
Vespertilioniformes	*Chaerephon plicatus*	153[†]	0
	Emballonura monticola	14[§]	0
	Ia io	7[ǂ]	0
	Miniopterus spp.	32[ǂ]	5
	Myotis altarium	2[ǂ]	0
	M. daubentoni	89[ǂ]	9
	M. ricketti	84[ǂ]	8
	Murina cyclotis	1[†]	0
	Nyctalus velutinus	1[ǂ]	0
	Scotophilus heathi	3[§]	0
	Scotophilus kuhlii	33[*], 20[ǂ], 98[†]	1[*], 0[ǂ], 0[†]
	Tadarida plicata	50[§]	0
	Taphozous melanopogon	4[*], 69[†]	0[*], 0[†]
	T. saccolaimus	1[*]	0
	T. theobaldi	121[†]	0

[*]Yob JM, et al. 2001; , [ǂ]Yan L, et al. 2008; [§]Wacharapluesadee S, et al. 2005; [†]Reynes JM, et al. 2005; [¶]Hayman DTS, et al. 2008; [Δ]Drexler JF, et al. 2009; [#]Ihele C, et al. 2007; [«]Epstein JH, et al. 2016

TABLE II – NIPAH VIRUS CASES 2001-2018

Morbidity and mortality due to Nipah or Nipah-like virus encephalitis in WHO South-East Asia Region, 2001-2018

Country: Bangladesh

Month/Year	Location	No. cases	No. deaths	Case Fatality Rate
April, May 2001	Meherpur	13	9	69%
January 2003	Naogaon	12	8	67%
Jan 2004 Apr 2004	Rajbari Faridpur	31 36	23 27	74% 75%
Jan- Mar 2005	Tangail	12	11	92%
Jan-Feb 2007 Mar 2007 Apr 2007	Thakurgaon Kushtia Pabna, Natore and Naogaon	7 8 3	3 5 1	43% 63% 33%
Feb 2008 Apr 2008	Manikgonj Rajbari	4 7	4 5	100% 71%
Jan 2009	Gaibandha, Rangpur and Nilphamari Rajbari	3 1	0 1	0% 100%
Feb-Mar 2010	Faridpur Faridpur, Rajbari, Gopalgonj, Kurigram,	8 8 1	7 7 1	87.50% 87.50% 100%
Jan-Feb 2011	Lalmohirhat, Dinajpur, Comilla Nilphamari, Faridpur, Rajbari	44	40	91%
Jan 2012	Joypurhat	12	10	83%
Jan- Apr 2013	Pabna, Natore, Naogaon, Gaibandha, Manikganj	24	21	88%
Jan-Feb 2014	13 districts	18	9	50%
Jan-Feb 2015	Nilphamari, Ponchoghor, Faridpur, Magura, Naugaon, Rajbari	9	6	67%

Country: India

Month/Year	Location	No. cases	No. deaths	Case Fatality Rate
Feb 2001	Siliguri	66	45	68%
Apr 2007	Nadia	5	5	100%
May 2018	Kerala	23	21	91%

WHO (World Health Organization). Morbidity and mortality due to Nipah or Nipah-like virus encephalitis in WHO South-East Asia Region, 2001-2018. Available at: http://www.who.int/csr/disease/nipah/en/.

TABLE III – VACCINE PLATFORMS

C.C. Broder et al. / Vaccine 34 (2016) 3525–353

Table 1
Advanced active vaccination and passive immunization platforms tested in Hendra virus and/or Nipah virus animal challenge models.

Platform	Viral antigen target or immunogen	Animal challenge model
Active vaccination		
Recombinant vaccinia virus	Nipah F and/or G glycoprotein	Hamster[a] (NiV)
Recombinant canarypox virus	Nipah F and/or G glycoprotein	Pig[b] (NiV)
Recombinant VSV	Nipah F and/or G glycoprotein	Ferret[c] (NiV), Hamster[d] (NiV), nonhuman primate[e] (NiV)
Recombinant AAV	Nipah G glycoprotein	Hamster[f] (NiV, HeV)
Recombinant measles virus	Nipah G glycoprotein	Hamster and nonhuman primate[g] (NiV)
Recombinant subunit	Hendra soluble G glycoprotein	Cat[h] (NiV), Ferret[i] (HeV, NiV), nonhuman primate[j] (HeV, NiV), horse[k] (HeV)
Passive immunization		
Human monoclonal antibody m102.4	Hendra/Nipah G glycoprotein	Ferret[l] (NiV) Nonhuman primate[m] (HeV, NiV)

[a] Hamsters immunized with NiV F and/or G glycoprotein encoding recombinant vaccinia viruses were protected against disease following intraperitoneal challenge with 10^3 PFU of NiV [137].
[b] Pigs immunized with NiV F and/or G glycoprotein encoding recombinant canarypox viruses were protected against intranasal challenge with 2.5×10^5 PFU of NiV [138].
[c] Ferrets immunized with NiV F and/or G glycoprotein encoding recombinant vesicular stomatitis virus (VSV) vectors were protected against lethal intranasal challenge with 5×10^3 PFU of NiV [141]
[d] Hamsters immunized with NiV F and/or G glycoprotein encoding recombinant vesicular stomatitis virus (VSV) vectors were protected against lethal intraperitoneal challenge with 10^5 $TCID_{50}$ of NiV [143]; or 6.8×10^4 $TCID_{50}$ of NiV [142].
[e] African green monkeys immunized with a NiV G encoding recombinant VSV vector were protected against lethal intratracheal challenge with 10^5 $TCID_{50}$ of NiV [156].
[f] Hamsters immunized with a NiV G encoding recombinant adeno-associated virus (AAV) vector were protected against lethal intraperitoneal with 10^4 PFU of NiV [139].
[g] Hamsters and African green monkeys immunized with a NiV G encoding recombinant measles virus vector were protected against lethal intraperitoneal challenge with 10^3 $TCID_{50}$ of NiV (hamsters) or 10^5 $TCID_{50}$ of NiV (monkeys) [140].
[h] Hendra virus soluble G glycoprotein (HeV-sG) used to immunize cats protects against lethal subcutaneous (500 $TCID_{50}$) [120] or oronasal (5×10^4 $TCID_{50}$) NiV challenge [145].
[i] HeV-sG used to immunize ferrets protects against lethal oronasal challenge with 5×10^3 $TCID_{50}$ of HeV [124] or 5×10^3 $TCID_{50}$ of NiV challenge [146].
[j] HeV-sG used to immunize African green monkeys protects against lethal intratracheal challenge with 10^5 $TCID_{50}$ of NiV [157] or 5×10^5 PFU of HeV [147].
[k] HeV-sG used to immunize horses protects against lethal oronasal challenge with 2×10^6 $TCID_{50}$ of HeV [15].
[l] A NiV and HeV cross-reactive G glycoprotein specific neutralizing human mAb (m102.4) protects ferrets against lethal oronasal challenge with 5×10^3 $TCID_{50}$ of NiV [125] or 5×10^3 $TCID_{50}$ of HeV (J. Pallister and C. Broder, unpublished) by post-exposure infusion.
[m] Human mAb m102.4 protects African green monkeys by post-exposure infusion following lethal intratracheal challenge with 4×10^5 $TCID_{50}$ of HeV [153] or lethal intratracheal challenge with 5×10^5 PFU of NiV [154].

TABLE IV – CURRENT VACCINE CANDIDATES

B.A. Satterfield et al. / Vaccine 34 (2016) 2971–2975

Table 1
Development status of current vaccine candidates.

Candidate name/ identifier: institution	Preclinical	Developers	Ref
Subunit vaccine			
HeV sG	X	Zoetis, Inc./USU	[16,18,34,39]
Vectored vaccines			
VSV-NiV_B F and/or G	X	UTMB	[17]
VSV-NiV_M G	X	CDC	[15]
VSV-NiV_M G	X	RML	[14,19]
VSV-NiV_M F and/or G	X	Yale University	[40]
VSV-HeV G:	X	TJU/RML	[41]
RABV-HeV G:	X	TJU/RML	[41]
ALVAC-F/G	X	CFIA-NCFAD	[20,42]
AAV-NiV_M G	X	INSERM	[43]
rMV-Ed-G	X	UoT	[44]
V-NiVG	X	USU	[45]
rLa-NiVG and/or rLa-NiVF	X	CAAS-SKLVB	[21]
Passive antibody transfer			
Polyclonal serum NiV F or G	X	INSERM	[46]
Mouse mAbs NiV F or G	X	INSERM	[47]
Human mAb m102.4 Henipah G	X	USU	[35,48]

Abbreviations: USU (Uniformed Services University of the Health Sciences); UTMB (University of Texas Medical Branch); CDC (Centers for Disease Control and Prevention); RML (Rocky Mountain Laboratories); TJU (Thomas Jefferson University); CFIA-NCFAD (Canadian Food Inspection Agency – Centre for Foreign Animal Diseases); Institut national de la santé et de la recherché médicale (INSERM); UoT (University of Tokyo); CAAS-SKLVB (Chinese Academy of Agricultural Sciences (CAAS) – State Key Laboratory of Veterinary Biotechnology (SKLVB)).

APPENDIX I: COUNTERMEASURES WORKING GROUP INSTRUCTIONS

Decision Model

We will use a decision model to assess potential countermeasures to stockpile. These countermeasures must significantly improve our ability to control and eradicate an outbreak of Nipah virus in a disease-free country. The decision model is a simple tool that will allow us to focus on critical criteria for the National Veterinary Stockpile, and rank the available interventions relative to each other. The decision model is available as a Microsoft Excel spread sheet, which has been prepared to quantitatively assess the rankings we assign to a set of selected criteria that will lead to the selection of the highest cumulative option. We can use as many criteria as we want but the objective is to get down to the ones that will make or break success. The criteria for each intervention will be selected by the Countermeasures Working Group, but a preliminary set has been identified to expedite the process. You are encouraged to review the criteria prior to coming to the meeting and be prepared to modify the criteria as needed with the working group. The following provides an example of criteria and assumptions for assessing vaccines.

Criteria

If a vaccine is going to be used as an emergency outbreak control tool for Nipah virus, then we need to know: 1) is it efficacious (does it effectively eliminate shedding or just reduce shed by a known log scale); 2) does it work rapidly with one dose (probably do not have time for a second dose); 3) whether it is available today from the perspective of having a reliable & rapid manufacturing process (need to know it can be up & running rapidly and will yield a predictable amount of vaccine; 4) can we get the product to the outbreak site rapidly & safely; 5) once at the site, can we get it into the target population rapidly; 6) type of administration- mass or injected, people and equipment to do the job become important); and 7) are diagnostics available to monitor success and or DIVA compliant. While cost is important, the cost of the vaccine in an outbreak will be small in comparison to the other costs. In addition, how fast the product can be made is important because that will have a big impact on how big a stockpile will be needed. Accordingly, you will see from the Excel sheets that have been prepared for vaccines that the following critical criteria and assignment of weights for each criterion are proposed.

Weight	Critical Criteria
10	Efficacy
6	Safety
8	One dose
6	Speed of Scale up
2	Shelf life
2	Distribution/storage
10	Quick Onset of Immunity
8	DIVA Compatible
2	Withdrawal
2	Cost to Implement

(b) (6) [redacted]

APPENDIX II – VACCINES ASSESSMENT

Experimental Veterinary Vaccines For Nipah Virus - USDA/ARS, 03-19-09								
	Rank each Intervention (2,4,6,8, or 10) as to its importance to making a decision, only one "10" rankings allowed							
Weight	**Critical Criteria**	CPV-G	CPV-F	VV-G	Soluble G			
10	Efficacy	6	4	2	6			
6	Safety	10	10	2	10			
8	One dose	4	4	4	2			
8	Manufacturing safety	8	8	6	8			
10	DIVA Compatible	8	8	8	8			
8	Manufacturing yield	8	8	8	6			
6	Rapid production	8	8	4	4			
4	Reasonable cost	6	6	4	2			
2	Short withdrawal	8	8	2	4			
8	Long shelflife	8	8	8	4			
Rank each Criteria 2,4,6,8 or10 on each criterion -- no more than two "10" rankings allowed								
	Critical Criteria	CPV-G	CPV-F	VV-G	Soluble G	0	0	0
	Efficacy	60	40	20	60	0	0	0
	Safety	60	60	12	60	0	0	0
	One dose	32	32	32	16	0	0	0
	Manufacturing safety	64	64	48	64	0	0	0
	DIVA Compatible	80	80	80	80	0	0	0
	Manufacturing yield	64	64	64	48	0	0	0
	Rapid production	48	48	24	24	0	0	0
	Reasonable cost	24	24	16	8	0	0	0
	Short withdrawal	16	16	4	8	0	0	0
	Long shelflife	64	64	64	32	0	0	0
	0	0	0	0	0	0	0	0
	Value	512	492	364	400	0	0	0

APPENDIX III – DIAGNOSTICS ASSESSMENT

Experimental Diagnostics For Nipah Virus - USDA/ARS, 03-19-09

Rank each Interven ion (2,4,6,8, or 10) as to its importance to you in making a decision, no more han one "10" rankings allowed

Weight	Critical Criteria	qPCR	conv PCR	field PCR	VI	penside	v ELISA	N ELISA	IgM ELISA	VNT	ps VNT	bind lum	block lum
10	Sensi ivity	10	10	8	8	4	10	4	8	8	8	8	8
8	Specificity	8	6	8	10	6	6	6	8	10	8	8	8
2	DIVA	8	8	8	8	8	2	10	6	2	2	8	2
6	multispecies	8	8	8	8	8	6	6	2	8	8	6	8
8	Validation to purpose	8	8	8	8	4	8	4	10	8	10	8	10
4	Speed of Scaleup	8	4	4	2	6	8	8	8	2	4	4	4
4	Throughput	8	2	2	2	4	8	8	8	2	4	6	6
4	Flock Side Test	2	2	10	2	10	2	2	2	2	2	2	2
10	Rapid Result	6	4	8	2	8	6	6	6	4	4	10	8
4	No need to Confirm	6	4	4	8	2	6	4	6	8	8	8	8
8	Easy to perform	8	6	6	4	8	8	8	6	6	6	8	8
8	safe to operate	8	8	6	2	6	8	8	8	2	8	8	8
8	Availability	8	8	2	2	2	6	8	4	2	6	4	4
6	Storage/Distribution	4	6	6	2	6	6	6	6	2	4	4	4
4	Low Cost to Implement	2	4	2	2	4	6	8	6	2	4	4	2

Rank each Criteria 2,4,6,8 or10 on each criterion -- no more han two "10" rankings allowed

Critical Criteria	qPCR	conv PCR	field PCR	VI	penside	v ELISA	N ELISA	IgM ELISA	VNT	ps VNT	bind lum	block lum
Sensi ivity	100	100	80	80	40	100	40	80	80	80	80	80
Specificity	64	48	64	80	48	48	48	64	80	64	64	64
DIVA	16	16	16	16	16	4	20	12	4	4	16	4
multispecies	48	48	48	48	48	36	36	12	48	48	36	48
Validation to purpose	64	64	64	64	32	64	32	80	64	80	64	80
Speed of Scaleup	32	16	16	8	24	32	32	32	8	16	16	16
Throughput	32	8	8	8	16	32	32	32	8	16	24	24
Flock Side Test	8	8	40	8	40	8	8	8	8	8	8	8
Rapid Result	60	40	80	20	80	60	60	60	40	40	100	80
No need to Confirm	24	16	16	32	8	24	16	24	32	32	32	32
Easy to perform	64	48	48	32	64	64	64	48	48	48	64	64
safe to operate	64	64	48	16	48	64	64	64	16	64	64	64
Availability	64	64	16	16	16	48	64	32	16	48	32	32
Storage/Distribution	24	36	36	12	36	36	36	36	12	24	24	24
Low Cost to Implement	8	16	8	8	16	24	32	24	8	16	16	8
Value	672	592	588	448	532	644	584	608	472	588	640	628

APPENDIX IV - CONTRIBUTORS

(b) (6)

(b) (6)


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(b) (6)

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From: (b) (6)
To: Laing, Eric; Broder, Christopher; (b) (6)
Subject: Henipavirus Gap Analysis Report - Updated
Date: Tuesday, December 18, 2018 6:12:32 AM
Attachments: Henipavirus Gap Analysis Report, Updated November 2018.pdf
image003.png
image005.png

Dear Colleagues,

Just a quick note to let you know that our gap analysis report was updated with new information on emerging henipaviruses and detection in bats in new geographical areas of Africa and Asia. Many thanks to (b) (6) at USUHS for their help in updating the report. The updated report is attached for your use. The reference and URL remain the same:

To cite this report:
Henipavirus Gap Analysis Workshop Report. 2018. U.S. Department of Agriculture, Agricultural Research Service, Washington, DC. http://go.usa.gov/xnHgR.

Also, you may have seen this already but our report was highlighted in Bat News – see below.

Thanks again everyone for all your help with this report and please send me updates in the future as needed. Next steps will be to share the report with funders of research to support the implementation of the research priorities identified in our report.

Wishing everyone a Merry Christmas, Happy Holidays, and success in 2019!

Best regards,

(b) (6)

[redacted]

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Wishing everyone a happy holiday and all the very best for 2019!

Best regards,
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Hendra virus

- Henipavirus Gap Analysis Workshop Report

White-nose syndrome

- How to vaccinate a wild bat
- Groundbreaking science at TRU aims to save bats
- Mammoth Cave scientists studying white-nose syndrome
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Other news

- Heat stress – media
- 'It's extremely cruel': backyard netting killing, maiming fruit bats
- Flying foxes detect new invaders
- MSU project to prevent bat-borne diseases wins $10 million grant
- 'Pest-controlling' bats could help save rainforests
- Betting on bats for genetic treasures
- National Wildlife Biosecurity Guidelines
- National Flying-Fox Forum – Presentations available

Publications

- *Polychromophilus melanipherus* and haemoplasma infections not associated with clinical signs

in southern bent-winged bats (*Miniopterus orianae bassanii*) and eastern bent-winged bats (*Miniopterus orianae oceanensis)*

- Alston virus, a novel paramyxovirus isolated from bats causes upper respiratory tract infection in experimentally challenged ferrets

- Slow growth and delayed maturation in a Critically Endangered insular flying fox (*Pteropus natalis*)

- Active and passive surveillance for bat lyssaviruses in Italy revealed serological evidence for their circulation in three bat species

- Lleida Bat Lyssavirus isolation in *Miniopterus schreibersii* in France

- Active sero-survey for European bat lyssavirus type-1 circulation in North African insectivorous bats

- Rabies & bats – publications

- Coronaviruses & bats – publications

- Bacterial resistance in bats from the Phyllostomidae family and its relationship with unique health

- Coordinated change at the colony level in fruit bat fur microbiomes through time

- Handling stress and sample storage are associated with weaker complement-mediated bactericidal ability in birds but not bats

- Mass-culling of a threatened island flying fox species failed to increase fruit growers' profits and revealed gaps to be addressed for effective conservation

- Publications – Other bat diseases

Hendra virus

Henipavirus Gap Analysis Workshop Report

Henipavirus Gap Analysis Workshop Report. 2018. U.S. Department of Agriculture, Agricultural

Research Service, Washington, DC. http://go.usa.gov/xnHgR

"…The United States Department of Agriculture (USDA) organized the first Nipah virus gap analysis workshop in Australia in 2009 with the support of the Australian Animal Health Laboratory (AAHL). The working group was charged by the USDA National Veterinary Stockpile Steering Committee with making recommendations on specific materials, commercially available and in the pipeline, which will ensure the United States has an arsenal of highly efficacious

countermeasures to control and mitigate the impact of an outbreak of Nipah virus. Nipah virus experts representing laboratories in South East Asia, Australia, Canada, and the United States were invited to participate and contributed to this report. The second workshop was organized in 2017 by the Special Pathogens Unit, National Centre for Foreign Animal Disease, Canadian Food Inspection Agency (CFIA)…The participants were charged with assessing available veterinary medical countermeasures to control and respond to a Nipah virus disease outbreak. In addition, the workshop participants agreed to update the gap analysis conducted at the AAHL in Geelong, Australia, in 2009." Download the report from the STAR-IDAZ website or at the link above.

White-nose syndrome

How to vaccinate a wild bat

22/11/2018 Scientific American Article: "This probably won't come as a surprise, but vaccinating wild bats is a difficult task. It's also an important one: many bat populations are now endangered by white-nose syndrome, a serious fungal disease that invades the skin of bats… There's a vaccine against the fungus, but this requires painstaking capture and manual application of the medicine. It would be far better to administer vaccine to many bats at once, if it were possible to spray the vaccine onto the bats as they enter and exit their dwellings. The vaccine would then be consumed by the animals as they groom the sprayed material from their fur. Which is why the National Wildlife Health Center, a unit of the U.S. Geological Survey, recently partnered with PARC, a Xerox company, to undertake a wildlife protection project in Madison, Wisconsin. The goal is to explore the use of new spraying technologies to treat wild bats with topical vaccines…"

Groundbreaking science at TRU aims to save bats

29/11/2018 BC Local News Article: "…Fontaine is working with a team of researchers… to stop White-Nose Syndrome (WNS) from decimating the western North American bat populations… They're using the principal of probiotics — the application of good bacteria — in order to prevent the fungus that causes WNS… The probiotic was developed in Dr. Xu's lab based on bacteria that Cheeptham and her team discovered on the wings of healthy bats. Lab tests have shown the probiotic is effective at inhibiting the growth of the deadly fungus… Results from this first small-scale study are expected within the next two weeks, and another larger study is expected to begin next spring…"

Mammoth Cave scientists studying white-nose syndrome

27/11/2018 Glasgow Daily Times Article: "Scientists at Mammoth Cave National Park are taking part in two studies regarding white-nose syndrome, or WNS… The study involves testing bacteria found on bats in caves in New Mexico and Arizona to see if the bacteria has antagonistic effects against WNS… MCNP scientists have been afraid the bacteria that could be used to suppress the growth of WNS could harm cave crickets, cave beetles and other creatures found inside Mammoth Cave, so they have been working with researchers in New Mexico and New York to conduct preliminary tests in petri dishes to see what effect the bacteria might have on cave organisms… The other study MCNP is involved with is using ultra-violet light to see how it might affect cave organisms…"

What secrets are hidden inside the call of a bat?

20/11/2018 Connecticut Public Radio Article: "The fungal disease white-nose syndrome has killed off millions of bats across America… Now, scientists are trying to learn more about the impact of this devastating disease, by listening to the calls of the bats left behind…"

Winter 2018/2019 bat submission guidelines and updates from the 2017/2018 white-nose syndrome surveillance season [USA]

29/12/2018 USGS National Wildlife Health Center Article : "Updated guidance from the USGS National Wildlife Health Center (NWHC) is now available for bat submissions for the 2018/2019 white-nose syndrome (WNS) surveillance season… Included are reference charts and an updated WNS Management Area map to assist submitters in identifying priority species and collecting appropriate samples for submission to a diagnostic laboratory. These guidelines support surveillance objectives of the WNS National Plan designed to identify new geographic locations and bat species impacted by *Pseudogymnoascus destructans* (*Pd*) and WNS… Surveillance conducted last season documented an expansion in the distribution of Pd… and an increase in the number of North American bat species on which the fungus has been detected. Specifically, WNS was confirmed in two new states (Kansas and South Dakota) and two additional Canadian provinces (Manitoba and Newfoundland)… Additionally, Pd in absence of clinical signs of WNS was detected on bats from Mississippi, Texas, and Wyoming…" The updated guidelines are available from the USGS website.

For Australian information on WNS including how to identify and report a suspect case of WNS and sample submission guidelines for veterinarians, go to the Wildlife Health Australia website.

White-nose syndrome – other publications

- Morisak K (2018). Variation of *Pseudogymnoascus destructans* spore loads and risk of human vectored transport. MSc thesis, University of Akron Abstract

- Bansal S. (2018). A bibliometric study of research output on white-nose syndrome. *Indian Journal of Information Sources and Services*, *8*(2), 95-98 Article [PDF]

- Martínková N et al (2018). Modelling invasive pathogen load from non-destructive sampling data. *bioRxiv*, 474817 Abstract [Pre-print, not peer reviewed]

Other news

Heat stress – media

To report flying-fox heat stress events, fill out the flying-fox heat-stress data form from the Lab of Animal Ecology, Western Sydney University. For alerts, go to the Flying-fox Heat Stress Forecaster.

A selection of media:

- 30/11/2018 The Guardian Article **Queensland flying fox species decimated by record heatwave:** "Thousands of threatened flying foxes have dropped dead due to heat stress brought on by extreme temperatures in far north Queensland this week. Conservationists and wildlife volunteers estimate more than 4,000 have perished this week during the record heatwave, which has seen temperatures in Cairns reach all-time highs of 42.6C. The species of flying fox affected is the spectacled flying fox, an endemic Queensland species found in north Queensland. It's currently listed as vulnerable under national environment laws but conservationists have been pushing to have the species up-listed to endangered because of declines in the population. Volunteer carers that have been counting dead animals and taking orphaned young into care say it is the first time the species has suffered mass deaths because of extreme heat…."

- 28/11/2018 The Australian Article **Qld heatwave decimates bat population:** "Thousands of heat-stressed bats are dropping from trees and creating a health hazard in far north Queensland, as a record-breaking heatwave blasts the region. About 3500 flying foxes are estimated to have perished since the furnace-like conditions began on Sunday. However, Trish Wimberley of the Australian Bat Clinic says that's a conservative estimate, with thousands more likely to perish before the heatwave ends…"

- 27/11/2018 Sunshine Coast Daily Article **Heatwave contributes to rise in bat bites:** "Cairns and Hinterland Hospital and Health Service have advised people to be wary of bats, as the heatwave sends them falling from trees and into biting range… He said some bats may be infected with the potentially deadly Australian bat lyssavirus (ABLV)…"

'It's extremely cruel': backyard netting killing, maiming fruit bats

5/12/2018 Sydney Morning Herald Article: "…Habitats and food sources of the fruit bat, also known as the grey-headed flying fox, have been so heavily encroached by urban sprawl and development, the animals are driven to backyard fruit trees. But the wide-holed nets used by backyard gardeners to protect their fruit trees are killing and maiming the native animals. And babies are dying because their injured, or dead, mothers are not returning to feed them… Wildlife Victoria has already responded to more than 600 bat rescue call-outs this year, with 77 of them being entanglement cases…"

Flying foxes detect new invaders

Biosecurity Queensland Article: "An innovative surveillance project is underway to see if flying fox camps can provide an early warning system for potentially invasive plant species. Biosecurity Queensland, in partnership with the City of Gold Coast and the Queensland Herbarium, are surveying vegetation around flying fox camps to establish an 'early warning surveillance system' for serious new weed species, particularly Miconia and Mexican bean trees… Flying foxes eat the fruit of a variety of plant species and digest seeds through their waste under their overnight camps. If we find high-risk new weeds growing under the camp, we know that the species is growing in gardens or bushland nearby. We can focus our public awareness and on-ground surveillance activity to a specific radius and find it before it has a chance to develop into a major problem…"

MSU project to prevent bat-borne diseases wins $10 million

grant

3/12/2018 Montana State University Article: “In an effort to prevent some of the world's most lethal diseases, an international research team spanning five continents and led by Montana State University will study bats in Australia, Bangladesh, Madagascar and Ghana. Raina Plowright, assistant professor in the Department of Microbiology and Immunology in MSU's College of Agriculture and College of Letters and Science, is leading a project to unravel the complex causes of bat-borne viruses that have recently made the jump to humans, causing concern among global health officials. The research team — which includes more than 20 scientists from Johns Hopkins, Cornell, Cambridge, UCLA, Penn State, Rocky Mountain Laboratories in Montana, Griffith University in Australia and five other universities and institutions — is supported by a $10 million cooperative agreement with the Defense Advanced Research Projects Agency…”

'Pest-controlling' bats could help save rainforests

11/12/2018 ScienceDaily Article: “A new study shows that several species of bats are giving Madagascar's rice farmers a vital pest control service by feasting on plagues of insects. And this, a zoologist at the University of Cambridge believes, can ease the financial pressure on farmers to turn forest into fields…”

Cited journal article: Kemp J et al (2019). Bats as potential suppressors of multiple agricultural pests: A case study from Madagascar. *Agriculture, Ecosystems & Environment*, 269, 88-96 Abstract

Betting on bats for genetic treasures

29/10/2018 Knowable Magazine Article: “Most of us think of bats only when it’s time to decorate for Halloween. But a large group of scientists finds them fascinating all year round — so much so that they’ve launched an ambitious research program, known as the Bat1K Project, to sequence the genomes of every one of the world's 1,300-odd bat species. And the payoffs could be surprisingly high…”

National Wildlife Biosecurity Guidelines

26/11/2018 WHA Article: “Wildlife Health Australia this week released an unprecedented and valuable resource to help Australians who work with wildlife; the National Wildlife Biosecurity Guidelines… CEO of Wildlife Health Australia, Rupert Woods said: “These new Guidelines draw together the latest information and insights on how wildlife workers in all fields and working across Australia can adopt best-practices in applying biosecurity controls to every aspect of their work. If everyone working with wildlife; from vets to government agencies, students to carers, adopt practices that protect biosecurity, this will be critical to protecting wild animal populations and communities, and Australia’s animal industries from new and emerging diseases.” The guidelines can be downloaded from the Wildlife Health Australia website (or with this direct link to the PDF). WHA has also a one-page information sheet.

National Flying-Fox Forum – Presentations available

The 3rd Annual National Flying-Fox Forum was held in Cairns on 8th November 2018. “This Forum follows on from successful events held in 2017 and 2016, bringing together over 100 dedicated individuals from all levels of government, non-government organisations, universities, environmental consultancies and community groups to explore the issues of flying-fox management

and conservation…" Presentations from the forum are now available to download from the Ecosure website.

Publications

Polychromophilus melanipherus and haemoplasma infections not associated with clinical signs in southern bent-winged bats (*Miniopterus orianae bassanii*) and eastern bent-winged bats (*Miniopterus orianae oceanensis)*

Holz PH et al (2018). *Polychromophilus melanipherus* and haemoplasma infections not associated with clinical signs in southern bent-winged bats (*Miniopterus orianae bassanii*) and eastern bent-winged bats (*Miniopterus orianae oceanensis*). *International Journal for Parasitology: Parasites and Wildlife,* 8, 10-18 Article [Open access]

Abstract: "…The southern bent-winged bat (*Miniopterus orianae bassanii*) is a critically endangered subspecies endemic to south-eastern Australia. As part of a larger study… southern bent-winged bats from several locations in Victoria and South Australia were captured and examined for the presence of the blood parasite, *Polychromophilus melanipherus*, and haemoplasmas (*Mycoplasma* sp.)... Both organisms were found in both subspecies… with no association between the probability of infection, body weight, abnormal blood parameters or any other indicators of ill health. However, Victorian southern bent-winged bats had heavier burdens of *P. melanipherus* than both the South Australian southern bent-winged bats and eastern bent-winged bats. Further investigations are required to determine if these differences are impacting population health."

Alston virus, a novel paramyxovirus isolated from bats causes upper respiratory tract infection in experimentally challenged ferrets

Johnson RI et al (2018). Alston Virus, a novel paramyxovirus isolated from bats causes upper respiratory tract infection in experimentally challenged ferrets. *Viruses*, 10(12), 675 Article[Open access]

Abstract: "Multiple viruses with zoonotic potential have been isolated from bats globally. Here we describe the isolation and characterization of a novel paramyxovirus, Alston virus (AlsPV), isolated from urine collected from an Australian pteropid bat colony in Alstonville, New South Wales. Characterization of AlsPV by whole-genome sequencing and analyzing antigenic relatedness revealed it is a rubulavirus that is closely related to parainfluenza virus 5 (PIV5)…. Oronasal challenge of ferrets resulted in subclinical upper respiratory tract infection, viral shedding in respiratory secretions, and detection of viral antigen in the olfactory bulb of the brain…."

Slow growth and delayed maturation in a Critically Endangered insular flying fox (*Pteropus natalis*)

Todd CM et al (2018). Slow growth and delayed maturation in a Critically Endangered insular flying fox (*Pteropus natalis*). *Journal of Mammalogy*, 99(6), 1510-1521 Abstract

Abstract: "Flying foxes (family Pteropodidae) have distinct life histories given their size, characterized by longevity, low reproductive output, and long gestation. However, they tend to decouple the age at which sexual maturity is reached from the age at which they reach adult dimensions. We examined growth, maturation, and reproduction in the Critically Endangered Christmas Island flying fox (*Pteropus natalis*) to determine the timing of sex-specific life cycle events and patterns of growth… Growth and maturation are even slower in *P. natalis* than in the few other *Pteropus* species studied to date. The slow growth and delayed maturation of *P. natalis* imply slower potential population growth rates, further complicating the recovery of this Critically Endangered single-island endemic."

Active and passive surveillance for bat lyssaviruses in Italy revealed serological evidence for their circulation in three bat species

Leopardi S et al (2018). Active and passive surveillance for bat lyssaviruses in Italy revealed serological evidence for their circulation in three bat species. *Epidemiology & Infection,* doi: 10.1017/S0950268818003072 Abstract [Open access]

Abstract: "The wide geographical distribution and genetic diversity of bat-associated lyssaviruses (LYSVs) across Europe suggest that similar viruses may also be harboured in Italian insectivorous bats. Indeed, bats were first included within the passive national surveillance programme for rabies in wildlife in the 1980s, while active surveillance has been performed since 2008. The active surveillance strategies implemented allowed us to detect neutralizing antibodies directed towards *European bat 1 lyssavirus* in six out of the nine maternity colonies object of the study across the whole country. Seropositive bats were *Myotis myotis*, *M. blythii* and *Tadarida teniotis*..."

Lleida Bat Lyssavirus isolation in *Miniopterus schreibersii* in France

Picard-Meyer E et al (2018). Lleida Bat Lyssavirus isolation in *Miniopterus schreibersii* in France. *Zoonoses and Public Health*, doi: 10.1111/zph.12535 Abstract

Abstract: "Bat rabies cases are attributed in Europe to five different Lyssavirus species of 16 recognized Lyssavirus species causing rabies. One of the most genetically divergent Lyssavirus spp. has been detected in a dead *Miniopterus schreibersii* bat in France… The analysis of the complete genome sequence confirmed the presence of Lleida bat lyssavirus (LLEBV) in bats in France with 99.7% of nucleotide identity with the Spanish LLEBV strain (KY006983)."

Active sero-survey for European bat lyssavirus type-1 circulation in North African insectivorous bats

Serra-Cobo J et al (2018). Active sero-survey for European bat lyssavirus type-1 circulation in North African insectivorous bats. *Emerging Microbes & Infections,* 7, 213 Article [Open access]

Article: "…In Africa, to date, three lyssaviruses have been identified in bats… Little is known about the circulation and distribution of insectivorous bat lyssaviruses in North Africa, as well as the impact such viruses may have on public health… The aim of this study was to assess the potential circulation of European bat lyssaviruses in Northern Africa from 2007 to 2012…"

Rabies & bats – publications

- Seetahal JF et al (2019). Of bats and livestock: The epidemiology of rabies in Trinidad, West Indies. *Veterinary Microbiology,* 228, 93-100 Abstract

- Reed M et al (2018). Novel mass spectrometry based detection and identification of variants of rabies virus nucleoprotein in infected brain tissues. *PLoS Neglected Tropical Diseases,* 12(12): e0006984 Article [Open access, uncorrected proof]

Coronaviruses & bats – publications

- Zheng Y et al (2018). Lysosomal proteases are a determinant of coronavirus tropism. *Journal of Virology*, *92*(24), e01504-18 Abstract

- Cui J et al (2018). Origin and evolution of pathogenic coronaviruses. *Nature Reviews: Microbiology,* doi: 10.1038/s41579-018-0118-9 Abstract

Bacterial resistance in bats from the Phyllostomidae family and its relationship with unique health

Sens-Junior H et al (2018). Bacterial resistance in bats from the Phyllostomidae family and its relationship with unique health. *Pesquisa Veterinária Brasileira,* 38(6), 1207-16 Abstract (English)

Abstract: "….The present paper has the purpose to identify the oral and perianal microbiota and to detect the bacterial resistance of frugivorous bats captured near communities inhabited by humans in the northwestern region of the state of Paraná…. All bat species studied had resistant strains, with a few of them presenting multi-resistance to antimicrobials… This is an issue and a future warning for unique health, since high percentages of resistance were found against antimicrobials broadly used, such as ampicillin, amoxicillin and amoxicillin+clavulonate."

Coordinated change at the colony level in fruit bat fur microbiomes through time

Kolodny O et al (2018). Coordinated change at the colony level in fruit bat fur microbiomes through

time. *Nature Ecology & Evolution*, 3, 116-124 Abstract

Abstract: “The host-associated microbiome affects individual health and behaviour, and may be influenced by local environmental conditions… Here, we investigate longitudinal changes in the fur microbiome of captive and free-living Egyptian fruit bats. We find that, in contrast to patterns described in humans and other mammals, the prominent dynamics is of change over time at the level of the colony as a whole...”

Handling stress and sample storage are associated with weaker complement-mediated bactericidal ability in birds but not bats

Becker D et al (2019). Handling stress and sample storage are associated with weaker complement-mediated bactericidal ability in birds but not bats. *Physiological and Biochemical Zoology,* 92(1), 37-48 Abstract

Abstract: “Variation in immune defense influences infectious disease dynamics within and among species. Understanding how variation in immunity drives pathogen transmission among species is especially important for animals that are reservoir hosts for zoonotic pathogens. Bats, in particular, have a propensity to host serious viral zoonoses without developing clinical disease themselves. The immunological adaptations that allow bats to host viruses without disease may be related to their adaptations for flight... A number of analyses report greater richness of zoonotic pathogens in bats than in other taxa, such as birds (i.e., mostly volant vertebrates) and rodents (i.e., nonvolant small mammals), but immunological comparisons between bats and these other taxa are rare. To examine interspecific differences in bacterial killing ability (BKA), a functional measure of overall constitutive innate immunity, we use a phylogenetic meta-analysis to compare how BKA responds to the acute stress of capture and to storage time of frozen samples across the orders Aves and Chiroptera...”

Mass-culling of a threatened island flying fox species failed to increase fruit growers’ profits and revealed gaps to be addressed for effective conservation

Florens FBV & Baider C (2018). Mass-culling of a threatened island flying fox species failed to increase fruit growers’ profits and revealed gaps to be addressed for effective conservation. *Journal for Nature Conservation,* doi: 10.1016/j.jnc.2018.11.008 Abstract

Abstract: “Human-wildlife conflicts (HWC) pose a growing threat to biodiversity worldwide and solutions can be as sound as the understanding of the HWC itself… In this context, Mauritius implemented what may be the first mass-culls of an already threatened native species when it culled the flying fox (*Pteropus niger*)… We synthesized the best literature available locally and also elsewhere in relevant situations, to critically appraise the setting, nature, timeline of events and outcome of both completed mass-culling campaigns to explore why and how they happened so as to help towards devising better approaches to such conflicts...”

Related news: 5/12/2018 Mongabay News Article: **Culls push endangered fruit bat closer to extinction in Mauritius**

Publications – Other bat diseases

- Nelson C (2018). New bat genome and immunity. *Lab Animal,* 47(7), p.185 Abstract

- Lau SKP et al (2018). Replication of MERS and SARS coronaviruses in bat cells offers insights to their ancestral origins. *Emerging Microbes & Infections,* 7, 209 Article [Open access]

- Balkema-Buschmann A et al (2018). Productive propagation of Rift Valley fever phlebovirus vaccine strain MP-12 in *Rousettus aegyptiacus* fruit bats. *Viruses*, *10*(12), 681 Abstract [Open access]

- Jacquet S et al (2018). Evolution of hepatitis B virus receptor NTCP reveals differential pathogenicity and species-specificities of hepadnaviruses in primates, rodents and bats. *Journal of Virology,* doi: 10.1128/JVI.01738-18 Abstract

- Xu Z et al (2018). Isolation and identification of a highly divergent Kaeng Khoi virus from bat flies (*Eucampsipoda sundaica*) in China. *Vector-Borne and Zoonotic Diseases*, doi: 10.1089/vbz.2018.2350 Abstract

- Ahmed W et al (2018). Marker genes of fecal indicator bacteria and potential pathogens in animal feces in a subtropical catchment. *Science of The Total Environment*, doi: 10.1016/j.scitotenv.2018.11.439 Abstract

- Nowak K (2018). African fruit bats as potential reservoir for zoonotic pathogens - the example of *Escherichia coli*. PhD thesis, Friei Universität Berlin Thesis

- Muñoz-Leal S et al (2018). New records of ticks infesting bats in Brazil, with observations on the first nymphal stage of *Ornithodoros hasei*. *Experimental and Applied Acarology,* doi: 10.1007/s10493-018-0330-3 Abstract

- Rosskopf SP et al (2019). *Nycteria* and *Polychromophilus* parasite infections of bats in Central Gabon. *Infection, Genetics and Evolution*, 68, 30-34 Abstract

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Wildlife Health Australia
****
(b) (6) [redacted]
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Wildlife Health Australia acknowledges the traditional owners of country throughout Australia and their continuing connection to land, sea and community. We pay our respects to them and their cultures and to their Elders past, present and future.

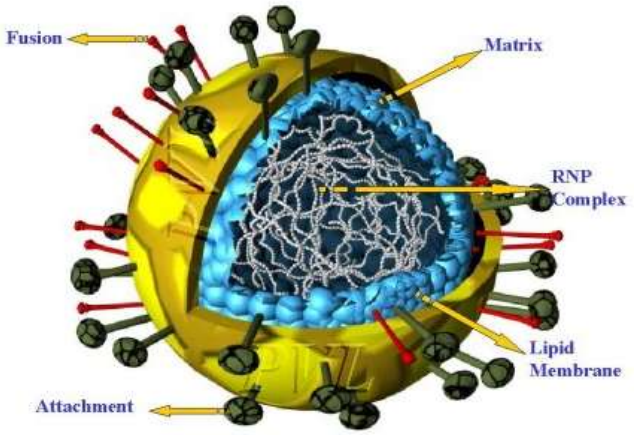
United States Department of Agriculture

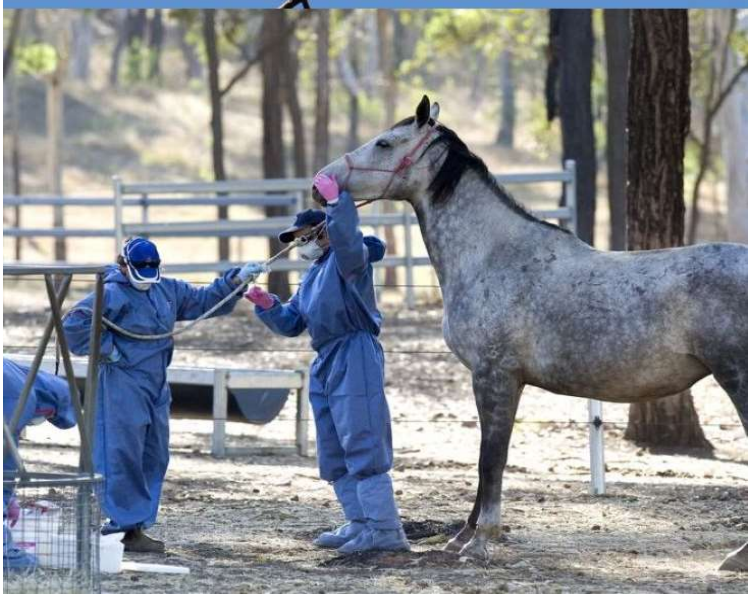
Agricultural Research Service

July 2018

Henipavirus Gap Analysis

Workshop Report





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EXECUTIVE SUMMARY

Henipavirus is the taxonomic genus for a group of viruses in the family Paramyxoviridae that includes *Hendra virus* (HeV) and *Nipah virus* (NiV). These viruses are zoonotic agents that are highly pathogenic in humans with case fatality rates of 40% to 70%. As such, these viruses are classified as Biosafety Level 4 (BSL-4) agents, requiring the highest level of laboratory biocontainment. Importantly, they have many of the physical attributes to serve as potential agents of bioterrorism, and are also considered emerging zoonotic pathogens with increasing geographical distribution in Australia, New Caledonia, Southeast Asia, and Madagascar.

Hendra virus first emerged in 1994 in Australia spilling over from bats to horses to humans, causing several disease outbreaks since with significant fatality rates. Nipah virus emerged in Malaysia in 1999, resulting in nearly 300 human cases with over 100 deaths.

The Nipah virus outbreak in Malaysia was especially concerning, causing widespread panic and fear because of the high mortality rate in people and the inability to control the disease initially. There were also considerable social disruptions and tremendous economic loss to an important pig-rearing industry. This highly virulent virus, believed to be introduced into pig farms by fruit bats, spread easily and silently among pigs and was transmitted to humans who came into close contact with infected animals. A NiV outbreak in Bangladesh in 2001 resulted from direct bat to human transmission via contaminated date palm juice with further spread within the human population. From 2001 to 2012, the World Health Organization (WHO) reported a total of 209 cases, with 161 deaths due to of NiV infections. In 2014, the WHO reported a NiV outbreak in fourteen districts of Bangladesh, resulting in 24 cases and 21 deaths. In 2015, three fruit bats tested positive for NiV in New Caledonia at the Noumea National Park, including three bats at the Noumea Zoo.

This gap analysis report focuses primarily on NiV and its potential impact on agricultural swine production. However, information is also provided on the threat henipaviruses pose to public health, both as emerging zoonotic agents and as potential agents of bioterrorism. Included in this report is scientific information on *Henipavirus* virology, epidemiology, pathogenesis, immunology, and an assessment of the available veterinary medical countermeasures to detect, prevent, and control disease outbreaks. Importantly, gaps are provided to inform research needs and priorities. Some of the major gaps and obstacles for disease control can be summarized as follows:

Diagnostics

The availability of safe laboratory diagnostic tests are limited. Virus isolation and serum neutralization assays require live NiV; thus, BSL-4 containment laboratories are required. Nucleic acid-based assays, such as RT-PCR are available, but genetic variation amongst henipaviruses are reported to impact sensitivity and real time RT-PCR may not be able to detect all divergent and novel henipavirus strains. Serological assays are limited in their ability to differentiate between known and unknown henipaviruses, as cross-reactivity to one or more known viruses is possible. Commercial diagnostic test kits are not available. International standards for NiV assay validation are needed. Gaps include a lack of positive experimental and field samples for test validation (or even evaluation) and there are restrictions on material transfer (e.g., obtaining animal samples that could be used to validate tests) due to biosecurity concerns. Low biosafety level reference sera

against various isolates are not yet available. There is a need for high throughput antibody assays for disease outbreaks, recovery and surveillance purposes. There is also a need to develop operator-safe diagnostics tests and reagents that can be produced in low biocontainment facilities.

Vaccines

There is currently a commercial vaccine available for horses, but there are no vaccines for swine or humans. There are several experimental vaccine candidates that may be safe and effective in swine and other domestic animals. However, all these vaccine candidates will require further research to establish their efficacy, and they will need to be fully developed to be licensed and stockpiled for rapid use in an emergency disease outbreak in swine.

Surveillance

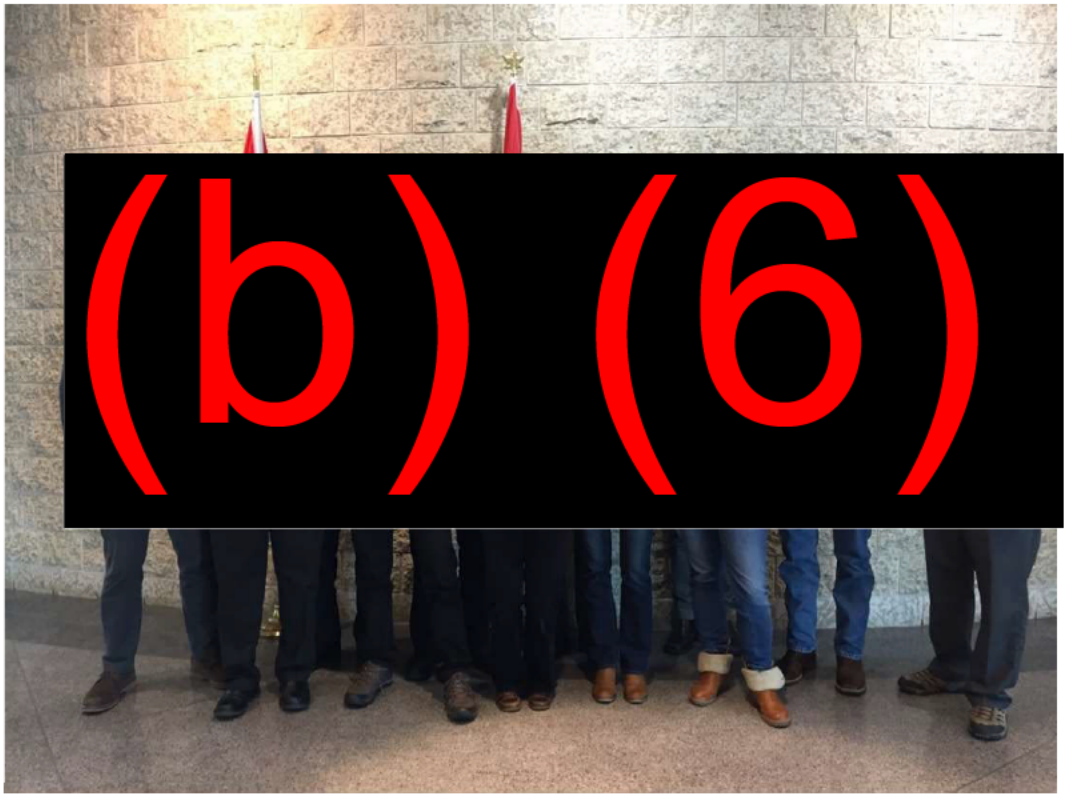
Surveillance is the first line of defense against a disease outbreak. Rapid and accurate detection affects the time when control measures can be implemented and affects the extent of the disease outbreak. Because of limitations with laboratory diagnosis, surveillance programs are dependent on the reporting of clinical signs in populations at risk. Diagnosis of NiV infections based on clinical presentation has a low positive predictive value as there are numerous etiologies for encephalitis in humans, and clinical signs in pigs are difficult to differentiate from many common endemic infectious diseases.

Depopulation

Depopulation is the primary countermeasure to reduce virus shedding and stop the spread of NiV in livestock. Disease outbreaks have shown that the control of NiV in pig populations through stamping out is complex due to the zoonotic nature of the agent. In addition, depopulation may be logistically difficult and may be impossible in a rapidly spreading outbreak in countries where there are pig dense regions with millions of pigs, such as the states of Iowa, North Carolina, and Minnesota in the United States, or South East China.

GROUP PICTURE

Henipavirus Gap Analysis Working Group, Winnipeg, Canada
November 14-17, 2017



The Nipah Virus Countermeasures Working Group, Geelong, Australia
March 17-19, 2009

GLOSSARY

APHIS: Animal and Plant Health Inspection Service, USDA, United States of America

ARS: Agricultural Research Service

AAHL: Australian Animal Health Laboratory

BSL-4: Biosafety Level 4

CDC: U.S. Centers for Disease Control and Prevention, HHS, United States of America

CFIA: Canadian Food Inspection Agency

DIVA: Differentiating between infected and vaccinated animals

ELISA: Enzyme-linked immunosorbent assay

FADDL: U.S Foreign Animal Disease Laboratory, Plum Island Animal Disease Center

FLI: Friedrich Loeffler Institute

GMP: good manufacturing practice

HeV: Hendra virus

HHS: Department of Human Health Services, United States of America

HSPD-9: Homeland Security Presidential Directive Nine

ICAR: Indian Council of Agricultural Research

Ig: Immunoglobulin

IEDCR: Institute of Epidemiology, Disease Control and Research in Bangladesh

MLV: Modified live virus vaccine

NAHLN: National Animal Health Laboratory Network, USA

NIHSAD: National Institute of High Security Animal Diseases, ICAR, India

NCFAD: National Center for Foreign Animal Disease, CFIA, Canada

NiV: Nipah virus

NiV-B: Nipah virus Bangladesh

NiV-M: Nipah virus Malaysia

NiV N: Nipah virus nucleoprotein

NVCWG: Nipah Virus Countermeasures Working Group

NVS: National Veterinary Stockpile

OIE: World Organisation for Animal Health

PCR: Polymerase Chain Reaction.

PPE: Personal Protective Equipment

RT-PCR: Reverse transcription-polymerase chain reaction

rRT-PCR: Real-time reverse transcription-polymerase chain reaction

sHeV G: recombinant soluble Hendra virus G protein

sNiV G: recombinant soluble Nipah virus G protein

USDA: United States Department of Agriculture, United States of America

INTRODUCTION

Nipah virus (NiV) is an emerging zoonotic virus. First isolated in pigs and people from an outbreak in Malaysia in 1998 (Ang *et al.* 2018), this emerging virus causes severe disease in humans. The source of transmission was determined to be from bats to pigs to humans, through close contact with infected animals. The virus is named after the location where it was first detected in Sungai Nipah, a village in the Malaysian Peninsula where exposed pig farmers became severely ill with encephalitis.

Nipah virus is closely related to another zoonotic virus called Hendra virus (HeV), formerly called Equine *Morbillivirus*, and named after the town where it first appeared in Australia. Hendra virus infection was first recognized in 1994, when it caused an outbreak of acute, fatal respiratory disease that killed 14 horses. Three human cases, leading to two deaths were recorded during the outbreak. The precise mode of virus transmission to the three Australian patients is not fully understood. All three individuals appear to have acquired their infection as a result of close contact with horses, which were ill and later died.

Although members of this group of viruses have only caused a few focal outbreaks, their ability to infect a wide range of animal hosts and to produce a high mortality rate in humans has made this emerging zoonotic viral disease a significant public health threat.

Certain species of bats of the genus *Pteropus* (fruit bats, also called flying foxes) are the principal natural reservoir hosts for NiV and HeV – see Table I. Bats are susceptible to infection with these viruses but do not develop disease. Fruit bats are distributed across an area encompassing Australia, Southeast Asia; including Indonesia, Malaysia, the Philippines and some of the Pacific Islands, the Indian subcontinent, and Madagascar (See Fig. 1). There is also growing evidence that viruses related to NiV and HeV circulate in non-pteropid fruit bats across the globe (Clayton, 2017).

The exact mode of transmission of henipaviruses is uncertain, but appears to require close contact with contaminated tissue or body fluids from infected animals. The role of domestic species other than pigs in transmitting NiV infection to other animals has not yet been determined. In 2014, an outbreak was reported in the Philippines involving the consumption of meat from NiV-infected horses, further expanding the potential routes of transmission for henipaviruses.

Despite frequent contact between fruit bats and humans there is no serological evidence of human infection among persons that are in contact with bats. Pigs were the apparent source of infection among most human cases in the Malaysian outbreak of NiV in 1998-1999. Nipah virus has continued to spillover over from animals with at least six outbreaks resulting in human fatalities in Bangladesh in 2013, one in India in 2014, and two in Bangladesh in 2015. The World Health Organization (WHO) had not reported any NIV cases 2016-2017, but in 2018 fourteen new cases and 12 deaths were reported in Kerala, India - See Table II.

The spread of henipaviruses to new geographical areas is a concern. In 2014, the Philippines reported an outbreak with a zoonotic paramyxovirus in horses and people. There is further evidence for broader distribution of NiV in pteropid fruit bats species. There is also growing evidence that viruses related to NiV and HeV also circulate in non-pteropid fruit bats worldwide.

BACKGROUND

Organization of the Gap Analysis Working Groups on Nipah Virus (2009 and 2017)

The United States Department of Agriculture (USDA) organized the first Nipah virus gap analysis workshop in Australia in 2009 with the support of the Australian Animal Health Laboratory (AAHL). The working group was charged by the USDA National Veterinary Stockpile Steering Committee with making recommendations on specific materials, commercially available and in the pipeline, which will ensure the United States has an arsenal of highly efficacious countermeasures to control and mitigate the impact of an outbreak of Nipah virus. Nipah virus experts representing laboratories in South East Asia, Australia, Canada, and the United States were invited to participate and contributed to this report. The second workshop was organized in 2017 by the Special Pathogens Unit, National Centre for Foreign Animal Disease, Canadian Food Inspection Agency (CFIA), in collaboration with BSL4ZNet and DISCONTOOLS (http://www.discontools.eu/). The participants were charged with assessing available veterinary medical countermeasures to control and respond to a Nipah virus disease outbreak. In addition, the workshop participants agreed to update the gap analysis conducted at the AAHL in Geelong, Australia, in 2009.

Reference Material

The following reports and websites are recommended:

OIE – World Organisation for Animal Health - Nipah in Animals
http://www.oie.int/en/animal-health-in-the-world/animal-diseases/Nipah-Virus/
Accessed July 22, 2018

FAO – Food and Agriculture Organization
Manual on the diagnosis of Nipah virus infection in animals
www.fao.org/DOCREP/005/AC449E/AC449E00.htm
Accessed July 22, 2018

CDC – Center for Disease Control and Prevention - Special Pathogens Branch
https://www.cdc.gov/vhf/nipah/index.html
Accessed July 22, 2018

WHO - World Health Organization
http://www.who.int/news-room/fact-sheets/detail/nipah-virus
Accessed July 22, 2018

Guidelines for Veterinarians Handling potential Hendra Virus infection in Horses (QDPI)
https://www.daf.qld.gov.au/__data/assets/pdf_file/0005/126770/2913_-Guidelines-for-veterinarians-handling-potential-Hendra-virus-infection-in-horses-V5.1.pdf
Accessed July 22, 2018

CFSPH – Center for Food Security and Public Health
Nipah Virus Infection
http://www.cfsph.iastate.edu/Factsheets/pdfs/nipah.pdf
Accessed July22, 2018

DEFINITION OF THE THREAT

The threat for a natural introduction of henipaviruses in the United States is low, but there is significant concern that henipaviruses could be used for nefarious purposes to harm agriculture and people. Both Hendra virus and Nipah virus are on the HHS and USDA list of overlap Select Agents and Toxins. Henipaviruses are listed as APHIS Tier 3 high-consequence foreign animal diseases and pests. Henipaviruses are promiscuous in their ability to cause severe morbidity in several animal species, including people, and human infections result in a very high mortality rate. The mortality rate in pigs is actually reported as about 2.5% in adult pigs – high morbidity, but low mortality. Mortality rates in humans range from 40% (Malaysia) to 75% (up to 100%) in Bangladesh. The animal reservoir includes several species of bats, and henipaviruses may thus be readily available in these wildlife reservoirs.

Infection in people

Between September 1998 and June 1999, a NiV outbreak in Malaysia resulted in severe viral encephalitis in 105 patients (Goh *et al.,* 2000; Epstein *et al.,* 2006). Ninety-three percent had had direct contact with pigs, usually within two weeks prior to the onset of illness, suggesting that there was direct viral transmission from pigs to humans and a short incubation period. The main presenting features were fever, headache, dizziness, and vomiting. Fifty-two patients (55%) had a reduced level of consciousness and prominent brain-stem dysfunction. Distinctive clinical signs included segmental myoclonus, areflexia and hypotonia, hypertension, and tachycardia. The initial cerebrospinal fluid findings were abnormal in 75% of patients. Antibodies against Hendra virus were detected in serum or cerebrospinal fluid in 76 percent of 83 patients tested. Thirty patients (32%) died after rapid deterioration in their condition. An abnormal doll's-eye reflex and tachycardia were factors associated with a poor prognosis. Death was probably due to severe brain-stem involvement. Neurologic relapse occurred after initially mild disease in three patients. Fifty patients (53%) recovered fully, and 14 (15%) had persistent neurologic deficits.

Unlike Malaysia, the NiV outbreaks in Bangladesh were strictly confined to human populations with significantly higher mortality rate (Hossain *et al.,* 2008). NiV outbreaks in Bangladesh have continued annually since 2008 resulting in a total of 207 reported cases, 152 of which were fatal resulting in a 70% mortality rate (Clayton,. 2017).

Infection in pigs

The NiV outbreak in Maylasia in 1999 was facilitated by the rapid spread of the virus in pig populations. Although some pigs demonstrated a febrile respiratory illness with epistaxis, dyspnoea, and cough, few animals exhibit neurological signs, and the majority of pigs had subclinical infections. There are no clinical signs in pigs that are specific for NiV infection. Both, apparently healthy pigs and pigs showing clinical signs shed significant amount of virus.

Economic impact

The NiV outbreak in Malaysia in 1999 destroyed the main market for Malaysian hogs in Singapore. The Malaysia outbreak resulted in an 80% drop in pork consumption in the domestic market. Over half the standing pig population in the country was culled to halt the outbreak. Half the pig farms went out of business. The cumulative economic losses based on government figures was estimated to be approximately $217 million USD.

Bioterrorism

NiV has many of the physical attributes needed for a biological weapon, including easy access to virus resulting from its wide distribution in nature and laboratories, easy to produce, easy to disseminate, and the potential for high morbidity and mortality in people.

GAP ANALYSIS

The following section summarizes what we know about henipaviruses, gaps in our knowledge, and the threat of bioterrorism.

VIROLOGY

The following summarizes our current knowledge of viral strains, taxonomy, reservoir, genome, morphology, determinants of virulence, host range, and tissue tropism.

Virus species

Nipah virus (NiV) was first isolated in 1999 from samples collected during an outbreak of encephalitis and respiratory illness among pig farmers. The name Nipah originated from Sungai Nipah, a village in the Malaysian Peninsula where pig farmers became sick. There are currently two genotypes identified: NiV-Malasia and NiV-Bangladesh. Different strains/genotypes of NiV have emerged: Malaysia, Bangladesh, and Cambodia. NiV Malaysia resulted in the culling of a million pigs and 250 human cases (106 fatal). NiV Bangladesh is associated with outbreaks in people (Clayton, 2017).

Hendra virus (HeV) was first isolated in 1994 from specimens obtained during an outbreak of respiratory and neurologic disease in horses and humans in Hendra, a suburb of Brisbane, Australia.

Cedar virus (CedPV) is a novel *Henipavirus* isolated from Australian bats, which appears to be non-pathogenic in lab animal experiments (Marsh et al. 2012).

Taxonomy

NiV and HeV are members of the family Paramyxoviridae, order *Mononegavirales*. Comparison of nucleic acid and deduced amino acid sequences with other members of the family confirms that NiV and HeV are members of the family Paramyxoviridae, but with limited homology with members of the *Morbillivirus*, *Rubulavirus* and *Respirovirus* genera (See Fig. 2). The name *henipavirus* was recommended for the genus of both HeV and NiV (Wang *et al.,* 2000). HeV appear to be less diverse that NiV but molecular epidemiology studies are needed to identify new isolates that may bridge the gap between HeV and NiV.

Reservoir

The natural reservoir of the henipaviruses are fruit bats mainly from the genus *Pteropus* (flying foxes).

Genome

The complete genomes of both HeV and NiV have been sequenced (Wang *et al.,* 2001). Henipaviruses have a large non-segmented genome comprised of single-stranded negative-sense RNA. Their genomes are 18.2 kb in size and contain six genes corresponding to six structural proteins. All genes are of similar size to homologues in the respirovirus and morbillivirus genera, with the exception of P which is 100-200 amino acids longer (See Fig. 3). Most of the increase in genome length is due to longer untranslated regions between genes, mainly at the 3' end of each gene. The role of these long untranslated regions are not understood. Henipaviruses employ an unusual process called RNA editing to generate multiple proteins from a single gene. The process involves the

insertion of extra guanosine residues into the P gene mRNA prior to translation. The number of residues added determines whether the P, V or W proteins are synthesized. The C protein is made via an alternative translational initiation mechanism. The functions of the V, W, and C proteins are unknown, but they may be involved in disrupting host antiviral mechanisms (see Immunology below). The function of the G protein is to attach the virus to the surface of a host cell via the major receptor ephrin B2, a highly conserved protein present in many mammals. G glycoprotein is the major neutralizing antigen and the target protein for vaccine development. X-ray crystal structure for NiV G complex with ephrin-B3 has been determined. This interaction is highly conserved between NiV and HeV. This interaction is a prime candidate for developing henipavirus specific therapeutics. The F protein fuses the viral membrane with the host cell membrane, releasing the virion contents into the cell. It also causes infected cells to fuse with neighboring cells to form large multinucleated syncytia.

The genome size and organization of CedPV is very similar to that of HeV and NiV. The nucleocapsid protein displays antigenic cross-reactivity with henipaviruses and CedPV uses the same receptor molecule (ephrin- B2) for entry during infection. Clinical studies with CedPV in *Henipavirus* susceptible laboratory animals confirmed virus replication and production of neutralizing antibodies although clinical disease was not observed. In this context, it is interesting to note that the major genetic difference between CedPV and HeV or NiV lies within the coding strategy of the P gene, which is known to play an important role in evading the host innate immune system. Unlike NiV and HeV, and almost all known paramyxoviruses, the CedPV P gene lacks both RNA editing and also the coding capacity for the highly conserved V protein (Marsh *et al.* 2012).

Morphology

Henipaviruses are pleomorphic ranging in size from 40 to 600 nm in diameter. They possess a lipid membrane overlying a shell of viral matrix protein. At the core is a single helical strand of genomic RNA tightly bound to the nucleocapsid (N) protein and associated with the large (L) and phosphoprotein (P) proteins, which provide RNA polymerase activity during replication. Embedded within the lipid membrane are spikes of fusion (F) protein trimers and attachment (G) protein tetramers.

Determinants of virulence, host range, and tissue tropism

Molecular determinants of virulence, host range and cell tropism have been extensively studied and are well understood for many paramyxoviruses. Infectivity is determined by the cell-attachment and fusion glycoproteins and the presence of appropriate P gene products modulate virulence by antagonizing the cellular interferon response.

Henipaviruses have a large host range, unlike other members of the Paramyxoviridae, which generally have a very narrow host range. The cell attachment protein, unlike many other members for the paramyxovirus subfamily, does not have haemagglutinating activity and as a consequence does not bind sialic acid on the surface of cells.

The receptor for henipavirus is present on many different cultured cell types from many different species. The receptors for HeV and NiV are the same and have been identified as ephrin-B2 and ephrin-B3. Ephrin-B2 or -B3 are highly conserved across vertebrate species and are members of a family of receptor tyrosine kinase ligands. Ephrin-B2 is highly expressed on neurons, smooth muscle, arterial endothelial cells and capillaries, which closely parallels the known tissue tropism of

HeV and NiV *in vivo.* Ephrin-B3 is also widely expressed but particularly in specific regions of the central nervous system and may facilitate pathogenesis in certain neural subsets.

Virology Research Priorities

- Molecular epidemiology and determinants of strain variation
- Need sequencing of henipaviruses from bats, especially Bangladesh
- Determine molecular basis for virulence

PATHOGENESIS

The following summarizes our current knowledge of viral pathogenesis, including routes of infection, tissue tropism, pathogenesis, clinical signs, and clinical pathology."\

NiV infections in humans and pigs are linked to contact with bats. Clinical signs in human cases indicate primarily involvement of the central nervous system with 40% of the patients in the Malysian outbreak having also respiratory syndromes, while in pigs the respiratory system is considered the primary virus target, with only rare involvement of the central nervous system.

Humans

The main histopathological findings include a systemic vasculitis with extensive thrombosis and parenchymal necrosis, particularly in the central nervous system (Wong *et al.,* 2002). Endothelial cell damage, necrosis, and syncytial giant cell formation are seen in affected vessels. Characteristic viral inclusions are seen by light and electron microscopy. Immunohistochemistry (IHC) analysis shows the widespread presence of NiV antigens in endothelial and smooth muscle cells of blood vessels (Hooper *et al*., 2001). Abundant viral antigens are also seen in various parenchymal cells, particularly in neurons. The brain appears to be invaded via the hematogenous route and virus has been isolated from the cerebrospinal fluid of patients with NiV encephalitis (Wong *et al*., 2002). Infection of endothelial cells and neurons as well as vasculitis and thrombosis seem to be critical to the pathogenesis of this new human disease.

NiV infection can rarely cause a late-onset encephalitis up to a couple of years following a non-encephalitic or asymptomatic infection, or a relapsed encephalitis in patients who had previously recovered from acute encephalitis (Wong *et al*., 2001; Goh *et al*., 2000; Tan *et al*., 2002).

Pigs

Experimental challenge studies in piglets conducted at the National Centre for Foreign Animal Diseases, Winnipeg, Canada, demonstrated neurological signs in several inoculated pigs (Weingartl *et al.,* 2005; Berhane et al., 2008; Weingartl, H.M., personal communication of unpublished data). The rest of the pigs remained clinically healthy. NiV was detected in the respiratory system (turbinates, nasopharynx, trachea, bronchus, and lung), the lymphoreticular system (endothelial cells of blood and lymphatic vessels), submandibular and bronchiolar lymph nodes, tonsil, and spleen, with observed necrosis or lymphocyte depletion in lymphoid tissues, most importantly in lymph nodes (Hooper et al., 2001, Weingartl et al., 2006; Berhane et al., 2008). NiV presence was confirmed in the nervous system of both sick and apparently healthy animals (cranial nerves, trigeminal ganglion, brain, and cerebrospinal fluid). No virus was detected urine, although NiV antigen was found in kidneys of field swine cases (Tanimura et al., 2004). This study suggests NiV invaded the porcine host central nervous

system via cranial nerves after initial virus replication in the upper respiratory tract, and later in the infection also by crossing the blood-brain barrier as a result of viremia. Additional information on NiV and HeV pathogenesis in pigs are summarized in Middleton and Weingartl, 2012.

Dogs

Middleton *et al*., 2017, conducted experimental infections with HeV in dogs and determined that the virus is not highly pathogenic in dogs but their oral secretions pose a potential transmission risk to people. The time window for potential oral transmission corresponded to the period of acute infection.

Bats

Pteropus spp. fruit bats have been identified as the reservoir hosts for henipaviruses . Henipaviruses have been isolated to date in bats from Australia (HeV), Asia (NiV), and recently serological evidence of infection in bats in Madagascar (Hayman D.T.S., *et al.,* 2008). Related henipaviruses have been detected serologically and by PCR in non-*Pteropus*, but related pteropodid bats in Central and West Africa, and in insectivorous bats in China, expanding the host and geographical range beyond *Pteropus.*

There is no significant pathology in bats, and the frequency of viral shedding from wild bats is rare, with prevalence ranging from (1%-3%) with temporal variation of infection and viral shedding observed among different bat populations (Gurley *et al.,* 2017 and Wacharaplusadee *et al.* 2010, 2016). Henipavirus isolation from bat excreta is challenging, potentially due to low viral load.

Pathogenesis Research Priorities

- Identify determinants of virulence in pigs
- Develop experimental infection models in bats to study shedding
- Comparative genomic studies of contemporaneous NiV strains collected from bats and humans during outbreaks.
- Expand knowledge of spectrum of henipaviruses in bat hosts in NiV hotspots (e.g. western Bangladesh & West Bengal India)
- Determine whether the innate immune system in bats is responsible for limiting viral replication
- Determine how the net reproductive value of henipaviruses are sustained in bats
- Determine how transmission effected within bats, and between bats and other species

IMMUNOLOGY

The following summarizes our current knowledge of NIV immunology, including innate and adaptive immune responses to wild-type virus, immune evasion mechanisms, and protective immunity.

Innate and adaptive immune responses to wild-type NiV

Viral RNA can be detected by both cytoplasmic and endosomal pattern recognition receptors (PRRs), resulting in innate immune Type I IFN induction/ and signaling pathways:

- Retinoic Acid-inducible Gene I (RIG- I)- recognizes 5’ triphosphorylated RNA
- Melanoma Differentiation Antigen 5 (Mda-5)-recognizes cytosolic dsRNA
- RNA-dependent Protein Kinase (PKR)- recognizes cytosolic dsRNA

- Toll-like Receptor (TLR) 3- recognizes endosomal dsRNA
- TLR 7-8- recognizes endosomal ssRNA

Immune evasion mechanisms

The NiV uses unusual processes called RNA editing and internal translational initiation to generate multiple proteins from the phosphoprotein (P) gene, resulting in 4 proteins (P, C, V, and W) that function in inhibiting Type I interferon pathways:

- NiV P, V, and W have individually been shown to bind STAT1 and STAT2, effectively preventing STAT1 phosphorylation in type I IFN-stimulated cells.
- The V protein localizes to the cytoplasm, while the W protein localizes to the nucleus.
- The C protein can partially rescue replication of an IFN-sensitive virus, but the mechanism is unknown.
- Nuclear localization of W enables it to inhibit both dsRNA and TLR 3 (IRF-3 dependent) IFN-β induction pathways.
- A single point mutation in the V protein abrogates its ability to inhibit of IFN signaling.
- The V proteins of paramyxoviruses interact with the intracellular helicase Mda-5, and inhibits its IFN-β induction, but not with RIG-I.
- NiV V, W, and P bind polo-like kinase (PLK) via the STAT1 binding domain (Ludlow *et al.,* 2008).
- The P, V, and W proteins of NiV Malaysia and NiV Bangladesh inhibit IFN-stimulated response element (ISRE), which have a role in inducing transcription of IFN-stimulated genes (ISGs). Some of these ISGs include IRF-7, 2'5' Oligoadenylate Synthetase (OAS), RnaseL, p56, and double-stranded RNA-induced protein kinase (PKR). These ISGs all contribute to the generation of an 'antiviral state' in the cell.

Protective immunity

The G and F protein induce neutralizing antibodies that protect against challenge. Recent evidence from vaccination challenge studies indicates that both serum neutralizing antibody and T cell-mediated immunity are needed for protection from Nipah virus infection in pigs (Protection against henipaviruses in swine requires both, cell-mediated and humoral immune response, B.S. Pickering, J.M. Hardham, G. Smith, E.T. Weingartl, P.J. Dominowski, D.L. Foss, D. Mwangi, C.C. Broder, J.A. Roth, H.M. Weingartl, Vaccine 34(40): 4777-4786, 2016

Research needs

- Innate immunity and immunosuppression
  - Need studies in NiV infected cells and animal models
  - Need to study infection in various cell types, including cells of the immune system and bat cells
  - Use infectious clone to study virulence determinants
  - Identify targets for antiviral agents
  - Cytokine response to infection in human and bat cell lines
  - Need to study the potential for type 1 interferon or other cytokines to provide early protection from Nipah virus infection, transmission and/or clinical signs.

- Protective Immunity
  - Need to better define correlates of protection
  - Study T lymphocyte subset responses and cellular targets (e.g., N)

EPIDEMIOLOGY

Certain species of fruit bats of the genus *Pteropus* are the principal natural reservoir hosts for NiV and HeV (see Table I). Bats are susceptible to infection with these viruses but do not develop disease. Oher zoonotic viruses like Ebola, Marburg, and SARS virus, have also been identified in various Pteropus spp. fruit bats (Angeletti et al., 2016). Fruit bats are distributed across an area encompassing Australia, Southeast Asia; including Indonesia, Malaysia, the Philippines and some of the Pacific Islands, the Indian subcontinent, and Madagascar (See Fig. 1). There is further evidence for broader distribution of NiV in pteropid fruit bats species across their range (Wacharapluesadee S. and Hemachudha T., 2007). There is also growing evidence that viruses related to NiV and HeV also circulate in non-pteropid fruit bats worldwide.

Hendra Virus

Hendra virus infection was first recognized in 1994 in Australia, when it caused an outbreak of acute, fatal respiratory disease that killed 14 horses. Three human cases, leading to two deaths were recorded during the outbreak. In 1995, a horse was infected with associated human cases. The precise mode of virus transmission to the three Australian patients is not fully understood. All three individuals appear to have acquired their infection as a result of close contact with horses, which were ill and later died.

There have been several recognized outbreaks in Australia since 1994. Hendra virus reemerged in 1999, 2004, and 2006-2010. All known HeV cases have occurred in Queensland or northern New South Wales. From 1994 to 2010, HeV was confirmed on 11 premises in Queensland and one premise in northern New South Wales. In Australia, GlobalincidentMap.com reported: 21 cases in 2011; 12 cases in 2012; 10 cases in 2013; four cases in 2014; three cases in 2015; one case in 2016; and four cases in 2017. Al cases have involved horses as an intermediate host along with some additional human infections, resulting in several fatalities. The Australian Veterinary Association's national president, Dr. Ben Gardiner, was quoted as stating "no state or territory was immune from the virus."

The natural reservoirs for HeV are flying foxes found in Australia. Bats are susceptible to infection with these viruses but do not develop disease.

Hendra virus infection has also been detected in two dogs that were in close contact with infected horses. Both dogs remained clinically normal with no history of related illness.

Updated statistics on HeV outbreaks, including locations, dates and confirmed human and animal cases may be found on the Australian Veterinary Association website (Assessed July 22, 2018).

Nipah Virus

Nipah virus is a recently-recognized, zoonotic paramyxovirus that causes severe disease and high fatality rates in people. Outbreaks have occurred in Malaysia, Singapore, India and Bangladesh, and a putative Nipah virus was also recently associated with human disease in the Philippines (Clayton,

2017). The following summarizes our current knowledge of NiV epidemiology taking into account disease outbreaks in Malaysia and Bangladesh.

Malaysia

Nipah virus was first described in 1999 in Malaysia. The outbreak in Malaysia resulted in over a million pigs culled, 800 pig farms demolished, 36,000 jobs lost, $120+ million exports lost, and over 300 human cases (106 fatal, ~35% mortality) in pig farmers (Chinese) and Singapore abattoir workers (Field *et al.,* 2001). The NiV outbreak in pigs was described as highly infectious, frequently asymptomatic, low mortality rate (~5%), with respiratory and neurological syndromes. The pig farm pattern of disease included 30% morbidity and 5% mortality with sows first affected, followed by weaners, growers and finishers. The duration of clinical disease on a farm lasted ~ 2 weeks with a sero-prevalence approaching 100% in some cases. The outbreak in Malaysian pigs was associated with an increased incidence of human viral encephalitis cases, strongly associated with pig farm workers, with temporal and spatial links to disease in pigs.

During the outbreak, evidence of NiV infection was found in domestic animals such as goats and cats, but especially dogs (Field *et al.,* 2001). After pig populations were destroyed, but before residents were allowed to return to their homes, studies were undertaken in the epidemic area to determine whether domestic animal populations maintained active infection in the absence of infected pigs (Mills *et al.,* 2009). Dogs were especially suspected because they live commensally with both pigs and humans. However, serologic screening showed that in the absence of infected pigs, dogs were not a secondary reservoir for NiV.

Although human-to-human transmission of NiV during the 1998-1999 outbreak in Malaysia was not reported, a small number of infected people had no history of contact with pigs, suggesting human-to-human transmission occurred in these cases (Clayton, 2017).

The reservoir and natural host of NiV was determined to be fruit bats. Fruit bats have a wide geographic distribution, high antibody prevalence (17-30%), but no apparent clinical disease. A NiV neutralizing antibody study (Yob *et al.,* 2001) from 237 wild-caught bats surveyed on Peninsular Malaysia April 1–May 7, 1999, found four different species of fruit bats, and one species of insectivorous bats, tested positive for NiV (see Table I).

The routes of NiV excretion in bats include urine, saliva, and foetal tissues and fluids but the exact modes of transmission have yet to be determined.

The drivers of the emergence of NiV in Malaysia were determined to be large piggery (30,000+) adjacent to primary forest/fruit bat habitat and a network of other large farms close by. The stages of emergence associated with the outbreak included a spillover from flying foxes to domestic pigs near Ipoh (see Fig. 4), where farming practices and high pig densities facilitated the dissemination of the infection. Transportation of pigs for commerce led to the southern spread of the outbreak with the amplifying pig host facilitating the transmission of the virus to humans.

The epidemic enhancement of the outbreak included the initial introduction of infection in a naive pig population resulting in a rapid epidemic peak, followed by burn-out and localized human infections. Subsequent introduction(s) into partially immune pig populations resulted in a lower epidemic peak

but prolonged duration and increased total number of infectious pigs, increasing the chances of spread to surrounding farms.

Bangladesh

Bangladesh experienced its first reported NiV outbreak in Siliguri and Naogaon in 2001 (Fig. 5). Unlike Malaysia, outbreaks in Bangladesh appeared to be strictly confined to human populations and significantly higher mortality rate. From 2001 to 2018, the WHO reported a total of 261 cases, with 198 deaths (76% mortality) due to NiV infection (see Table II).

The transmission of NiV to humans in Bangladesh was determined to be associated with drinking date palm juice, considered a delicacy in this region of the world. In the Tangail outbreak of 2005, it was estimated that persons drinking raw date palm sap had a 7.0 odds ratio of developing a NiV infection when compared to controls (95% confidence level, 1.6).

NiV cases in Bangladesh have been seasonal, with human cases reported between the months of January and April. This coincides with the season for collecting date palm sap, late November through April. However, there is significant heterogeneity in the number of spillovers detected by district and year that remains unexplained. Cortes et al., in 2018 analyzed data from all 57 spillovers occurring during 2007–2013 and found that temperature differences explained 36% of the year-to-year variation in the total number of spillovers each winter, and that distance to surveillance hospitals explained 45% of spatial heterogeneity. January, when 40% of the spillover events occurred, was the month with the lowest mean temperature during every year of the study.

Bats are recognized as a nuisance and frequently drink the juice, defecate into juice, and occasionally drown in the palm sap collecting pot. Measures have been put in place to prevent bats access to the sap collecting pot, which has been very effective in reducing the spread of NiV from bats to humans in Bangladesh.

India

In 2001, an outbreak occurred within a hospital in Siliguri, West Bengal. Nosocomial transmission likely occurred, though it is unknown how primary cases were infected. Another outbreak in 2007 was reported in Nadia, West Bengal. Consumption of date palm sap was identified as the likely route of infection of primary cases there. In May of 2018, another outbreak was reported in Kerala. A total of 85 cases were reported in these three outbreaks in 2001, 2007, and 2018, with 62 deaths (73% mortality) due to NiV infection (see Table II).

In 2012, Yadav et al. surveyed the Indian states of Maharashtra and West Bengal to evaluate the presence of viral RNA and IgG against NiV in different bat populations belonging to the species Pteropus giganteus, Cynopterus sphinx and Megaderma lyra. The authors found NiV RNA in Pteropus bat thus suggesting it may be a reservoir for NiV in India.

Philippines

In 2014, the Philippines reported an outbreak with a zoonotic paramyxovirus in horses and people that is very closely related to NiV based on sequence analysis. Virus isolation was unsuccessful so it was impossible to confirm that there was transmission from presumably bats to horses, from horses to people, and also human to human (Ching P.K., *et al.*, 2015; Clayton, 2017).

New Caledonia

In 2015, three fruit bats tested positive for NiV in New Caledonia at the Noumea National Park, including three bats at the Noumea Zoo.

Research needs

- Improved understanding of infection dynamics in flying foxes: modes of transmission, immune response, evidence of disease, and the implications of co-infection with NiV and other henipaviruses
- Better understanding of co-circulation of different strains / species of henipaviruses within Pteropus populations and the effect of waning herd immunity on outbreaks.
- Other animals such as infected dogs and cats need to be further studied to determine their potential role in the transmission of NiH .
- Improved understanding of infection dynamics in humans: modes of transmission, implications of genetic diversity of the virus for infection, transmission & pathogenicity
- Research into bat populations: additional research regarding bat distributions & ecological impacts
- Research aimed at improving the capacity to diagnose henipavirus infections and improve human health outcomes
- Research into infection and clinical signs in pigs in Bangladesh and potential for pig to human and human to pig transmission.

BIOTERRORISM

The following summarizes the rationale for considering NiV as a potential agent of bioterrorism.

NiV is classified by CDC as a Category C pathogen – emerging pathogens that could be engineered for mass dissemination in the future. Category C include pathogens are readily available, easy to produce, easy to disseminate, and have the potential for high morbidity and mortality with major health impact.

NiV has many of the physical attributes to serve as a potential agent of bioterrorism. The outbreak in Malaysia caused widespread panic and fear because of its high mortality and the inability to control the disease initially. There were considerable social disruptions and tremendous economic loss to an important pig-rearing industry. This highly virulent virus, believed to be introduced into pig farms by fruit bats, spread easily among pigs and was transmitted to humans who came into close contact with infected animals. From pigs, the virus was also transmitted to other animals such as dogs, cats, and horses.

Nipah Virus Bioterrorism Threat Assessment

Virology

- Reverse genetic methods are available for negative strand RNA viruses, including Nipah, and all genomic sequence data is available.
- Many laboratories are actively engaged in research programs on the cell biological properties of the henipaviruses.
- Virus can be amplified to reasonably high unconcentrated titers (>10^7). Several cell culture lines can be used, Vero cell use most often reported, and wild-type virus can be grown and harvested from cell cultures.
- A major constraint in handling Nipah is the requirement for BSL4 facilities; , however, potential terrorists may not respect this need.
- Inactivation of virus can be achieved with a variety of agents typically used for envelope viruses; but extensive environmental stability testing not reported.
- Vaccines and passively-delivered countermeasures are under development both for human and veterinary use. A commercial Hendra virus vaccine is available for horses, and the soluble G protein based vaccine has shown experimental efficacy against Nipah virus in nonhuman primates.
- Bats are sold (often live) in markets throughout their range, providing a potential source of virus; and serological tests are available for identifying henipaviruses

Economic Impact

- Destroyed the main market for Malaysian hogs in Singapore
- ~80% drop in pork consumption in the domestic market.
- Over half the standing pig population in the country was culled to halt the outbreak.
- Half the pig farms went out of business.
- During the outbreak cumulative economic losses based on government figures >$217 million USD.
- Cumulative government costs in operations and lost revenues >$298 million USD.
- Other countries in South East Asia often have a higher pig density than Malaysia. China, with approximately half of the pigs in the world, is especially vulnerable to an economic and public health disaster if NiV were to emerge and be rapidly transmitted between pigs and from pigs to people.

Epidemiology and Clinical Disease

- In outbreaks to date henipaviruses do not appear to be highly infectious. Infection requires close contact with secretions of diseased animals. Many infections can be mild to asymptomatic.
- In the initial 1998-99 outbreak the virus was *initially misdiagnosed* as Japanese Encephalitis; amplification occurred from veterinary reuse of needles in immunization programs to control JE, increasing outbreak severity and extent.
- Time from exposure to signs of infection averages ~2 weeks for humans and seroconversion occurs within a month of onset (dose / route dependent).
- Transmission directly to the vascular system could occur through bites from infected animals or broken skin exposed to secretions of infected animals.

- It is quite likely that an outbreak in animals would result in transmissions to humans.
- An outbreak of Nipah pneumonia or ARDs-like disease with human-to-human transmission as demonstrated in the Bangladesh outbreak could cause significant mortality. Nipah could cause more severe or different disease presentations in older or sick populations.

Viral Transmission

- Deliberate release of virus in some manner is possible.
- Aerosol delivery might transmit the disease effectively to domestic animals, but the environmental requirements for maintaining virus stability are not well known.
- Transmission to humans through consumption of contaminated food has been documented.
- The veterinary reuse of needles in the Japanese Encephalitis immunization campaign and in artificial insemination may have been a factor in the near 100% infection level of Nipah in pigs observed on affected farms.
- Deliberate contamination of veterinary needles could initiate an outbreak in susceptible domestic animals.
- Human-to-human transmission through travel has not been documented, but is possible.
- Transport of infected pigs on trucks was a transmission route in the Malaysian outbreak. Generalizing-- transportation of infected humans on crowded airplanes, buses or trains could also transmit the disease. Human cases have been transported to highly populous cities (e.g. Dhaka) where risk of exposure and spread among the public is increased.

Summary

- Nipah (henipaviruses) can be isolated from animal hosts.
- Several species of fruit bats, including *Pteropus spp.* widely distributed throughout Southeast Asia. The live animals are sold in food markets.
- A Nipah outbreak in swine producing areas can cause an economic crisis, even if human cases do not occur.
- Nipah virus can be amplified in permissive cell cultures (e.g., Vero cells) providing adequate laboratory facilities are available (Biosafety Level 4), although a bioterrorist group would not be restricted from growing the virus because of the lack of BSL-4 facilities.
- Effective aerosol delivery is likely possible but unpredictable on the basis of publicly available information. General unknowns are-- titers necessary for infection, virion stability in vitro, and how infectious the virus would be with this delivery.
- Effective surveillance programs, particularly in pig farming areas, are the best defense for early detection and containment of infection, whatever the source.

SUMMARY OF OBSTACLES TO PREVENTION AND CONTROL

The 2017 gap analysis working group determined that the following countermeasures were important but several weaknesses were identified.

DIAGNOSIS

NiV and HeV are zoonotic paramyxoviruses capable of causing severe disease in humans and animals. These viruses require biosafety level 4 (BSL-4) containment. The availability of safe laboratory diagnostic tests is limited. Sequence variation effects molecular diagnostics; both Clifton Beach (2007) and Redlands (2008) reported that Hendra virus strains failed in AAHL Hendra virus specific real-time PCR. Most published diagnostic PCRs only detect HeV or NiV, but not both. There is a need for a more general PCR to detect divergent and novel strains. Pan-paramyxovirus PCR assays exist and are in use to detect henipaviruses, but limitations in sensitivity limit diagnostic value. The USAID PREDICT program previously used its pan-paramyxovirus PCR assay for surveillance in more than 20 countries in Africa and Asia. Virus isolation and serum neutralization assays require live NiV. There is a need for diagnostics that can be used safely in the laboratory. There is a need for rapid nucleic acid-based assays that can detect all henipaviruses. There is also a critical need for improved antibody-based assays for disease outbreaks and disease surveillance. Importantly, there is a need to develop operator-safe diagnostic tests for which reagents can be produced without requiring high containment facilities.

Currently there is no expectations that validated tests will become available for livestock (or other species) in the near future. Nothing has been done in terms of test harmonization since 2009; however, a number of technology transfers have occurred: from AAHL to laboratories in Asia (Malaysia mainly); limited transfer from NCFAD to India (Bhopal High Containment Animal Health Laboratory); limited transfer from AAHL to the FLI and bilateral transfers between NCFAD and FLI.

VACCINATION

There is currently a commercially available vaccine for horses but no vaccines for swine or human vaccines. The goal for a HeV vaccine for horses is to vaccinate horses in areas at risk for transmission from bats to horses in order to prevent bat to horse transmission and subsequent horse to human transmission. The goal for a NiV vaccine for swine is to have a large stockpile of vaccine available for rapid use in an outbreak situation to prevent swine to swine, swine to human, and perhaps human to swine transmission to control the outbreak. A large stockpile of NiV vaccine, or vaccine antigen concentrate, for rapid emergency use in swine to control a potential outbreak that spreads too quickly to be stamped out in swine dense areas is needed. The vaccine should be licensed in the U.S., E.U or Australia for stockpiling as well as in the countries where NiV is endemic in bats. The stockpile should be available for use internationally where ever it may be needed.

SURVEILLANCE

Passive surveillance is the primary and most economical method used. Passive surveillance in commercial swine herds based on clinical signs has many weaknesses due to the difficulty of differentiating NiV from many common endemic infectious diseases of pigs; e.g., classical swine

fever, porcine reproductive and respiratory syndrome, pseudorabies, swine enzootic pneumoniae, and porcine pleuropneumonia.

In the case of infections in swine where recognition of Nipah symptoms is less likely, surveillance activities must be based on diagnostic testing to supplement surveillance based on clinical signs.

Active surveillance programs are expensive and would have to rely on direct diagnostic tests such as viral isolation and nucleic acid-based assays but available tests have significant weaknesses and have not been validated.

Rapid confirmation of cases is essential. Knowledge on serological cross-reactions with other henipaviruses and/or morbilliviruses in bats is improving. There is an urgent need to establish diagnostic capacity for Nipah virus in countries that are most likely to experience spillovers from the bat reservoirs.

DEPOPULATION

Depopulation is the primary countermeasure to reduce virus shedding and stop the spread of Nipah virus in swine. Recent outbreaks have shown that the control of Nipah virus in pig populations through stamping out is complex due to the zoonotic nature of the agent and may be very expensive, particularly in areas with high pig densities. Because Nipah virus spreads rapidly and silently in pigs, a large number of animals would need to be pre-emptively culled if an outbreak occurred in the U.S, or in other swine dense countries in order to minimize the virus spread in the vicinity of infected herds. Thus, this method of control would have significant financial implications due to the culling of thousands or millions of animals.

COUNTERMEASURES ASSESSMENT

ASSUMPTIONS

The following captures assumptions made by the gap analysis working group to assess potential countermeasures to enhance our ability to contain and eradicate an outbreak of NiV.

Situation
Countermeasures assessed for worst case scenario: A coordinated intentional distribution of NiV-contaminated material in a high density highly populated pig region of the United States.

Target Population
Countermeasures assessed for target pig production segments in priority order:

1. Backyard pigs
2. Comprehensive commercial swine operations (farrowing, nursery, and finishing)
3. Commercial indoor farrowing operations
4. Large intensive indoor pig farms
5. Valuable commercial genetic swine stock

Scope of Outbreak
Countermeasures assessed for multiple outbreaks occurring simultaneously in backyard pigs, three farrowing commercial operations, a finishing pig commercial operation, a sow replacement operation, and evidence of infection in feral swine.

DECISION MODEL

The quantitative Kemper-Trego (KT) decision model was used to assess available vaccines and diagnostics. For the criteria and weights used to assess NiV vaccines and diagnostics (See Appendices II, III).

Criteria
The following critical criteria were selected to enable the comparison of countermeasures using a pertinent and valid analysis, as follows:

<u>Vaccines</u>

- Efficacy
- Safety
- One dose
- Manufacturing safety
- DIVA compatible
- Manufacturing yield
- Rapid production
- Reasonable cost
- Short withdrawal period

- Long shelf life

Diagnostics
- Sensitivity
- Specificity
- DIVA detection
- Multispecies
- Validation to purpose
- Speed of scale-up
- Throughput
- Pen-side test
- Rapid result
- No need for a confirmatory test
- Easy to perform
- Safe to operate
- Availability
- Storage/Distribution
- Low cost to implement
- Perform at BSL-2
- Does not require use of live virus to prepare reagents

Weight

Each criterion was weighted to allow a quantitative comparison of the impact of the selected interventions (See Appendices II and III).

Product profile

To ensure a consistent and meaningful assessment, the desired product profile (i.e., the benchmark) was identified for each countermeasure:

Desired Vaccine Profile

1. Highly efficacious: prevent transmission; efficacy in all age animal target hosts, including maternal antibody override; cross protection across all henipavirus strains; quick onset of immunity; multiple animal target hosts; one year duration of immunity
2. Safe in all age animal target hosts; no reversion to virulence for live vaccines
3. One dose
4. Safe vaccine strain for manufacturing
4. DIVA compatible
5. Manufacturing method yields high number of doses
6. Rapid speed of production and scale-up
7. Reasonable cost
8. Short withdrawal period for food consumption
9. Long shelf life

Desired Diagnostic Test Profile

1. Detect all henipavirus
2. Identify Nipah virus specific strains
3. Direct tests for control and eradication
4. Indirect tests for post-control monitoring
5. Rapid test
6. >95% specificity
7. >95% sensitivity
8. Pen-side test
9. DIVA Compatible
10. Field validated
11. Easy to perform/easily train NAHML's personnel
12. Scalable
13. Reasonable cost
14. Operator safe
15. Reagents can be produced in low containment

Values

The values assigned for each of the interventions reflect the collective best judgment of members of the gap analysis working groups (See Appendices I and II)

VACCINES

The human infections in the 1999 outbreak in Malaysia were linked to transmission of NiV from pigs. Accordingly, a swine vaccine able to prevent virus transmission would be an important tool to safeguard commercial swine operations and people at risk. In addition, since henipaviruses have a very broad host range, a vaccine that is efficacious in multiple susceptible animal species would be desirable. Although the 2017 gap analysis working group determined that there are still no NiV commercial vaccines available, there are several vaccine candidates that may be safe and effective in swine and other domestic animals that were recently reviewed in: (Weingartl H.M., 2015; Broder, C.C., *et al, 2016*; and Satterfield, B.A., *et al.,* 2016). After these reviews were published, a manuscript was published demonstrating the efficacy of a virus-like-particle (VLP) Nipah virus vaccine in hamsters for inducing virus neutralizing antibodies and protection from challenge (Walpita P., *et al.,* 2017). Another manuscript was published that concluded that an adjuvanted Hendra soluble G vaccine in pigs induced neutralizing antibody titers considered to be protective against Nipah virus without detectable T cell-mediated immunity to Nipah, which did not protect from challenge with Nipah virus. However, pigs that had been previously challenged with a low dose of NiV developed neutralizing antibodies and cell-mediated immune memory and were protected from a high challenge dose of NiV. The conclusion of this manuscript was that both virus neutralizing antibodies and cell-mediated immunity were necessary for protection from NiV challenge (Pickering B.S., *et al.,* 2016). Subsequent unpublished research demonstrated that a different adjuvant used with the soluble Hendra G vaccine caused the induction of both high tittered virus neutralizing antibody and detectable T cell-mediated immunity in pigs to NiV. Challenge studies were not conducted (J.A. Roth, personal communication). All of these vaccine candidates would need further research and development to be licensed, and would need to be made available as a stockpile for rapid use in an emergency if an outbreak in swine were to occur that could not be effectively stamped out. A swine vaccine would

also be needed if the Nipah virus were to mutate to be efficiently transmitted between people and between people and pigs.

Summary

- Vaccination against NiV has been successfully demonstrated
- Experimental henipavirus vaccines can prevent clinical disease
- Experimental henipavirus vaccines elicit systemic and mucosal immunity
- Experimental henipavirus vaccines prevent viral replication in target tissues
- HeV commercial vaccine Equivac® HeV does not cross protect against NiV infection in swine
- Henipavirus vaccines appear to be effective in several mammalian animal species

Assessment of Commercial Vaccines

A commercial vaccine (Equivac® HeV) against Hendra virus approved for use in horses (Middleton D.J. *et al.,* 2014) was registered by Zoetis in Australia in 2015. A six month booster dose is required for full protection, followed by annual vaccination. The vaccine is also approved for pregnant mares. There is currently no Nipah virus vaccine approved for swine. Likewise, there is no vaccine against Hendra virus (or Nipah virus) approved for human use.

Assessment of Experimental Vaccines

The working group felt that limited information was available to assess and contrast experimental vaccines that have been reported in the literature. Experimental animal vaccines under investigation are summarized in Table I. Experimental vaccines for humans are summarized in Table II. Several of the working group members have directly or indirectly been involved in the research associated with these vaccines so that an assessment could be made (See Appendix I). The following describes some of the most promising experimental vaccine technologies.

1) Canarypox-vectored NiV Vaccines

The ALVAC canarypox virus-based recombinant vaccine vector (Taylor *et al.,* 1994) was used to construct two experimental NiV vaccines (Weingartl *et al.,* 2006). These experimental vaccines were engineered by Merial.

The first construct carries the gene for NiV attachment glycoprotein G (ALVAC-G). The second construct carries the NiV fusion protein F (ALVAC-F).

The efficacy of both the ALVAC-G and ALVAC-F were tested in pigs either as monovalent vaccine or in combination (ALVAC-G/F). The vaccine dose used was 10(8) PFU. The vaccine regimen was two doses administered 14 days apart. Both non-vaccinated controls and vaccinated pigs were challenged with 2.5 x 10(5) PFU of NiV two weeks later.

The combined ALVAC-F/G vaccine induced the highest levels of neutralization antibodies. Despite the low neutralizing antibody levels induced by ALVAC-F all vaccinated animals were protected against challenge. Virus was not isolated from the tissues of any of the vaccinated pigs post-challenge, and a real-time reverse transcription (RT)-PCR assay detected only small amounts of viral

RNA in several samples. In challenge control pigs, virus was isolated from a number of tissues or detected by real-time RT-PCR. Vaccination of pigs with the ALVAC-F/G stimulated both type 1 and type 2 cytokine responses. No virus shedding was detected in vaccinated animals, in contrast to challenge control pigs, from which virus was isolated from the throat and nose.

Based on the data generated in this one study, both the ALVAC-G or the combined ALVAC-F/G vaccine appears to be a very promising vaccine candidate for swine.

2) Soluble G Henipavirus Vaccine

HeV and NiV infect cells by a pH-independent membrane fusion event mediated by their attachment (G) and fusion (F) glycoproteins. Scientists at the Uniformed Services University of the Health Sciences in Bethesda, Maryland, in collaboration with the Australian Animal Health Laboratory characterized HeV- and NiV-mediated fusion activities and detailed their host-cell tropism characteristics. These studies suggested that a common cell surface receptor was utilized by both viruses. To further characterize the G glycoprotein and its unknown receptor, soluble forms of HeV G (sG) were constructed by replacing its cytoplasmic tail and transmembrane domains with an immunoglobulin kappa leader sequence coupled with an S-peptide tag (sG) to facilitate purification and detection. Expression of sG was verified in cell lysates and culture supernatants by specific affinity precipitation. Analysis of sG by size exclusion chromatography and sucrose gradient centrifugation demonstrated tetrameric, dimeric, and monomeric species, with the majority of the sG released as a disulfide-linked dimer. Immunofluorescence staining revealed that sG specifically bound to HeV and NiV infection-permissive cells. The scientists further reported that administration of sG to rabbits can elicit a potent cross-reactive neutralizing antibody response against infectious HeV and NiV (Bossart *et al.* 2005).

Experimental subunit vaccine formulation containing either HeV sG or NiV sG were evaluated as potential NiV vaccines in the cat model. Two cats were immunized with HeV sG and two cats were immunized with NiV sG. Immunized animals and two additional naïve controls were then challenged subcutaneously with 500 TCID50 of NiV. Naive animals developed clinical disease 6 to 13 days post-infection, whereas none of the immunized animals showed any sign of disease (Mungall *et al.,* 2006).

In a subsequent experiment, an experimental subunit formulation containing HeV sG and CpG adjuvant was evaluated as a potential NiV vaccine in the cat model. Vaccinated animals demonstrated varying levels of NiV-specific Ig systemically and importantly, all vaccinated cats possessed antigen-specific IgA on the mucosa. Upon oronasal challenge with NiV (50,000 TCID50), all vaccinated animals were protected from disease although virus was detected on day 21 post-challenge in one animal. (McEachern *et al.,* 2008).

A recent publication demonstrated that an adjuvanted Hendra soluble G vaccine in pigs induced SN antibody titers considered to be protective against Nipah virus without detectable T cell-mediated immunity to Nipah which did not protect from challenge with Nipah virus. Pigs which had been previously challenged with a low dose of Nipah developed SN antibodies and cell-mediated immune memory and were protected from a high challenge dose of Nipah virus. The conclusion of this manuscript was that both SN antibodies and cell-mediated immunity were necessary for protection from Nipah virus challenge (Protection against henipaviruses in swine requires both, cell-mediated and

humoral immune response, B.S. Pickering, J.M. Hardham, G. Smith, E.T. Weingartl, P.J. Dominowski, D.L. Foss, D. Mwangi, C.C. Broder, J.A. Roth, H.M. Weingartl, Vaccine 34(40): 4777-4786, 2016). Subsequent unpublished research demonstrated that a different adjuvant used with the soluble Hendra G vaccine caused the induction of both high tittered SN antibody and detectable T cell-mediated immunity in pigs to Nipah virus. Challenge studies were not conducted (J.A. Roth, personal communication).

3) Vaccinia-vectored NiV Vaccine

The NYVAC vaccinia virus-based recombinant vaccine vector (Tartaglia *et al.,* 1992) was used to construct an experimental NiV vaccine where the vaccinia virus expresses both the NiV glycoproteins G and F (Guillaume *et al.,* 2004). This experimental vaccine was engineered by the Pasteur Institute.

Scientists at the Pasteur Institute in collaboration with University of Malaysia scientists showed that both of the NiV glycoproteins G and F when expressed as vaccinia virus recombinants induced an immune response in hamsters that protected against a lethal challenge with NiV. Furthermore, this team of scientists demonstrated passive transfer of antibody induced by either of the glycoproteins protected the animals.

DIAGNOSTICS

The gap analysis working group determined that the availability of validated diagnostic tests for surveillance, early detection, and recovery during a NiV outbreak were critical to minimize the spread of disease and reduce the economic and public impact.

Currently the diagnosis of NiV infection is by virus isolation, detection of viral RNA, or demonstration of viral antigen in tissue collected at necropsy. Specific antibody detection can also be useful, particularly in pigs where NiV infection may go unnoticed. Demonstration of specific antibody to NiV in either animals or humans is of diagnostic significance because of the rarity of infection and the serious zoonotic implication of NiV transmission.

Summary

- Antibody response to NiV take at least 14 days and therefore early diagnosis based on serology will be less reliable than antigen or molecular tests
- Recombinant N-ELISA will likely not pick up all infected pigs
- The concept of a pen-side test is attractive, but the development and regulation of such a test will be extremely challenging

Assessment of Laboratory Diagnostic Tests (See Appendix II)

Details in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2015, Chapter 2.1.14 Hendra and Nipah Virus Diseases, provides recommendations for the following tests.

Identification of the agent

1. Virus isolation and characterization
    1.1. sampling and submission of specimens
    1.2. isolation in cultured cells
    1.3. Identification: immunostaining and Immuno EM

2. Viral identification: differentiation of HeV and NiV
   2.1 comparative immunostaining
   2.2. immunofluorescence
   2.3. microtiter neutralization

3. Molecular methods
   3.1. real-time RT-PCR
   3.2. Conventional RT-PCR and Sanger sequencing

4. Immunohistochemistry

Serological tests
1. Virus neutralization tests
2. Enzyme-linked immunosorbent assay
3. Bead-based assays

Histopathology
1. Veterinary diagnostic labs might use histopathology to make the first diagnosis
2. NiV does not produce pathognomonic lesions, but a generalized vasculitis with fibrinoid necrosis in several tissues (e.g. lung and kidneys) is characteristic; NiV might be considered in the initial differential diagnosis by experienced veterinary pathologists.

Assessment of Available Diagnostic Tests

Australia, Canada, and Germany have diagnostic capability for henipaviruses in livestock; India (e.g. NIHSAD) is building its veterinary diagnostic capability; U.S. veterinary diagnostic laboratories do not have diagnostic capability to detect NiV in livestock, although the Center for Disease Control (CDC) in Atlanta, Georgia, is an OIE collaborating center for NiV.

Currently, there are no expectations of validated tests for livestock (or other species). Nothing has been done in terms of test harmonization for serological, antigen, or nucleic acid detection assays; however, successful technology transfers have taken place, as follows: from AAHL to laboratories in Asia (Malaysia mainly); limited transfer from NCFAD to India (Bhopal High Containment Animal Health Laboratory); limited transfer from AAHL to FLI and bilateral transfers between NCFAD and FLI.

Serologic testing plays an important role in the diagnosis and detection of NiV infections. Serologic tests are the most straightforward and practical means to confirm acute cases of disease and serologic evidence of infection is used in screening programs for reservoir hosts and domestic animals. However, serological assays are limited in their ability to differentiate between known and unknown henipaviruses, as cross-reactivity to one or more known viruses is possible. Both serum neutralization and Luminex assays have shown positive reactivity to both NiV and HeV in bats where the presence of a yet-to-be characterized henipavirus could not be ruled out.

Several standard and new experimental technologies that are currently being used or considered for the detection of NiV in the laboratory or as pen-side tests for field use. Shedding of NiV in oral fluids starts early post-infection and rope sampling could prove convenient for collecting samples that could be used to test larger numbers (i.e., pen tests) of pigs. Suitability of oral fluid samples for various test

platforms should be investigated. There is a need to develop a formalized worldwide structure for test validation and ring trials (i.e., inter-laboratory comparisons).

The following describes some of the most promising diagnostic platforms with potential application for NiV detection.

1) Quantitative (q) real-time PCR

Real-time PCR is a sensitive and useful approach to the detection of henipavirus genome in specimens. Due to its nature, rRT-PCR may not be able to detect all divergent and novel henapivirus strains, although adaptation of molecular tests to new virus variants could be rapid. Test methods and primers used depend on the technology platform and associated chemistry being used in individual laboratories. Test procedures have been described by different laboratories (Mungall *et al.,* 2006; Wacharapluesadee and Hemachudha, 2007; Guillaume *et al*., 2004; Chang *et al*., 2006; Feldman *et al*., 2009).

The AAHL has developed a quantitative real-time PCR to detect NiV or HeV RNA synthesis. The most commonly targeted amplification regions are directed against the N gene (Feldman et al., 2009).

RT-PCR targeting the N gene of NiV will detect both, NiV-M and NiV-B, with somewhat lower sensitivity for NiV-B. Confirmatory RT-PCR targeting the F gene specific only for NiV-B has therefore been developed (publication in preparation; H.M. Weingartl, personal communication).

2) Conventional PCR

Classical RT-PCR followed by sequencing may be more successful in detecting novel henipavirus strains. Combination of both approaches may need to be considered. Genomic RNA detection can be performed on blood or serum samples collected from live animals as well as tissues from dead animals. RNA is extracted using an RNA extraction kit [e.g., RNeasy mini kit (Qiagen)]. Extracted total cellular RNA is first subjected to first-stand cDNA synthesis using a reverse transcriptase kit [e.g., SensiScript (Qiagen)] and a reverse transcriptase primer. The resulting cDNA is amplified using a Master Mix PCR kit (Qiagen) and primers that are designed to target HeV and NiV positive-sense mRNA from the N, M and G genes and negative-sense genomic viral RNA (vRNA) at the N/P, M/F and F/G gene junctions.

3) Field PCR

Not available. Isothermal real-time RT-PCR is promising as a field deployable assay.

While this will be costly and not be practical to have in large numbers, it is worth considering having the capabilities to establish in several strategically located regions across the nation to response rapidly in an emergency situation. Technically it will not be difficult to achieve if there is the will and financial support.

4) *Virus isolation (VI)*

Virus isolation in permissive cell culture is considered the "gold standard" for isolating all strains of henipaviruses. Virus isolation greatly facilitates identification procedures and definitive diagnosis should be undertaken where operator safety can be guaranteed. Isolation is especially relevant in any new case or outbreak, particularly in countries or geographical areas where infection by NiV or HeV has not been previously documented. Implication of wildlife species as natural hosts of the viruses requires positive serology, PCR or virus isolation from wild-caught animals (Daniels *et al.,* 2007). The range of tissues yielding virus in natural and experimental cases include the brain, lung, kidney and spleen (Crameri G., *et al.* 2002).

Henipaviruses grow rapidly to high titers in a large number of cell lines. African green monkey kidney (Vero) and rabbit kidney (RK-13) cells have been found to be particularly susceptible. A CPE usually develops within 3 days, but two 5-day passages are recommended before judging the attempt unsuccessful. After low multiplicity of infection, the CPE is characterised by formation of syncytia that may, after 24–48 hours, contain over 60 or more nuclei. Syncytia formed by NiV in Vero cell monolayers are significantly larger than those created by HeV in the same time period. Although the distribution of nuclei in NiV-induced syncytia early in infection resembles that induced by HeV, with nuclei aggregated in the middle of the syncytia, nuclei in mature NiV-induced syncytia are distributed around the outside of the giant cell (Hyatt *et al.*, 2001).

Very low virus load in bats makes isolation very difficult. Linfa Wang and colleagues at the AAHL have increased sensitivity of cell lines by "rational engineering," consisting of a single point mutation in ephrinB2 resulting in enhanced affinity for NiV.

5) *Pen-side test*

Not yet developed.

While the concept is attractive, it is a huge challenge technically and in regulatory sense, especially considering how presumable false positive results would be handled.

6) *N and G ELISA*

Indirect recombinant N- ELISA and G-ELISA have been developed, and are now in the stage of diagnostic evaluation (Fisher K., *et al.*, 2018). The N-ELISA protocol was transferred to HSADDL (India) and validated and used for surveillance (Kulkarni *et al.,* 2016).

Problems with specificity (i.e., false positives) could arise. For example, swine sero-surveillance in West Bengal, India, appears to be negative; however, 8/328 samples tested positive (i.e., presumably false positive) using the anti-N antigen ELISA antibody detection test. Evaluation of the indirect IgG ELISA based on the recombinant NiV-N antigen using swine samples from Canada yielded similar results, including an indirect IgG ELISA based on the G glycoprotein. In Canadian context, the problem is the diagnostic specificity, with 5% false positives, resulting in the decision to complement with the G-ELISA. Only sera positive on both tests are considered

positive. Confirmatory testing may be required, if this was to be the first case reported in non-endemic area.

A diagnostic test for differentiating infected from vaccinated animals (DIVA) would have to most likely target the N antigen, or alternatively P gene coded products depending on the level of expression and antigenicity in animals, and the number of reactors in non-endemic areas.

The N ELISA assay could fulfill DIVA requirements if the canarypox vectored NiV-G-NiV-F vaccine is used because antibodies to N would only occur after NiV infection.

7) *IgM ELISA*

The U.S Center for Disease Control and Prevention (CDC) developed an IgM ELISA for human serology. Detection of IgM was used to confirm recent infection with NiV in both Malaysia and Bangladesh. NiV-infected cells that have been inactivated by gamma irradiation are used as antigens.

In theory the same can be done for different animal species as long as we have the right anti-species antibodies. For bats, that is still a challenge.

8) *Virus neutralization test (VNT)*

VNT serves as the traditional gold standard of serological investigations. The VNT requires live virus and thus BSL-4 containment facilities are required (Crameri *et al.,* 2002). It has proven to be a very valuable specific and sensitive tool in the diagnosis of NiV.

VNT rely on quantification methods. Three different procedures are available to titer HeV and NiV. In the traditional plaque and microtiter assay procedures, the titer is calculated as plaque forming units (PFU) or the tissue culture infectious dose capable of causing CPE in 50% of replicate wells ($TCID_{50}$), respectively.

In an alternative procedure, the viruses are titrated on Vero cell monolayers in 96-well plates and after 18–24 hours, foci of infection are detected immunologically in acetone-fixed cells using anti-viral antiserum (Crameri G., *et al.* 2002). The virus titre is expressed as focus-forming units (FFU)/ml.

Neutralisation assays using these three methods are described in the OIE Manuel of Diagnostic Tests and Vaccines for Terrestrial Animals.

Virus quantification procedures should be conducted at BSL4. A new version of the differential neutralisation test has been recently described, which avoids the use of infectious virus by the use of ephrin-B2-bound biospheres (Bossart *et al.,* 2007). Although the test has yet to be formally validated, it appears to have the potential to be a screening tool for use in countries without BSL4 facilities.

9) Pseudotype virus plaque reduction neutralization test (PRNT)

The standard plaque reduction neutralization assay (PRNT) used to detect NiV and HeV must be performed in BSL-4 containment and takes several days to complete. The CDC and the AAHL have modified the PRNT by using recombinant Vesicular Stomatitis Virus (VSV) derived from the cDNA of VSV Indiana to construct pseudotype particles expressing the F and G proteins of NiV (pVSV-NiV-F/G) as target antigens (Chang *et al.,* 2006; Tamin *et al.,* 2009; Kaku *et al*., 2009). This rapid assay can be performed at BSL-2. The PRNT was evaluated using serum samples from outbreak investigations and more than 300 serum samples from an experimental NiV vaccination study in swine. The results of the neutralization assays with pVSV-NiV-F/G as antigen showed a good correlation with those of standard PRNT. The PRNT titers give an indication of protective immunity. Therefore, this new method has the potential to be a rapid and cost-effective diagnostic method, especially in locations that lack high containment facilities, and will provide a valuable tool for basic research and vaccine development. A similar assay has been developed by the Japanese-Australian group (Kaku et al., 2009), which proved to be as specific as the VNT and much more sensitive than VNT.

10) Binding Luminex Assay

Sera are tested for antibodies binding to recombinant soluble G (sG) proteins in a Luminex® multiplexed microsphere binding assay. The sG proteins retain their ability to bind the cellular receptor molecule, indicating their native conformation is maintained, which is important for the detection of neutralizing antibodies. For bat sera, median fluorescence intensities (MFI) readings of $\geq$200 are considered positive. Three times the average background reading of negative sera is was used as a cut-off for the binding assay.

11) Luminex® multiplexed nucleic acid detection assay

Foord et al, 2012, reported microsphere suspension array systems enable the simultaneous fluorescent identification of multiple separate nucleotide targets in a single reaction using commercially available oligo-tagged microspheres (Luminex® MagPlex-TAG) to construct and evaluate multiplexed assays for the detection and differentiation of HeV and NiV. Assays were developed to target multiple sites within the nucleoprotein (N) and phosphoprotein (P) encoding genes. The relative specificities and sensitivities of the assays were determined using reference isolates of each virus type, samples from experimentally infected horses, and archival veterinary diagnostic submissions. Results were assessed in direct comparison with an established qPCR. Foord reported the microsphere array assays achieved unequivocal differentiation of HeV and NiV and the sensitivity of HeV detection was comparable to qPCR, indicating high analytical and diagnostic specificity and sensitivity.

12) Luminex® proprietary multiplex bead-based immunoassay

Currently, the Luminex® proprietary multiplex bead-based immunoassay testing platform for the detection of anti-G antibodies is used for bat surveillance at the AAHL, and by other research investigators. Luminex® technology detects antibodies to recombinant soluble G (sG) proteins from NiV and HeV in a multiplexed microsphere binding assay. Since the glycoprotein specific

antibody response to both NiV and HeV can be measured simultaneously, this assay can differentiate between the serologic responses to NiV and HeV.

13) Blocking Luminex® Assay

This is an extension of the Binding Luminex Assay, developed as a surrogate VNT in the sense that it measures antibodies that block the binding of the soluble henipavirus G protein to the ephrin-B2 receptor molecule. It is highly specific, but needs further validation with field samples.

DEPOPULATION

Preemptive culling of herds in the neighborhood of an infected herd is an effective and even indispensable measure in the control of a NiV epidemic in areas with high pig densities. The purpose of this measure is to prevent infection of new herds, which would generate massive infectious virus production, and thus to reduce the virus infection load in an area. This reduced infection load subsequently results in a reduction of the between-herd virus transmission. However, recent outbreaks have shown that the control of Nipah virus in pig populations through stamping out is complex due to the zoonotic nature of the agent. In addition, depopulation may be logistically difficult and very expensive in swine dense area, and would not be effective if the Nipah virus mutates to become easily transmitted between people and from people to pigs. Depopulation will not be possible in situation like those that occurred in Bangladesh in which NiV was transmitted from bats to humans without an amplifying host. Depopulation of swine may be impossible in a rapidly spreading outbreak in a pig dense region with hundreds of millions of swine, such as in southeast China (Vergne T. *et. al*. 2017).

SURVEILLANCE

The initial expression of NiV in U.S swine would be variable and unpredictable due to the myriad of host factors and the broad diversity of virulence among strains of henipaviruses. Different surveillance strategies will be required to detect the different clinical manifestations.

For acute infection, surveillance activities can be based on clinical signs, but signs are unlikely to be noticed by producers and practitioners. It would be prudent to develop surveillance activities based on diagnostic testing to supplement surveillance based on clinical signs.

The following surveillance programs are in place to meet the objective of rapid detection of henipaviruses in Malaysia and Australia:

1. Population-based passive reporting of suspicious NiV cases. Efforts to enhance reporting will be focused on high risk areas.
2. Laboratory-based surveillance of serum and tissue submitted from sick pigs.

There is no diagnostic capability for henipaviruses in United States veterinary diagnostic laboratories due to the lack of BSL-4 laboratory space. The only diagnostic capability for henipaviruses in the U.S is the Center for Disease Control and Prevention (CDC). There are no active or passive surveillance programs. Henipavirus suspect samples would be sent to the CDC, the OIE reference laboratory at the

Australian Animal Health Laboratory, or the National Canadian Foreign Animal Disease Center, in Winnipeg, Canada.

DRUGS

There are no licensed anti-viral drugs available to treat people or animals against Henipaviruses.

DISINFECTANTS

People: Soaps and detergents.

Fomite disinfection: Sodium hypochlorite to supply 10,000 ppm chlorine or Virkon.

PERSONAL PROTECTIVE EQUIPMENT (PPE)

PPE should be suitable to prevent farm-to-farm virus spread by diagnostic or vaccination teams.

RECOMMENDATIONS

RESEARCH

The 2017 gap analysis working group recommends the implementation of the following research priorities.

Viral Pathogenesis

- Determine early events of NiV infection, immune evasion and identify determinants for virulence and host susceptibility

Immunology

- Characterize the antibody and cell-mediated immune response to NiV infection and vaccination
- Develop the basic knowledge of the mechanisms NiV uses to evade the innate immune response
- Characterize the ability of interferons to inhibit virus replication and shedding early in infection.

Vaccine Discovery and Development Research.

- Implement comprehensive vaccine research program to deliver next generation NiV vaccines and specifically design strategies for control in priority susceptible hosts
- Investment in Nipah vaccine development needs to include conducting studies to demonstrate safety and efficacy necessary for licensure by authorities in countries that may have an emergency need for vaccine in swine.

Diagnostics

- Develop a panel of reference standards for both molecular and serologic tests that can be made available to all of the laboratories performing diagnostic tests for henipaviruses. This panel should also include monoclonal antibodies and recombinant antigens that would be readily available as low biosecurity BSL-2 reagents.
- Develop a formalized structured worldwide network for reference panel development and assay validation and harmonization.
- Develop and validate broadly reactive PCR assays targeting highly conserved genetic targets within the henipaviruses. Evaluate the relative sensitivity and specificity of the currently used PCR assays.
- Develop and validate field tests (both protein- and nucleic acid-based) to detect henipaviruses.
- Explore new antigen detection assays, including antigen capture, Loop Mediated Isothermal Amplification Protocol (LAMP) suitable for resource limited situations, and nanotechnology.
- Develop species specific reagents to improve the quality of serologic assays.
- Evaluate the relative sensitivity and specificity of molecular and serologic tests, especially new serologic tests that could replace serum neutralization titers (SNT) and meet DIVA (differentiate infected from vaccinated animals) requirements.
- Explore the use of serological assays based on recombinant antigens that could be produced at BSL-2. Classical serological tests using low biosecurity (recombinant) reagents produced at BSL-2 facilities could be developed reasonably quickly and at a reasonable cost.
- Develop species independent serologic assays using recombinant antigens.

Epidemiology

- The epidemiology of NiV in disease outbreaks needs to be assessed and modeled on the level of the individual pig, the herd, and the demographics of the region.
- Epidemiological investigations should be performed on the implementation of emergency vaccination and the use of 'DIVA' and other diagnostic tests to detect infected pigs in vaccinated populations
- Risk assessments need to be performed with regard to control or spread of henipaviruses
- The epidemiological evaluation of wildlife needs to be carried out in order to improve the risk estimates of outbreaks in domestic animal and human populations

PREPAREDNESS

Many of the countermeasures discussed in this report will require preparation and integration in a coordinated disease control program and funding for a stockpile for use in an emergency response plan for an outbreak of NiV infection. The Henipavirus gap analysis working group recommends investing in the implementation of the following preparedness plan to ensure the effective use of the countermeasures in the NVS:

- See the Ausvetplan: https://www.animalhealthaustralia.com.au/our-publications/ausvetplan-manuals-and-documents/ Assessed July 22, 2018
- See Guidelines for Veterinarians Handling potential Hendra Virus infection in Horses (QDPI): https://www.daf.qld.gov.au/__data/assets/pdf_file/0005/126770/2913_-Guidelines-for-veterinarians-handling-potential-Hendra-virus-infection-in-horses-V5.1.pdf Accessed July 22, 2018

Surveillance

Routine surveillance for NiV is now limited to serologic screening of pigs in several Southeast Asian countries.

- Develop a regional surveillance strategy, including laboratory, to detect spillovers of NiV into domestic and agricultural animals.
- Determine the optimal surveillance strategy to detect circulation of NiV in the bats reservoirs and other wild life.
- Improve surveillance capacity to detect henipaviruses in high risk countries.
- Establish a formal laboratory network for henipavirus surveillance that includes standardized specimen collection, laboratory testing scheme, quality control, specimen referral and accreditation.

Biosecurity

Design NiV-specific on-farm biosecurity programs to implement in a disease outbreak situation.

Personal Protective Equipment and Decontamination

- See Australian procedures https://www.dpi.nsw.gov.au/__data/assets/pdf_file/0003/494202/Hendra-virus-ppe-procedures.pdf Assessed July 22, 2018

- FAO (Food and Agriculture Organization) – Manual on the Diagnosis of Nipah Virus in Animals: Chapter 2: Working safely with Nipah Virus
  http://www.fao.org/docrep/005/AC449E/ac449e05.htm#bm05
  Assessed July 22, 2018

Depopulation and Disposal

Develop plans for handling disposal of animals infected with a zoonotic agent, including an emergency plan to dispose of infected swine and decontaminate facilities and equipment determined to be infected.

- FAO (Food and Agriculture Organization) – Manual on the Diagnosis of Nipah Virus in Animals: Chapter 5: Control and eradication
  http://www.fao.org/docrep/005/AC449E/ac449e08.htm#bm08

CONCLUSION

The threat of an outbreak with a henipavirus in the United States due to a natural transmission from a reservoir host is very low since the reservoirs are known to be bats in South East Asia, South Asia, and Asia. However, an outbreak that is not controlled in swine or in people in Asia could result in infection being introduced accidentally into North America or Europe. There is considerable concern that henipaviruses could be used as a weapon of mass destruction (WMD) because they have many of the characteristics of the ideal biological weapon, including causing one of the highest mortality rate in people known for an infectious disease. The possibility of an intentional criminal spread at least in short clusters of terrorist attacks is a distinct possibility, for example by aerosolization in confined public spaces, or through infection of pigs. Surveillance brings challenges and weaknesses of diagnostic methods may impede the early detection of an outbreak in the United States. There are no commercially available diagnostic tests and although laboratory tests are available they have not been field validated. Depopulation is the primary method to eradicate NiV but present very high risks since henipaviruses are BSL-4 zoonotic agents. There are commercially available vaccines for horses, but none for swine and people. Accordingly, the gap analysis working group recommends investing in the research and development of countermeasures and ensure their use and integration in planning for preparedness and future control campaigns. Priority should be given to funding research to improve surveillance, diagnostics, and vaccines. Specific goals include 1) improving diagnostic tests to rapidly identify new disease outbreaks; 2) epidemiological research to better understand virus transmission in wildlife and maintain a passive surveillance program in high risk commercial livestock operations; and 3) develop safe and effective vaccines specifically designed for control and eradication. The Unites States should stockpile NiV vaccines when they become available for use in contact herds to create a buffer zone as an additional control measure to prevent the spread of henipaviruses should an outbreak ever occur.

FIGURES

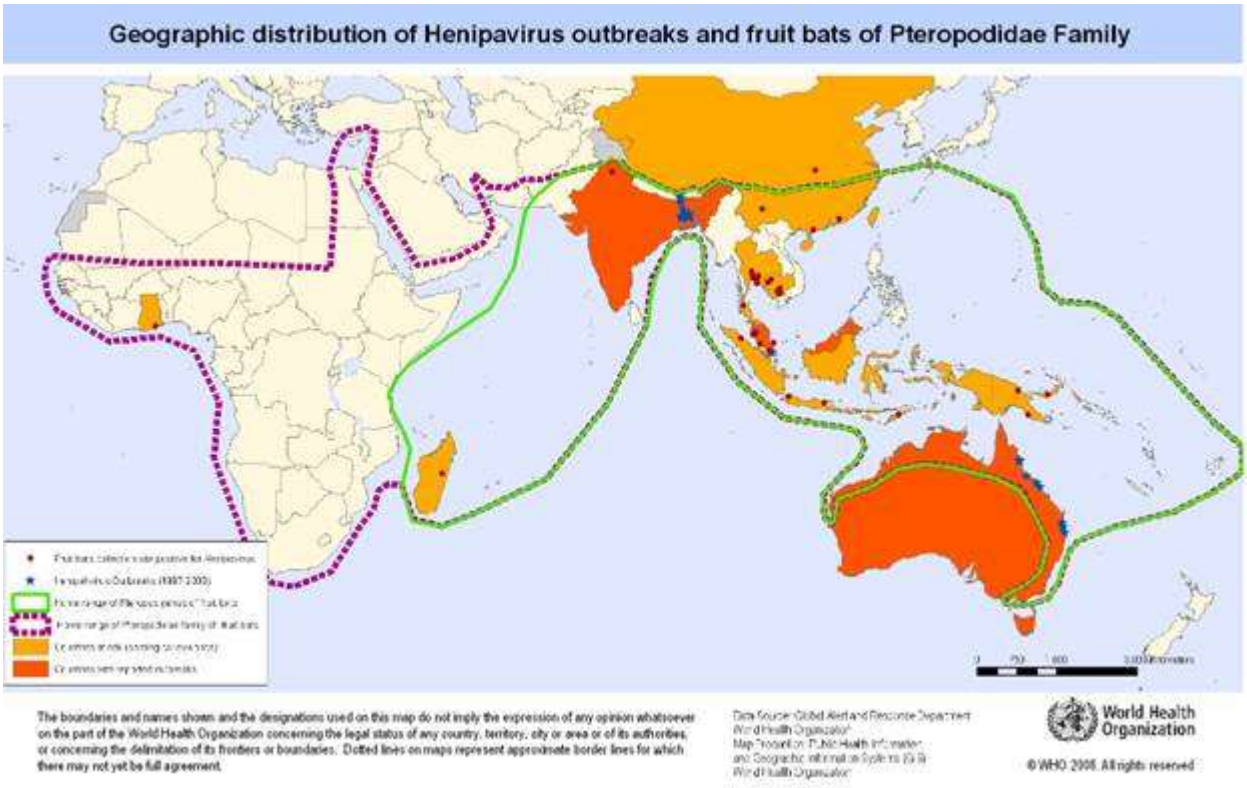


Figure 1: Geographic distribution of fruit bats of the Pteropoditae family. WHO: Nipah virus infections: http://www.who.int/csr/disease/nipah/en/ (Assessed July 22, 2018)

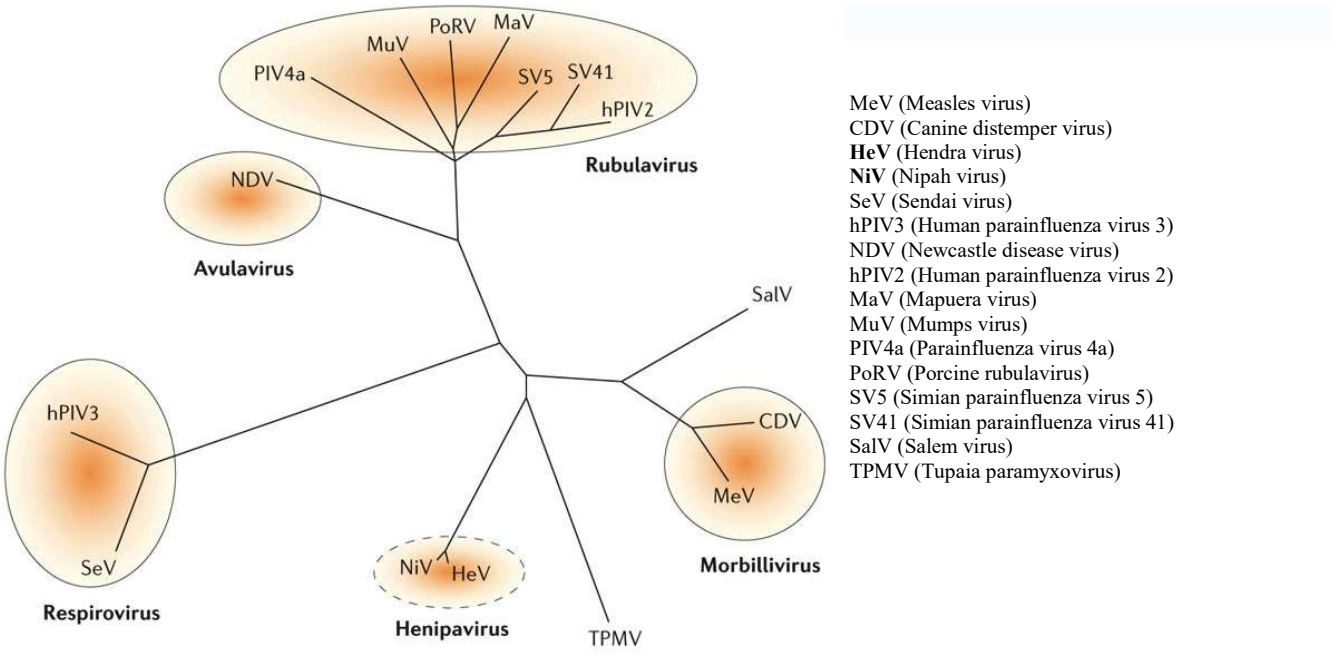


Figure 2: Phylogenetic tree based on alignment of deduced amino acid sequence of the N-gene of selected *Paramyxovirinae* subfamily members (Eaton *et al*, 2006. Nature Reviews Microbiology 4:25-35).

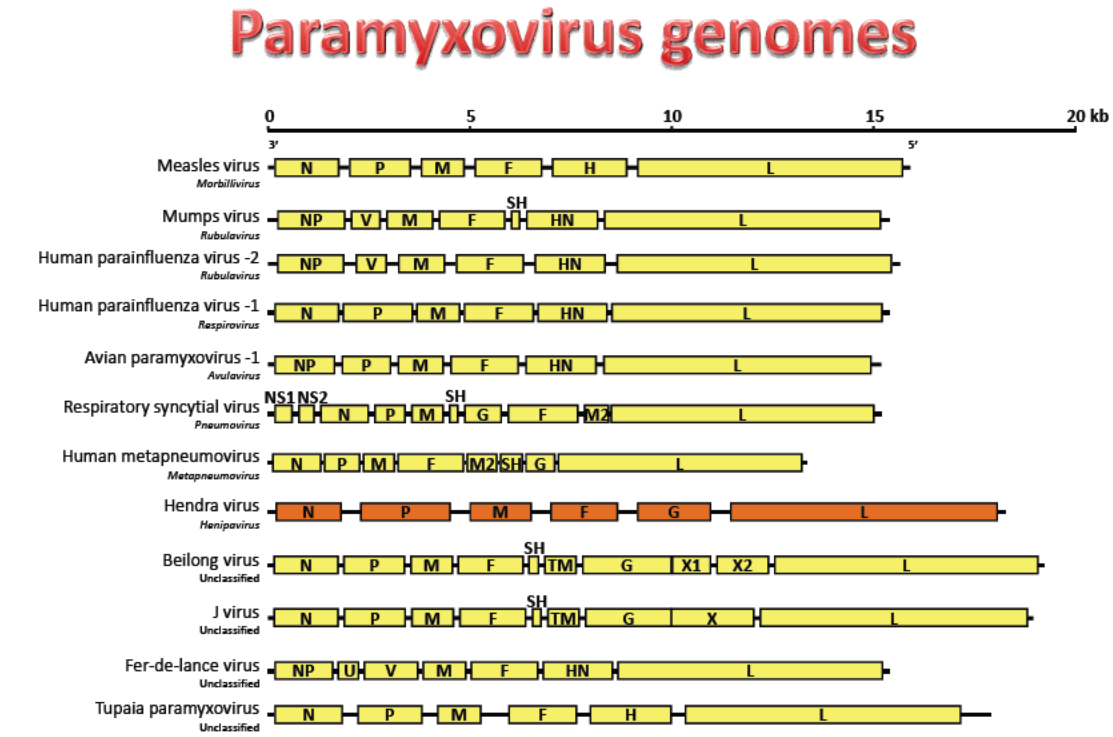


Figure 3: Comparison of *Paramyxoviridae* viruses genomes (Provided by Glen Marsh, AAHL)

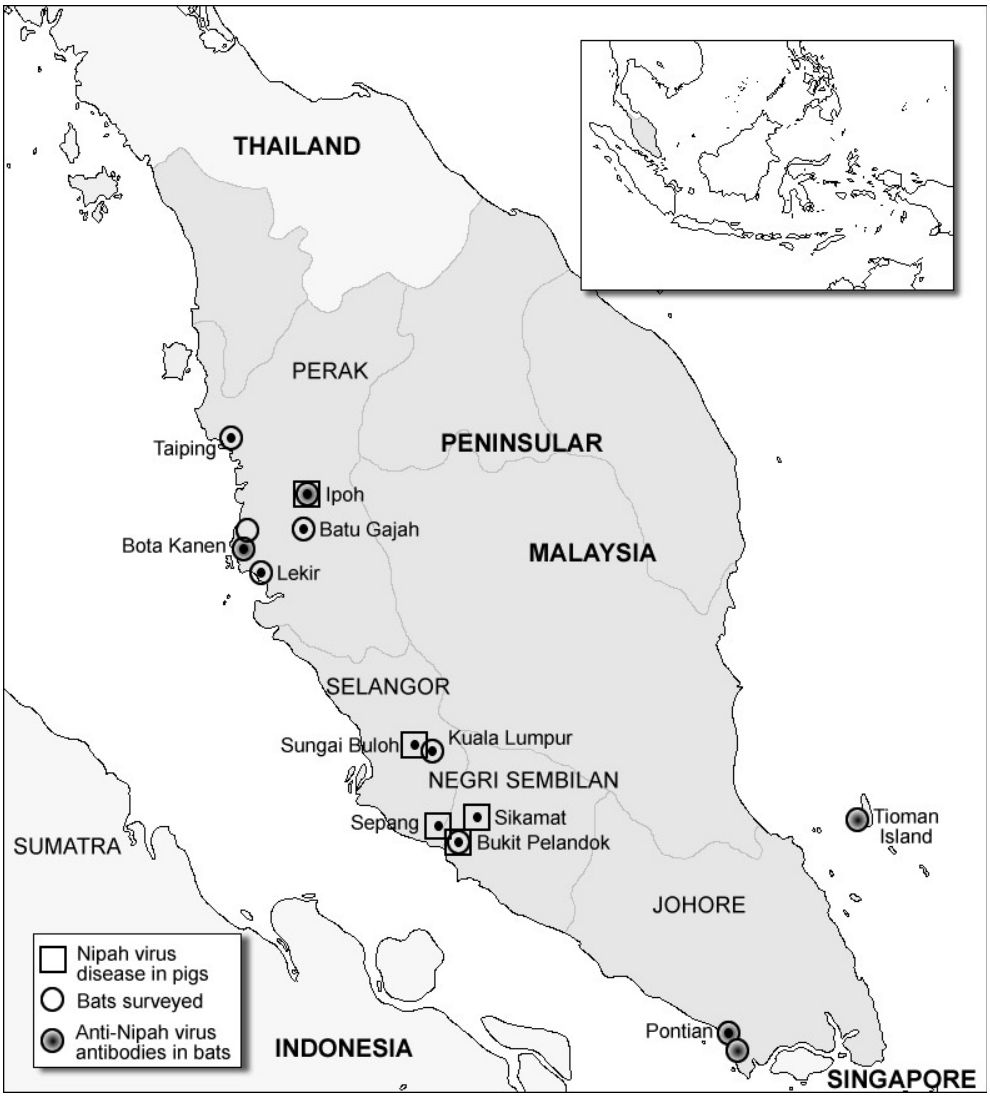


Figure 4: Descriptive map of NiV in Malaysia (Yob *et al.,* 2001)

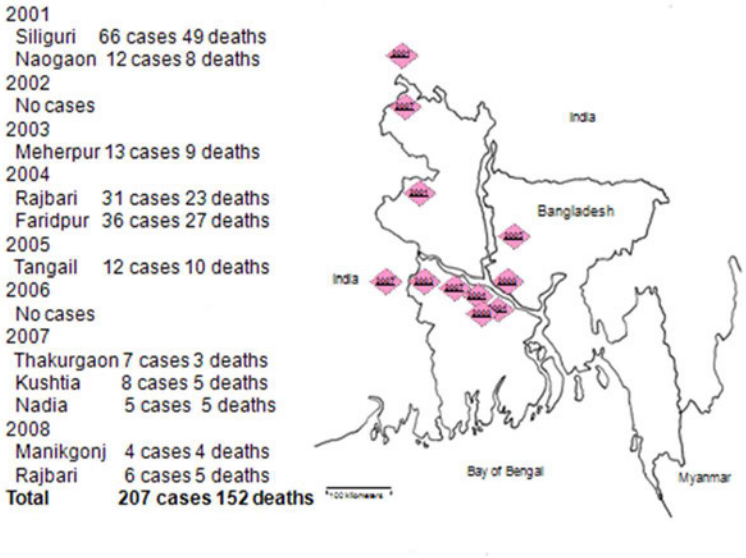


Figure 5: Epidemiology of Nipah Virus Infections in Bangladesh (Source: Steve Luby, icdd,b)

TABLE I: NIPAH VIRUS INFECTION IN BATS

Species	No. of bats	No. Positive (%)
Megachiroptera (fruit bats)		
Cynopterus brachyotis	56	2 (4)
Eonycteris spelaea	38	2 (5)
Pteropus hypomelanus	35	11 (31)
Pteropus vampyrus	29	5 (17)
Cynopterus horsfieldi	24	0
Ballionycterus maculata	4	0
Macroglossus sobrinus	4	0
Megaerops ecaudatus	1	0
Microchiroptera (Insectivorous bats)		
Scotophilus kuhlii	33	1 (3)
Rhinolophus affinis	6	0
Taphozous melanopogon	4	0
Taphozous saccolaimus	1	0
Hipperosiderus bicolor	1	0
Rhinolophus refulgens	1	0
Total	237	21

Source: Yob *et al.,* 2001

TABLE II – NIPAH VIRUS CASES 2001-2018

Morbidity and mortality due to Nipah or Nipah-like virus encephalitis in WHO South-East Asia Region, 2001-2018

Country: Bangladesh

Month/Year	Location	No. cases	No. deaths	Case Fatality Rate
April, May 2001	Meherpur	13	9	69%
January 2003	Naogaon	12	8	67%
Jan 2004 Apr 2004	Rajbari Faridpur	31 36	23 27	74% 75%
Jan- Mar 2005	Tangail	12	11	92%
Jan-Feb 2007 Mar 2007 Apr 2007	Thakurgaon Kushtia Pabna, Natore and Naogaon	7 8 3	3 5 1	43% 63% 33%
Feb 2008 Apr 2008	Manikgonj Rajbari	4 7	4 5	100% 71%
Jan 2009	Gaibandha, Rangpur and Nilphamari Rajbari	3 1	0 1	0% 100%
Feb-Mar 2010	Faridpur Faridpur, Rajbari, Gopalgonj, Kurigram,	8 8 1	7 7 1	87.50% 87.50% 100%
Jan-Feb 2011	Lalmohirhat, Dinajpur, Comilla Nilphamari, Faridpur, Rajbari	44	40	91%
Jan 2012	Joypurhat	12	10	83%
Jan- Apr 2013	Pabna, Natore, Naogaon, Gaibandha, Manikganj	24	21	88%
Jan-Feb 2014	13 districts	18	9	50%
Jan-Feb 2015	Nilphamari, Ponchoghor, Faridpur, Magura, Naugaon, Rajbari	9	6	67%

Country: India

Month/Year	Location	No. cases	No. deaths	Case Fatality Rate
Feb 2001	Siliguri	66	45	68%
Apr 2007	Nadia	5	5	100%
May 2018	Kerala	14	12	86%

WHO (World Health Organization). Morbidity and mortality due to Nipah or Nipah-like virus encephalitis in WHO South-East Asia Region, 2001-2018. Available at: http://www.who.int/csr/disease/nipah/en/. (Accessed on July 22, 2018).

TABLE III – VACCINE PLATFORMS

C.C. Broder et al. / Vaccine 34 (2016) 3525–353

Table 1
Advanced active vaccination and passive immunization platforms tested in Hendra virus and/or Nipah virus animal challenge models.

Platform	Viral antigen target or immunogen	Animal challenge model
Active vaccination		
Recombinant vaccinia virus	Nipah F and/or G glycoprotein	Hamster[a] (NiV)
Recombinant canarypox virus	Nipah F and/or G glycoprotein	Pig[b] (NiV)
Recombinant VSV	Nipah F and/or G glycoprotein	Ferret[c] (NiV), Hamster[d] (NiV), nonhuman primate[e] (NiV)
Recombinant AAV	Nipah G glycoprotein	Hamster[f] (NiV, HeV)
Recombinant measles virus	Nipah G glycoprotein	Hamster and nonhuman primate[g] (NiV)
Recombinant subunit	Hendra soluble G glycoprotein	Cat[h] (NiV), Ferret[i] (HeV, NiV), nonhuman primate[j] (HeV, NiV), horse[k] (HeV)
Passive immunization		
Human monoclonal antibody m102.4	Hendra/Nipah G glycoprotein	Ferret[l] (NiV) Nonhuman primate[m] (HeV, NiV)

[a] Hamsters immunized with NiV F and/or G glycoprotein encoding recombinant vaccinia viruses were protected against disease following intraperitoneal challenge with 10^3 PFU of NiV [137].
[b] Pigs immunized with NiV F and/or G glycoprotein encoding recombinant canarypox viruses were protected against intranasal challenge with 2.5×10^5 PFU of NiV [138].
[c] Ferrets immunized with NiV F and/or G glycoprotein encoding recombinant vesicular stomatitis virus (VSV) vectors were protected against lethal intranasal challenge with 5×10^3 PFU of NiV [141]
[d] Hamsters immunized with NiV F and/or G glycoprotein encoding recombinant vesicular stomatitis virus (VSV) vectors were protected against lethal intraperitoneal challenge with 10^5 $TCID_{50}$ of NiV [143]; or 6.8×10^4 $TCID_{50}$ of NiV [142].
[e] African green monkeys immunized with a NiV G encoding recombinant VSV vector were protected against lethal intratracheal challenge with 10^5 $TCID_{50}$ of NiV [156].
[f] Hamsters immunized with a NiV G encoding recombinant adeno-associated virus (AAV) vector were protected against lethal intraperitoneal with 10^4 PFU of NiV [139].
[g] Hamsters and African green monkeys immunized with a NiV G encoding recombinant measles virus vector were protected against lethal intraperitoneal challenge with 10^3 $TCID_{50}$ of NiV (hamsters) or 10^5 $TCID_{50}$ of NiV (monkeys) [140].
[h] Hendra virus soluble G glycoprotein (HeV-sG) used to immunize cats protects against lethal subcutaneous (500 $TCID_{50}$) [120] or oronasal (5×10^4 $TCID_{50}$) NiV challenge [145].
[i] HeV-sG used to immunize ferrets protects against lethal oronasal challenge with 5×10^3 $TCID_{50}$ of HeV [124] or 5×10^3 $TCID_{50}$ of NiV challenge [146].
[j] HeV-sG used to immunize African green monkeys protects against lethal intratracheal challenge with 10^5 $TCID_{50}$ of NiV [157] or 5×10^5 PFU of HeV [147].
[k] HeV-sG used to immunize horses protects against lethal oronasal challenge with 2×10^6 $TCID_{50}$ of HeV [15].
[l] A NiV and HeV cross-reactive G glycoprotein specific neutralizing human mAb (m102.4) protects ferrets against lethal oronasal challenge with 5×10^3 $TCID_{50}$ of NiV [125] or 5×10^3 $TCID_{50}$ of HeV (J. Pallister and C. Broder, unpublished) by post-exposure infusion.
[m] Human mAb m102.4 protects African green monkeys by post-exposure infusion following lethal intratracheal challenge with 4×10^5 $TCID_{50}$ of HeV [153] or lethal intratracheal challenge with 5×10^5 PFU of NiV [154].

TABLE IV – CURRENT VACCINE CANDIDATES

B.A. Satterfield et al. / Vaccine 34 (2016) 2971–2975

Table 1
Development status of current vaccine candidates.

Candidate name/ identifier: institution	Preclinical	Developers	Ref
Subunit vaccine			
HeV sG	X	Zoetis, Inc./USU	[16,18,34,39]
Vectored vaccines			
VSV-NiV_B F and/or G	X	UTMB	[17]
VSV-NiV_M G	X	CDC	[15]
VSV-NiV_M G	X	RML	[14,19]
VSV-NiV_M F and/or G	X	Yale University	[40]
VSV-HeV G:	X	TJU/RML	[41]
RABV-HeV G:	X	TJU/RML	[41]
ALVAC-F/G	X	CFIA-NCFAD	[20,42]
AAV-NiV_M G	X	INSERM	[43]
rMV-Ed-G	X	UoT	[44]
V-NiVG	X	USU	[45]
rLa-NiVG and/or rLa-NiVF	X	CAAS-SKLVB	[21]
Passive antibody transfer			
Polyclonal serum NiV F or G	X	INSERM	[46]
Mouse mAbs NiV F or G	X	INSERM	[47]
Human mAb m102.4 Henipah G	X	USU	[35,48]

Abbreviations: USU (Uniformed Services University of the Health Sciences); UTMB (University of Texas Medical Branch); CDC (Centers for Disease Control and Prevention); RML (Rocky Mountain Laboratories); TJU (Thomas Jefferson University); CFIA-NCFAD (Canadian Food Inspection Agency – Centre for Foreign Animal Diseases); Institut national de la santé et de la recherché médicale (INSERM); UoT (University of Tokyo); CAAS-SKLVB (Chinese Academy of Agricultural Sciences (CAAS) – State Key Laboratory of Veterinary Biotechnology (SKLVB)).

APPENDIX I – VACCINES ASSESSMENT

Experimental Veterinary Vaccines For Nipah Virus - USDA/ARS, 03-19-09

Rank each Intervention (2,4,6,8, or 10) as to its importance to making a decision, only one "10" rankings allowed

Weight	Critical Criteria	CPV-G	CPV-F	VV-G	Soluble G			
10	Efficacy	6	4	2	6			
6	Safety	10	10	2	10			
8	One dose	4	4	4	2			
8	Manufacturing safety	8	8	6	8			
10	DIVA Compatible	8	8	8	8			
8	Manufacturing yield	8	8	8	6			
6	Rapid production	8	8	4	4			
4	Reasonable cost	6	6	4	2			
2	Short withdrawal	8	8	2	4			
8	Long shelflife	8	8	8	4			

Rank each Criteria 2,4,6,8 or10 on each criterion -- no more than two "10" rankings allowed

Critical Criteria	CPV-G	CPV-F	VV-G	Soluble G	0	0	0
Efficacy	60	40	20	60	0	0	0
Safety	60	60	12	60	0	0	0
One dose	32	32	32	16	0	0	0
Manufacturing safety	64	64	48	64	0	0	0
DIVA Compatible	80	80	80	80	0	0	0
Manufacturing yield	64	64	64	48	0	0	0
Rapid production	48	48	24	24	0	0	0
Reasonable cost	24	24	16	8	0	0	0
Short withdrawal	16	16	4	8	0	0	0
Long shelflife	64	64	64	32	0	0	0
0	0	0	0	0	0	0	0
Value	512	492	364	400	0	0	0

APPENDIX II – DIAGNOSTICS ASSESSMENT

Experimental Diagnostics For Nipah Virus - USDA/ARS, 03-19-09

Rank each Interven ion (2,4,6,8, or 10) as to its importance to you in making a decision, no more than one "10" rankings allowed

Weight	Critical Criteria	qPCR	conv PCR	field PCR	VI	penside	v ELISA	N ELISA	IgM ELISA	VNT	ps VNT	bind lum	block lum
10	Sensi ivity	10	10	8	8	4	10	4	8	8	8	8	8
8	Specificity	8	6	8	10	6	6	6	8	10	8	8	8
2	DIVA	8	8	8	8	8	2	10	6	2	2	8	2
6	mul ispecies	8	8	8	8	8	6	6	2	8	8	6	8
8	Validation to purpose	8	8	8	8	4	8	4	10	8	10	8	10
4	Speed of Scaleup	8	4	4	2	6	8	8	8	2	4	4	4
4	Throughput	8	2	2	2	4	8	8	8	2	4	6	6
4	Flock Side Test	2	2	10	2	10	2	2	2	2	2	2	2
10	Rapid Result	6	4	8	2	8	6	6	6	4	4	10	8
4	No need to Confirm	6	4	4	8	2	6	4	6	8	8	8	8
8	Easy to perform	8	6	6	4	8	8	8	6	6	6	8	8
8	safe to operate	8	8	6	2	6	8	8	8	2	8	8	8
8	Availability	8	8	2	2	2	6	8	4	2	6	4	4
6	Storage/Distribution	4	6	6	2	6	6	6	6	2	4	4	4
4	Low Cost to Implement	2	4	2	2	4	6	8	6	2	4	4	2

Rank each Criteria 2,4,6,8 or10 on each criterion -- no more than two "10" rankings allowed

Critical Criteria	qPCR	conv PCR	field PCR	VI	penside	v ELISA	N ELISA	IgM ELISA	VNT	ps VNT	bind lum	block lum
Sensi ivity	100	100	80	80	40	100	40	80	80	80	80	80
Specificity	64	48	64	80	48	48	48	64	80	64	64	64
DIVA	16	16	16	16	16	4	20	12	4	4	16	4
mul ispecies	48	48	48	48	48	36	36	12	48	48	36	48
Validation to purpose	64	64	64	64	32	64	32	80	64	80	64	80
Speed of Scaleup	32	16	16	8	24	32	32	32	8	16	16	16
Throughput	32	8	8	8	16	32	32	32	8	16	24	24
Flock Side Test	8	8	40	8	40	8	8	8	8	8	8	8
Rapid Result	60	40	80	20	80	60	60	60	40	40	100	80
No need to Confirm	24	16	16	32	8	24	16	24	32	32	32	32
Easy to perform	64	48	48	32	64	64	64	48	48	48	64	64
safe to operate	64	64	48	16	48	64	64	64	16	64	64	64
Availability	64	64	16	16	16	48	64	32	16	48	32	32
Storage/Distribution	24	36	36	12	36	36	36	36	12	24	24	24
Low Cost to Implemen	8	16	8	8	16	24	32	24	8	16	16	8
Value	672	592	588	448	532	644	584	608	472	588	640	628

APPENDIX III - CONTRIBUTORS

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From: (b) (6)
To: (b) (6) ; "Broder, Christopher"; (b) (6)

Subject: RE: Henipavirus Gap Analysis Report
Date: Thursday, August 9, 2018 8:01:14 AM
Attachments: Henipavirus Gap Analysis Report, August 2018.docx
image003.png
image005.png

Dear Colleagues,

Thanks to (b) (6) for helping update Section 6 of the diagnostic section. I'm attaching here the report one more time for those of you that have not had a chance to look at it yet. I plan on posting the report on the USDA website next week so please take a quick look as indicated in my message below.

Thank you.

(b) (6)

From: (b) (6)
Sent: Monday, July 30, 2018 8:13 AM
To: (b) (6) ; 'Broder, Christopher' (b) (6)
Subject: Henipavirus Gap Analysis Report

Dear Colleagues,

Please find attached the subject report for your review and input. It's taken me a while to update the gap analysis report, but really appreciated the help received from (b) (6) . I would like to post the report on the USDA website as soon as possible (a lot of people have been asking me for the report), so I would really appreciate if you could take a quick look in the sections that interest you and edit and supplement with new information and references where needed.

At a minimum, there are three areas that definitely need your attention.

1) The Recommendation section on "Preparedness" on Page 40 is weak as I had to delete all the reference materials previously provided by (b) (6) because they have all been "deleted" from the AAHL website. I tried finding other relevant information on the internet, unsuccessfully. Perhaps this section should be deleted? Or you may be able to improve it?

2) The one area that requires special technical attention for sure is the diagnostic section, especially "6) *N and G ELISA" on Page 34.* I think this section needs to clearly differentiate antibody detection versus antigen detection ELISAs, which is not clear as currently written. I think we also need to add information on assays that have recently been developed that can differentiate NiV and HeV; e.g., publications from Chiang et al (2013), and Fisher et al (2018).

3) Lastly, I think the research recommendations on Page 39 could be improved with additional research priorities to fill the many gaps identified in the report.

As previously mentioned when we were all in Canada, this report will be distributed to stakeholders and funders of research (globally), as well as industry, and government agencies that have interest in stockpiling veterinary medical countermeasures – so your critical view of the report is essential as it reflects our collective expert opinion.

Thank you so much for your help and contributions.

Best regards,

(b) (6)

[redacted]

Get more information:

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(b) (5)

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From:	(b) (6)
To:	Eric Laing
Cc:	Chris Broder
Subject:	Re: Georgia and GHERI
Date:	Thursday, July 26, 2018 5:41:16 PM
Attachments:	EcoHealth Alliance BEP proposal 2018.docx ATT00002.bin

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Hey (b) (6),

Thanks, sorry things have been crazy and I haven't gotten much in way of writing done yet.

The general plan for the Liberia/CIV project is to screen human and bat sera for Filos and Henipas only. Wondering if the assays will capture this one too? http://punchng.com/new-ebola-virus-found-in-sierra-leone-govt-says/ https://twitter.com/EcoHealthNYC/status/1022567864656699392 Late breaker, more to come.

As we discussed, we're hoping to use your existing Bio-plex panels, and to export samples to USU for analysis. Still a lot to figure out - e.g. if we should have any capacity building piece to this, and also adding in some "predictive risk mapping" with the results, but by tomorrow afternoon should have this scoped out more. Can you guys advise on the cost per specimen and labor costs, so I can think about the number of specimens and sampling design a little more?

Also, attached is the language I have from one of our previous (unfunded) BEP proposals that I'll modify. Please feel free to track changes and flesh out the methods a little more or change them if needed.

Cheers,
(b) (6)

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From: (b) (6)
To: Broder, Christopher
Cc: (b) (6)
Subject: Re:
Date: Friday, January 26, 2018 2:10:17 PM
Attachments: EHA_GLO_18__001_PROJECT SUMMARY.pdf

Hi (b) (6),

Please find the Project Summary from the submission. Please let me know if you need anything else.

Thanks,
(b) (6)

[redacted]

On Fri, Jan 26, 2018 at 1:44 PM, (b) (6) wrote:

> hi (b) (6)
>
> Could you please send me the info from the state dept grant that went in last week?
>
> title, dates, number?
>
> thanks
> (b) (6)
>
> [redacted]

(b) (6)

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From: (b) (6)
To: (b) (6) eric.laing_usuhs; (b) (6)
Cc: (b) (6)
Subject: Thailand EID-SEARCH Meeting this week August 2/3
Date: Monday, July 31, 2023 9:28:36 AM
Attachments: Thailand Y4 Scope of Work_v02_(b) (6).docx

Dear All,

This is a reminder of the Thailand EID-SEARCH Meeting **this Wednesday (9 PM NYC) and Thursday (8 AM BKK)** when we'll discuss the Y4 Scope of Work and check on a few items:

- Year 4 project Scope of Work in Thailand (please see the attached Word document with edits from both EHA and TRC-EIDCC teams)
- Update on the Mpox concept notes (budget)
- Update on papers
- Others

Look forward to the discussion.

Dear Dr. (b) (6) - Thank you for sharing the edits. I didn't include the PPT slides in the email since there is budget information that you may want to present from your side at the meeting.

All the best,
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From: (b) (6)
To: Laing, Eric
Subject: R01 summary responses and follow-up
Date: Monday, July 17, 2023 11:36:02 AM
Attachments: 1R01AI179865-01.pdf
1R01AI179865-01.pdf

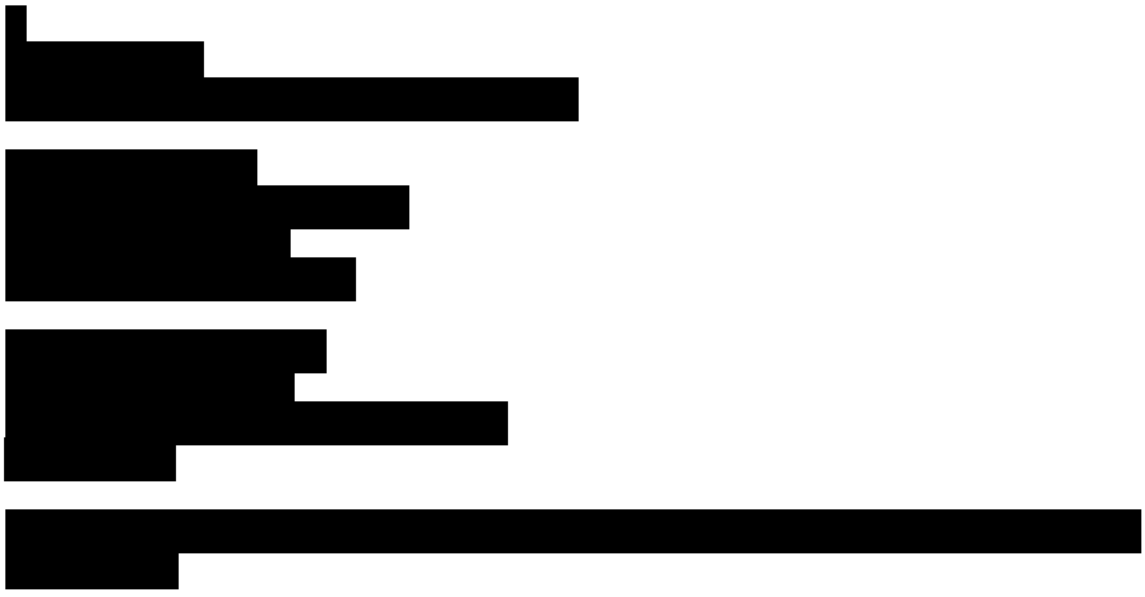
Hi (b) (6)

I have the summary response to R01 submission (attached). I'm not sure if you got it. I'm still processing it. One reviewer really hated it. I think the other critiques are addressable but fundamentally I think we need to be able to demonstrate the feasibility with a preliminary version and figure out how carve out the time in current/smaller projects to do so.

I have a very busy rest of the month, but propose that we regroup in August to think about plans for next steps/resubmission.

A couple of other things:
- On August 1 at 4PM, we have the team from NIH ImmPort giving a talk at EHA's regular "methods and models" meeting. I'll send along an invite if you or any of your lab are interested in attending.
- I'm also considering submitting a proposal to this NIH software development call: https://grants.nih.gov/grants/guide/rfa-files/RFA-AI-23-038.html, for creating R packages that would implement the both current and in-development serology analysis methods. It says "No wet-lab technologies or new data collection will be supported," but I'd include you and your team in it for data/testing/consultation.

Best,
(b) (6)



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From: (b) (6)
To: Laing, Eric; Christopher Broder
Cc: (b) (6); Broder, Christopher
Subject: Re: Thesis review - cattle farm chapter 3
Date: Thursday, March 2, 2023 4:51:47 PM
Attachments: Chap 3 Serology_draft8.docx
Table on prev of neighbouring countries.docx
Ag reactions for DTRA livestock and archived pigs.xlsx

Hi (b) (6),

Thank you for going through the chapter early. I appreciate the many great suggestions and helpful feedback.

Kindly see attached for the updated draft and attachment for the table of seroprevalence of filovirus and henipavirus in neighbouring countries mentioned in 3.2.3 Sample Size.

I'm also reattaching the breakdown of antigen reaction which I sent to you and Jon last time for (b) (6), happy to hear from you.

Thank you for guiding me,
(b) (6)

From: (b) (6)
Sent: Tuesday, February 28, 2023 9:05 AM
To: (b) (6)
Cc: (b) (6)
Subject: Re: Thesis review - cattle farm chapter 3

Hi (b) (6),

I'm looping in Dr. (b) (6) for additional comments.

(b) (6)

On Mon, Feb 27, 2023 at 11:38 AM Laing, Eric (b) (6) wrote:

> Hi (b) (6)
>
> I enjoyed reading the chapter and seeing the totality of your thesis work. Excellent work! I've made some edits, suggestions, and comments. Feel free to send back updates or questions if you need more clarification.
>
> (b) (6)
>
> [redacted]

(b) (6)

On Sun, Feb 26, 2023 at 6:46 PM (b) (6) wrote:

Hi (b) (6),

Thank you so much.

(b) (6)

From: Laing, Eric (b) (6)
Sent: Monday, February 27, 2023 6:54:30 AM
To: (b) (6)
Cc: (b) (6)
Subject: Re: Thesis review - cattle farm chapter 3

Hi (b) (6),

I'll finish by today.

(b) (6)

On Mon, Feb 27, 2023 at 2:40 AM (b) (6) wrote:

Hi (b) (6),

The due date is 13 March. I think after 4th March is still doable. But if you have time to scheme through the results section and point out any problems before the 4th would be awesome too.

Safe travels!

Thanks,
(b) (6)

From: Laing, Eric (b) (6)
Sent: Thursday, February 23, 2023 1:28 PM
To: (b) (6)

Cc: (b) (6) [redacted]

Subject: Re: Thesis review - cattle farm chapter 3

Hi (b) (6),

Email received. Can I have a few days to review this, what's a due date? I'm traveling now and time is limited thru March 4. But if this is critical before then I can do my best.

- (b) (6)

[redacted]

On Wed, Feb 22, 2023 at 6:01 PM (b) (6) [redacted] > wrote:

> Dear (b) (6) [redacted],
>
> This version is a newer version with more details on the positive & negative controls + results of USU's naive livestock sera.
>
> If you have not reviewed the previous version, please review this one.
>
> Looking forward to hearing from you.
>
> Thanks!
> (b) (6)
>
> ---
>
> **From:** (b) (6)
> **Sent:** Monday, February 20, 2023 7:07 AM
> **To:** (b) (6) [redacted]
> **Cc:** (b) (6) [redacted]
> **Subject:** Thesis review - cattle farm chapter 3
>
> Hi (b) (6) [redacted],
>
> Hope the both of you are well.

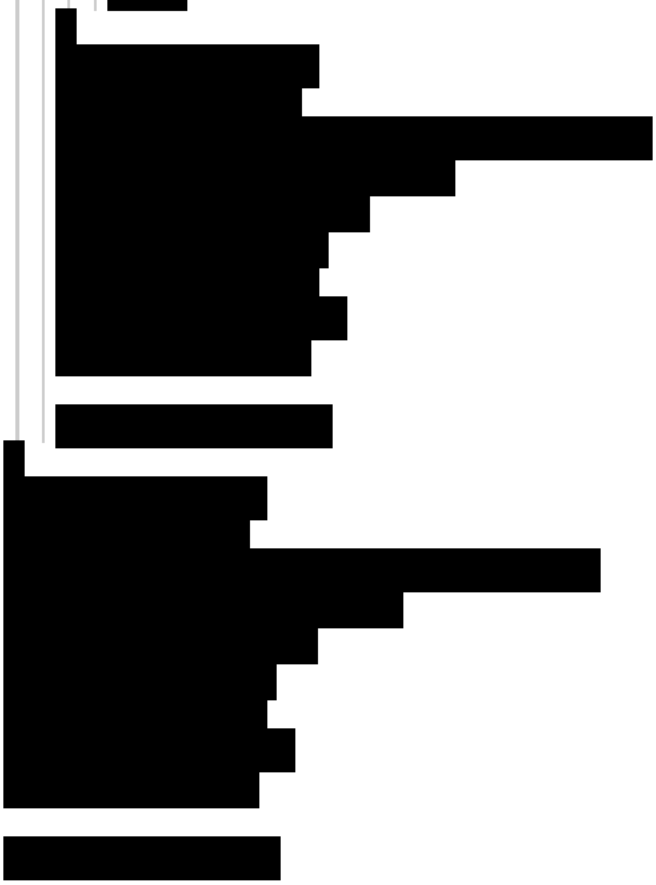
Attached is a chapter from my thesis that covers the cattle farm project + its Appendix for your review.
Please ignore the green highlights as those are for me to double-check when I compile the thesis.

(b) (6), should I add the MFI baseline for the livestock samples that you've conducted in USU?

Appreciate if you could review it by end of this month if possible. Will send the archived pig chapter as soon as I finalize it with Prof (b) (6).

Thank you.

Kind regards,
(b) (6)



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From: Laing, Eric (b) (6)
To: (b) (6)
Subject: Fwd: Subaward Proposal Dr. Laing
Date: Saturday, February 18, 2023 9:30:01 AM
Attachments: RR_Budget(b) (6)_DTRAv3.pdf
USU subaward budget (WAB-Net 2) v4.xlsx
DTRA_WABNet2_(b) (6)_Budget Justificationv4.pdf
Current and Pending Support Template (WAB-Net 2)_(b) (6)v2.pdf
BioSketch_Laing_DTRA.pdf
DTRA_WABNet2(b) (6)_LOSv2(b) (6).pdf
Facilities_DTRA_(b) (6).pdf
HJF LETTER OF INTENT_(b) (6)_FDC021723.pdf
Key Personnel and Performance Site_(b) (6)_DTRA (5).pdf
image001.png

Hey(b) (6) - your email isn't addressed correctly.

Please confirm receipt of this email.

(b) (6)

---------- Forwarded message ---------
From: (b) (6)
Date: Fri, Feb 17, 2023 at 4:28 PM
Subject: Subaward Proposal Dr. Laing
To (b) (6)
Cc: (b) (6) Eric Laing (b) (6)

Dear Mr. (b) (6),

On behalf of Dr. (b) (6) of USUHS, I attach the following documents for your review:

- Budget—RR and Excel,
- Budget Justification,
- Current and Pending Support-Dr (b) (6),

Biosketch-Dr. (b) (6),
- Letter of Support—Dr. (b) (6) and Dr. (b) (6),
- Facilities/Equipment,
- Key Person/Performance site information,
- Letter of Intent.

If you have any questions or concerns, please let me know. Thank you for this opportunity to collaborate with Dr. (b) (6) and EcoHealth Alliance.

Best,

(b) (6)

(b) (6)

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From: (b) (6)
To: (b) (6)
Cc: Eric Laing
Subject: Re: Submission
Date: Thursday, February 16, 2023 7:41:08 AM
Attachments: BioSketch(b) (6) _DTRA.pdf
Current and Pending Support Template (WAB-Net 2)(b) (6) .pdf
DTRA_WABNet2_(b) (6) _LOSv2.pdf
Facilities_DTRA_(b) (6) .pdf
DTRA_WABNet2_(b) (6) _Budget Justificationv2.pdf

Hello (b) (6),

I don't anticipate any major changes .... But here is what we have. Note - there is a fee added to the project total costs. It is very minimal and we will include the language in the justification. Also, getting a countersignature from the chair, we should have that this morning also.

Thanks much .... (b) (6)

On Wed, Feb 15, 2023 at 4:28 PM (b) (6) wrote:

> Hi (b) (6),
>
> Could you please send me the biosketch, C&P, facilities, letter of support, and budget justification for (b) (6) by tomorrow? Please let me know if that's not feasible. Thank you!
>
> Best,
>
> (b) (6)
> [redacted]
>
> [redacted]
>
> [redacted]
>
> [redacted]
>
> On Mon, Feb 13, 2023 at 3:52 PM (b) (6) wrote:
>
> > Hi (b) (6),
> >
> > Those are the correct documents, and I have not received any of them yet.
> >
> > (b) (6)
> > [redacted]
> >
> > [redacted]

(b) (6) [redacted]

[redacted]

On Mon, Feb 13, 2023 at 12:46 PM (b) (6) [redacted] wrote:

> Hello (b) (6),
>
> Please confirm the following is necessary:
>
> *Budget
> *Budget Justification
> *Current & pending support
> *Biosketch
> *Letter of support
> *Facilities / Equipment
>
> Have you received any of the documents yet?
>
> Thanks much ..... (b) (6)
>
> --
> (b) (6) [redacted]
>
>

--
(b) (6) [redacted]

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DEFENSE THREAT REDUCTION AGENCY
BROAD AGENCY ANNOUNCEMENT
HDTRA1-14-24-FRCWMD-BAA
Amendment 17
December 2022

Research and Development Directorate (RD)
Enabling Capabilities Department (RD-EC)

Fundamental Research to Counter Weapons of Mass Destruction (C-WMD)

Original Posting Date: March 2015

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OVERVIEW INFORMATION

Agency Name:

Defense Threat Reduction Agency (DTRA)
Research and Development (RD) Directorate
Enabling Capabilities Department (EC)
8725 John J. Kingman Road, MS 6201
Fort Belvoir, VA 22060-6201

Funding Opportunity Title: Fundamental Research to Counter Weapons of Mass Destruction (FRCWMD) Broad Agency Announcement (BAA)

Announcement Type: This is an amended announcement of this funding opportunity. This BAA is in effect from March 2015 through September 2024. It is anticipated that a majority of the actions funded from this announcement will be in the form of grants; however, other instruments such as contracts, cooperative agreements (CAs) or other transactions agreements (OTAs) may also be awarded from this announcement. Submissions for this BAA may occur in two ways: 1) in response to the published topics detailed in Attachment 1 or 2) to a general thrust area as described in Section 1.5.

In general, all topic-specific and general thrust area submissions require pre-coordination in accordance with the guidelines in Section 1.5 and Section 4.2.1. DTRA reserves the right to waive the pre-coordination requirement for topics on a case-by-case basis; and will state the waiver applies within the individual topic description. If a pre-application white paper is received without prior coordination, DTRA may not review it. From the date of the disposition email the applicant has 63 days to submit the pre-application white paper. If the submission is not feasible within this 63-day window, the abstract must be coordinated again to ensure the interest in the white paper remains.

The evaluation of all submissions will be conducted in two phases. Phase I is for receipt and evaluation of pre-application white papers in direct response to a published topic or by invitation based on the assessment of the idea by the Technical POC. Phase II is for receipt and evaluation of invited proposal applications. Invitation to the Phase II, invited proposal submission, will be based on the evaluation results of the Phase I pre-application white paper.

Funding Opportunity Number: HDTRA1-14-24-FRCWMD-BAA

Catalog of Federal Domestic Assistance (CFDA) Number: 12.351

Dates: This BAA is open continuously from March 2015 through September 2024. Published topics will include instructions on any topic-specific opening and closing dates as well as any topic-specific limitations on award types, dollar values, and eligibility. Submissions to a general thrust area may occur at any time this BAA is in effect. Applicants should take care to note requirements for pre-coordination of an abstract.

ADDITIONAL OVERVIEW CONTENT

Research, educational program, or other effort proposals are sought from accredited degree-granting colleges and universities. Research, educational program, or other effort proposals are also sought from industrial, commercial (including small businesses), and not-for-profit research entities. DTRA strongly encourages and may give preference to pre-application white papers and proposals that demonstrate a significant contribution (significant is defined as a minimum of

30% of total value) by one (1) or more universities.

All submissions (pre-application white papers and invited proposals) must be made in accordance with the submission instructions in this BAA through www.grants.gov using the application packages linked with this BAA (under the "Package" tab) on www.grants.gov. Applicants are responsible for ensuring compliant and final submission of their pre-application white papers and proposal applications. Any submission that does not conform to the requirements outlined in the BAA and in the invitation for proposal may not be reviewed or considered further at the discretion of DTRA.

Pre-application white papers may be evaluated any time after receipt. Invitations for full proposal submission may occur any time after the pre-application white paper evaluation and will be limited to available program funds.

Efforts may be proposed for up to five (5) years. Awards may be for a base period of one (1) year with four (4) additional years as possible options, a base period of two (2) years with three (3) additional years as possible options, or a base period of three (3) years with two (2) additional years as possible options. Applicants should take care to propose the most logical mix of base and option years for the scope of work. Further, the base period should yield a logical completion point for the work. In cases where option years are proposed, decisions regarding exercising those options will be based on the evaluation of the work accomplished in the base period. Pre-application white papers and proposals that outline scope and effort for only the base period and do not propose options are also acceptable; however, the Government reserves the right to invite option years for awards that originally only included a base period.

Grants may range from small dollar value (e.g., $25K) up to $1M annually (total, including both direct and indirect costs) depending on the nature and the scope of work. Payments on grants will be made in advance, subject to the conditions described in 2 CFR 200.305. Funding amounts for contracts, CAs, and other procurement instruments will be considered on a case-by-case basis. Thirty (30)-60 individual awards are anticipated each year.

Any assistance instrument awarded under this announcement will be governed by the award terms and conditions, which conform to DoD's implementation of OMB circulars applicable to financial assistance. This includes DoD implementation of OMB guidance in 2 CFR part 200, "Uniform Administrative Requirements, Cost Principles, and Audit Requirements for Federal Awards."

1. FUNDING OPPORTUNITY DESCRIPTION

1.1. DTRA safeguards America and its allies from weapons of mass destruction (WMD) and provides capabilities to reduce, eliminate, and counter the threat and effects from chemical, biological, radiological, nuclear, and high yield explosives. DTRA seeks to identify, adopt, and adapt emerging, existing and revolutionary sciences that may demonstrate high payoff potential to Counter-WMD (C-WMD) threats. This BAA is an extramural endeavor that combines the fundamental research, educational program, or other effort needs appropriate for basic or applied research funding of DTRA and other DoD interests.

This announcement solicits ideas and topic-based pre-application white papers for long-term challenges that offer a significant contribution: to the current body of knowledge, to the understanding of phenomena and observable facts, to significantly advance revolutionary technology, to new concepts for technology application, or that may have impact on future C-WMD threat reduction, expertise, or capabilities.

A portion of this effort is expected to be devoted to awards for science, technology, engineering and mathematics education programs with a C-WMD focus; such as, but not limited to postdoctoral fellowships, stipends, degrees, visiting scientist programs, student exchange programs, and development of accredited C-WMD curricula.

1.2. Fundamental research means basic and applied research in science and engineering, the results of which ordinarily are published and shared broadly within the scientific community, as distinguished from proprietary research and from industrial development, design, production, and product utilization, the results of which ordinarily are restricted for proprietary or national security reasons.

Contracted Fundamental Research includes research performed under grants, contracts (awards), or OTAs that are (a) funded by budget Category 6.1 (Basic Research), whether performed by universities or industry or (b) funded by budget Category 6.2 (Applied Research) performed on-campus at a university. Fundamental research provides for science and technology (S&T) research and early applied development. It seeks to lower performance risk to a manageable level and facilitate transition and funding to capability end-state programs.

1.3. Technology Readiness Levels (TRLs) provide a systematic metric/measurement system that supports assessments of the maturity of a particular technology and the consistent comparison of maturity between different types of technology. Fundamental research may be defined within the first four (4) TRLs.

1.4. This BAA seeks optimum approaches to meet DTRA fundamental research objectives. The Government encourages pre-application white papers and proposals that span a wide spectrum of research to expand fundamental scientific knowledge in response to specific topics and to the more general thrust areas. The Government reserves the right to award any combination of approaches which offer the best overall value to the Government and to oversee any and all processes and approaches once ongoing.

1.5. Thrust Areas 1-7 are described below. When a specific set of topics has been identified, these detailed needs will be described in Attachment 1 along with any topic-specific submission instructions, deadlines, anticipated award structure, and funding requirements. Otherwise, pre-application white papers and proposals may be written against one of the general thrust area descriptions.

DTRA may not review any pre-application white papers without prior coordination of the idea with the appropriate thrust area- e-mail address (Section 7). Applicants should note that there is extremely limited funding available for many of the thrust areas; Thrust Areas 1-5 are not currently accepting abstracts for pre-coordination. Pre-application white papers will only be accepted from the coordinated abstracts under limited circumstances.

1.5.1. **Thrust Area 1—*Science of WMD Sensing and Recognition:*** The science of WMD sensing and recognition investigates the fundamental understanding of materials that demonstrate measurable changes when stimulated by radiation or particles from WMD in the environment. This involves the exploration and exploitation of interactions between materials and various electromagnetic phenomena, molecules, nuclear radiation, and particles. Furthermore, these interactions and the specific form of recognition they offer are used for the subsequent generation of information, providing knowledge of the presence, identity, and quantity of material or energy in the environment that may be significant. The goal of this thrust area's portfolio is to advance the following capabilities: location, identification and characterization of radiological-nuclear (RN) materials; detection of RN materials at significant stand-off distances; and the reduction of the technical nuclear forensics timeline. Thrust Area 1 is currently not interested in research focusing on the sensing of explosives or the detection of Improvised Explosive Devices (IEDs). **Thrust Area 1 is NOT currently accepting abstracts for pre-coordination.**

1.5.2. **Thrust Area 2—*Network Sciences:*** The fundamental science of network sciences includes advancing knowledge of complex disparate but interdependent networks critical to military operations where WMD-related robustness, resiliency, recovery of, and informational and operational utility is required. It includes response, resilience, and recovery of interdependent, multi-layered physical networks after exposure from electromagnetic pulse and other nuclear weapons effects, rapid discovery and analyzing low-observable WMD-related information from large, disparate WMD-related data sets from multiple types of networks, and to develop theories and representations for low observable WMD-related radical ideation from social networks. **Thrust Area 2 is NOT currently accepting abstracts for pre-coordination.**

1.5.3. **Thrust Area 3—*Science for Protection:*** Fundamental science for protection involves advancing knowledge in physical, biological, and engineering sciences to protect personnel, sensitive electronic systems, and structural infrastructure from the effects of weapons of mass destruction. Protection includes both passive and active defense against threats. Approaches include advanced highly-ordered materials and nanomaterials to hardening infrastructure and facilities against blast, nuclear events, or other CBRNE effects; exploring new methods to experimentally and computationally simulate the effects of a nuclear event; investigations of the interaction of radiation with sensitive electronics and systems as well as development of novel materials and methods that are robust against radiation effects; novel methods to protect personnel from the physical, radiological, and nuclear effects of WMDs; and the study of biological systems, including intact structures, metabolic products, or discrete components and pathways, as applied to protection of U.S. Forces during operations in areas actually or potentially contaminated by radiation. For protection of personnel the areas of interest include development of radiation countermeasures to prevent biological damage associated with exposure to ionizing radiation and development of novel biologically-based or -produced detection systems for wide area surveillance to determine the nature, extent, and distribution of contamination. **Thrust Area 3 is NOT currently accepting abstracts for pre-coordination.**

1.5.4. **Thrust Area 4—*Science to Defeat WMD:*** Fundamental science for significantly improving energetic materials for use against WMD facilities and systems with minimal collateral effects from post-blast WMD release, for deeper penetration to deny the adversary sanctuary of WMD, and for predictable modeling of counter-WMD munitions and simulation of in-theater scenarios with accurate lethality calculations. **Thrust Area 4 is NOT currently accepting abstracts for pre-coordination.**

1.5.5. **Thrust Area 5—*Science to Secure WMDs:*** Fundamental science to support securing WMD includes: revolutionary means to safely handle, transport, control access, or eliminate WMD components and weapons; new physical or other methods to monitor compliance to support future agreements or treaties; and, exploring phenomena and means that facilitate reduction of nuclear or non-nuclear WMD proliferation pathways. This includes focus on: science principles to assist tagging, tracking, location to secure WMD; novel means to mark and read objects in order to secure inventories; remote or unattended monitoring to understand the nature of objects (e.g., is it nuclear, biological, chemical or conventional?); monitoring to detect intrusion, diversion or substitution, tampering, and other adverse activity; and, understanding of both physical and life science environmental signatures as witnesses of WMD-related activity. The ability to secure WMD may impact either verification of treaties, or control of WMD outside treaty regimes. **Thrust Area 5 is NOT currently accepting abstracts for pre-coordination.**

1.5.6. **Thrust Area 6—*Cooperative Counter WMD Research with Global Partners:*** Cooperative fundamental research to reduce the global threat of WMD in collaboration with a broad range of global research partners. This thrust area involves exploratory basic and applied research that will address opportunities to reduce, eliminate, and counter WMD across the Chemical, Biological, Radiological, Nuclear, and High Explosive (CBRNE) spectrum. Efforts in this area will develop strong international relationships which will foster a smooth transition of program ownership to the partnering country. The goal is to improve international collaboration to detect, characterize, and report WMD, and to advance partner nation sustainment through a culture of long-term cooperation and scientific responsibility for such programs. Multi-disciplinary, multinational research in science, technology, engineering, and mathematics development will be conducted to promote transparency through quality research publications and continual dialogue between scientists/engineers and young researchers.

The Cooperative Biological Engagement Program (CBEP), a component of the DoD Cooperative Threat Reduction (CTR) Program, recognizes the danger to U.S. and global health security posed by the risk of outbreaks of dangerous infectious diseases, whether natural or manmade. Consistent with the national and departmental strategies, CBEP strives to address this risk by promoting best practices in biological safety and security, improving partner country capability to safely and rapidly detect and report dangerous diseases, and establish and enhance international research partnerships that focus on informing the disease surveillance network. The desired end state for CBEP engagements is the development of sustainable partner country capabilities to:

- Employ responsible bio-risk management best practices and principles,
- Conduct a modern and effective disease surveillance mission,
- Independently sustain engagement with, and effectively compete for funding within, the international scientific community,

- Comply with World Health Organization (WHO) International Health Regulations (IHR) and World Organization for Animal Health (OIE)/U.N. Food and Agriculture Organization (FAO) reporting guidelines, and
- Promote the One Health Concept.

The goals and objectives of CBEP international research partnerships are to:

- Goal 1: Support Biosurveillance, Biosafety and Biosecurity (BS&S) Capability Building Efforts
    - Objective 1: Inform and enhance operational biosurveillance strategies and implementation through improved understanding of endemic disease burden and pathogen biology.
    - Objective 2: Institutionalize responsible biorisk management best practices with partner country scientists.
- Goal 2: Engage Partner Country Scientists in Hypothesis-Driven Research
    - Objective 1: Support local, national, and regional priorities for understanding and mitigating human and animal disease risk (e.g., small, focused projects within individual countries linked by broad, integrating projects that include regional partners).
    - Objective 2: Improve international collaborations to survey, detect, characterize, and report disease.
- Goal 3: Promote One Health Initiative
    - Objective 1: Emphasize the nexus of human health, animal health, and the environment, and seek to further understand the mechanisms and factors involved in disease transmission.
    - Objective 2: Advance partner country sustainment of global health security initiatives.
- Goal 4: Foster an International Culture of Responsible and Ethical Conduct in Biological Research
    - Objective 1: Transition to a culture of responsibility and ethical conduct in biological research through thoughtful experimental design and good laboratory practices that result in high-quality data, and active participation in professional societies and the peer-review process.
    - Objective 2: Train partner country researchers to think critically in the pursuit of ethical research and to be competitive in soliciting funding from the international scientific community.

Ultimately, the techniques, procedures, and approaches must be sustainable for the partner country and linked with appropriate training in order to promote global health security, reinforce norms of safe and responsible conduct, obtain timely and accurate insight on current and emerging infectious disease risks, and transform the international dialogue on biological threats.

CBEP research projects are not determined by or limited to specific biological agents, but must be plausibly linked to pathogens of security concern and aimed at measurably supporting threat reduction objectives that:

- Enhance partner country's/region's capability to identify, consolidate, and secure collections of pathogens and diseases of security concern in order to prevent the sale, theft, diversion, or accidental release of such pathogens and diseases.
- Enhance partner country's/region's capability to rapidly and accurately survey, detect, diagnose, and report biological terrorism and outbreaks of pathogens and diseases of security concern in accordance with international reporting requirements.

Region-specific areas of interest are described in CBEP Regional Science Plans. Examples of general CBEP research areas of interest include: Epidemiology (e.g. studies measuring disease prevalence and incidence), Pathogen Biology, Pathogen Characterization, Assay Adaptation and Optimization, Microbial Ecology within a Public Health Context, and Preventative Strategies and Countermeasures. For clarification, medical countermeasure development (i.e., development of diagnostic tools, vaccines, therapeutics) is supported by many other U.S. Government or international agencies and is generally not supported by CBEP; however, research projects may inform medical countermeasure development and support validation and verification testing (e.g., as part of proficiency testing, pilot studies/testing, or exercises, etc.). Additionally, CBEP does ***not*** generally support research with common disease agents such as HIV/AIDS, malaria, and tuberculosis where other U.S. agencies have dedicated missions to do so; however, the program may choose to capitalize on opportunities to leverage research on these diseases to further CBEP goals, for example by testing samples collected under the auspices of other programs. CBEP also **will not** support research which poses risks to the overall threat reduction mission of CBEP, Dual-Use Research of Concern, or related activities (i.e., *in vivo* pathogenicity studies, virulence studies, animal passaging, etc.).

CBEP is interested in collaborative research engagements with foreign partners in any one of the following regions: Countries of the Former Soviet Union (FSU) (specifically, Armenia, Azerbaijan, Georgia, Kazakhstan, and Ukraine), Africa (including, but not limited to, Kenya, Tanzania Uganda, South Africa), Southeast Asia (including, but not limited to, Cambodia, Indonesia, Laos, Malaysia, Philippines, Thailand), and Middle Eastern /South Asian countries (including, but not limited to, Afghanistan, Iraq, India and Pakistan). CBEP encourages proposers to develop projects in conjunction with foreign institutions in CBEP-engaged countries.

1.5.7. **Thrust Area 7—*Fundamental Science for Chemical and Biological Defense:*** Fundamental science for chemical and biological (CB) defense includes science and technology research that advances knowledge in physical and life sciences to defend and counter chemical and biological WMD that could be used against our Nation's warfighters. Fundamental research efforts enable capabilities such as development of improved detection devices for traditional and nontraditional chemical agents; development of diagnostics for existing and emerging infectious disease threats; increasing knowledge and improved capabilities for development of new or improved medical and material countermeasures to CB threats for both pre- and post-exposure scenarios; enhanced personal protection against, modeling of, prevention of, or decontamination of CB threats; and providing effective elimination strategies via non-kinetic approaches for threat agent destruction, neutralization and/or sequestration.

1.6. This BAA, in addition to any amendments issued in conjunction with this BAA, will be posted to the Grant Opportunities Website (https://www.grants.gov), the System for Award Management website (https://sam.gov/), and the DTRA website (https://www.dtra.mil). The

DTRA website is not the official sites; applicants are responsible for monitoring both sam.gov and www.grants.gov. Posted amendments supersede all previous versions of the BAA. Note that topics will be listed in Attachment 1 and will be added/closed with Amendments to this BAA.

1.7. All coordination and communication between applicants and the Government will be conducted using the e-mail address associated with this BAA, specified in Section 7. Applicants should include both the administrative email and the relevant thrust area email address. DTRA will not release employee personal contact information.

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2. AWARD INFORMATION

2.1. Award Types. The full range of flexible procurement instruments available to DTRA are possible results from this announcement, including but not limited to contracts, grants, CAs, and OTAs; however, grants will likely be the predominant procurement instrument. Each of the applicable procurement instruments offer different advantages, liabilities and responsibilities for applicants and the Government.

Applicants must specify in their submittal their recommended approach (e.g. contract, grant, CA, or OTA); however, the Government reserves the right to negotiate and award the types of procurement instruments determined most appropriate under the circumstances. If warranted, portions of resulting awards may be segregated into pre-priced options.

Except for OTAs, the Government actions under this BAA shall adhere to the requirements of the Federal Acquisition Regulation (FAR), Defense Federal Acquisition Regulation Supplement (DFARS) and/or DoD Grant and Agreement Regulations (DoDGARS), as appropriate for the type of instrument. DoDGARs can be accessed online at http://www.ecfr.gov/cgi-bin/text-idx?SID=e5d686f6760f3274b3368f36723fbb7e&mc=true&tpl=/ecfrbrowse/Title32/32CIsubchapC.tpl . See also 32 Code of Federal Regulations (CFR) 22, which can be accessed online at http://www.ecfr.gov/cgi-bin/text-idx?rgn=div5;node=32%3A1.1.1.3.16 . Any assistance instrument awarded under this announcement will be governed by the award terms and conditions, which conform to DoD's implementation of OMB circulars applicable to financial assistance.

On average, DTRA expects to award 30-60 individual awards each year. The predominance of awards will be grants. Payments on grants will be made in advance, subject to the conditions described in 2 CFR 200.305.

2.2. Scope of Awards. Awards may range from focused, exploratory projects with a high risk approach in innovative research in subjects with potential for high impact to C-WMD science to comprehensive programs of innovative research in an interdisciplinary area with potential for high impact.

Awards may have multiple Co-Principal Investigators (Co-PIs) and subawards. Authors of pre-application white papers and proposals should detail the contribution of all Co-PIs and any subawards to the C-WMD scientific impact.

Preference will be given to projects where undergraduate and/or graduate students, and/or postgraduate students are supported by the awards. Details regarding the participation of the students and the value of the research to the students as part of the pre-application white paper

and full proposal are expected. Additional guidance regarding student and/or postgraduate student participation may be provided in the published topics or in communications with the applicant to include the coordination of the abstract or in the debrief summary of the pre-application white paper. Any specific guidance provided in a topic or to an applicant supersedes the information provided herein.

2.3. Subawards. Subawards (subgrants and/or subcontracts) are permitted. Subawards may be used to carry out a portion of the research or efforts. Awards may have multiple subawards. Awards will be made by a single award, e.g., grant or contract, to the lead organization. All subawards are the responsibility of the award recipient; exceptions will not be made.

For submissions made to Thrust Area 6 and associated topics, there is no limitation on subawards. DTRA will review and consider the proposed subawards for all pre-application white papers and proposals on a case-by-case basis. The prime awardee will be responsible for transferring funds to the subawardee. Applicants are reminded that priority is given to projects with the main locus of activity in the region-of-interest, so budgets should be allocated accordingly. Preference will be given to proposals where the subaward component to the region-of-interest partner(s) represents more than half of the award value (as measured in U.S. dollars).

2.4. Award Values. Grants resulting from submissions to Thrust Areas 1-7, including topics associated with these thrust areas, may range from small dollar value (e.g., $25K) up to $1M annually (total, including both direct and indirect costs) depending on the nature and the scope of work. Contracts, CAs, and OTAs will be considered on a case-by-case basis. All awards are subject to the availability of funds. Additional guidance regarding award values may be provided in the published topics or in communications with the applicant to include the coordination of the abstract or in the debrief summary of the pre-application white paper. Any specific guidance provided in a topic or to an applicant supersedes the information provided herein. Funding for participation in this program is highly competitive and the cost of proposed research should strictly be maintained as detailed herein or as indicated in the invitation instructions.

2.5. Period of Performance and Award Structure. Efforts for Thrust Areas 1-7, including topics associated with these thrust areas, may be proposed for up to five (5) years. Awards may be for a base period of one (1) year with four (4) additional years as possible options, a base period of two (2) years with three (3) additional years as possible options, or a base period of three (3) years with two (2) additional years as possible options. Additional guidance regarding award structure may be provided in the published topics or in communications with the applicant to include the coordination of the abstract or in the debrief summary of the pre-application white paper. Any specific guidance provided in a topic or to an applicant supersedes the information provided herein.

Applicants should take care to propose the most logical mix of base and option years for the scope of work. Further, the base period should yield a logical completion point for the work. In cases where option years are proposed, decisions regarding exercising those options will be based on the evaluation of the work accomplished in the base period.

DTRA is flexible on the award structure unless otherwise specified in the published topics or in communications with the applicant to include the coordination of the abstract or in the debrief summary of the pre-application white paper. Applicants should take care to clearly label the tasks and anticipated outcomes for the base and option years in the pre-application white papers

and the proposals. Pre-application white papers and proposals that outline scope and effort for only the base period and do not propose options are also acceptable; however, the Government reserves the right to invite option years for awards that were originally awarded with only a base period.

2.6. The Government Accountability Office, in its report GAO-16-14, WOMEN IN STEM RESEARCH: Better Data and Information Sharing Could Improve Oversight of Federal Grant-making and Title IX Compliance, December 3, 2015, recommended that the DoD collect certain demographic and career information to be able to assess the success rates of women who are proposed for key roles in applications in science, technology, engineering, or mathematics disciplines. To enable this assessment, DTRA will include with each Phase II application package the Research and Related Senior/Key Person Profile (Expanded) form and the Research and Related Personal Data form.

2.7. The Government does not anticipate the need to provide any hardware or software to execute the proposed research. However, DTRA will review and consider any hardware/software requests for all pre-application white papers and proposals on a case-by-case basis.

2.8. The Government reserves the right to fund all, some, or none of the proposals submitted; may elect to fund only part of any or all proposals; and may incrementally fund any or all awards under this BAA. The Government also reserves the right to request applicants make any changes necessary to submitted full proposals to increase the feasibility of making the proposal fundable. Applicants may decline to participate in any revisions to application packages requested by DTRA.

2.9. The Government may offer funding for any full proposals or portions of proposals at any time during the lifetime of this BAA.

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3. ELIGIBILITY INFORMATION

3.1. Pre-application white papers and proposals submitted for this BAA will be considered from the following U.S. and Foreign Institutions as follows:

- Accredited degree-granting colleges, universities, and academic institutions.
- Industrial and commercial entities, including small businesses.
- Not-for-profit entities with a portfolio predominantly in research and foreign government laboratories. Proof of 501(c)(3) status from the Internal Revenue Service may be required. For foreign-based establishments entirely based outside the U.S. and/or its territories, proof of not-for-profit status may be required. Foreign based government laboratory equivalents include those residing in the Ministry of Defense, Ministry of Health, Ministry of Agriculture, Ministry of Education and Science and Food Safety Agencies.

DTRA strongly encourages and may give preference to pre-application white papers and proposals that demonstrate a significant contribution (significant is defined as a minimum of 30% of total value) by one (1) or more universities. Applicants should note that university participation may be mandatory for some published topics. Additional guidance regarding university participation may be provided in the published topics or in communications with the

applicant to include the coordination of the abstract or in the debrief summary of the pre-application white paper. Any specific guidance provided in a topic or to an applicant supersedes the information provided herein.

The following entities may not participate as prime awardees nor furnish Principal Investigators (PIs) in awards made under this BAA but may act as collaborators, including as Co-PIs, and/or subawardees:

- Federal Academic organizations (e.g., Naval Postgraduate School).
- Federal laboratories (including DoD and Department of Energy (DOE)).
- U.S. Government agencies.
- DoD-sponsored Federally Funded Research and Development Centers (FFRDCs) specified in the Defense Federal Acquisition Regulation Supplement (DFARS) 235.017-1 (http://farsite.hill.af.mil/VFDFARA.HTM) and click on 'DFARS Part 35'.
- DOE-sponsored FFRDCs.

Note: Federal laboratories (including DoD and DOE) and FFRDCs are eligible to submit abstracts (when required), pre-application white papers, and proposals in response to the Government Call (HDTRA1-16-24-FRCWMD-Call). However, a FFRDC (other than the DoD FFRDCs specified in DFARS 235.017-1) must have authorization from its sponsoring agency in accordance with FAR 35.017-1. Eligibility requirements under the Call are subject to change. See http://www.dtrasubmission.net and after logging in, follow the link to the 'FY16-24 Fundamental Research to Counter Weapons of Mass Destruction (C-WMD) Government Call'.

3.2. Cost Sharing or Matching. In general, cost sharing or matching is not required for applications to this announcement. However, DTRA reserves the right to require cost sharing or matching on a case-by-case basis. Such instances will be specifically detailed in the published topics or in communications with the applicant to include the coordination of the abstract or in the debrief summary of the pre-application white paper.

3.3. Other. DTRA uses the System for Award Management (SAM) to exclude recipients ineligible to receive Federal awards. SAM can be accessed online at http://sam.gov (Reference 2 CFR 1125).

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4. APPLICATION AND SUBMISSION INFORMATION

4.1. Address to Request Application Package. This announcement contains all information required to submit a pre-application white paper and invited proposal.

4.1.1. The required application packages for the pre-application white papers and for the invited proposals are posted with this announcement. Note that each thrust area (as outlined in Section 1.6) and each topic (as outlined in Attachment 1) has a unique application package posted with this BAA. The application package corresponding to both: a.) the thrust area or topic of interest and b.) the phase, should be used for submission of pre-application white papers and invited full proposals.

4.1.2. The application packages posted to www.grants.gov consist of the forms as detailed in

Table 2.

Form Name	Phase I Pre-Application White Paper	Phase II Invited Proposal
SF-424 (R&R) Application for Federal Assistance Form	Required	Required
RR Budget Form	N/A	Required
R&R Subaward Budget Attachment(s) Form(s)	N/A	If Applicable
Research & Related Senior/Key Person Profile Form (Expanded)	N/A	Required
RR Personal Data	N/A	Required
Research & Related Other Project Information	N/A	Required
Disclosure of Lobbying Activities (SF-LLL)	N/A	If Applicable
Attachments Form	N/A	Required

Table 2: Forms. The instructions for completing each of these forms may be found online at the following web link: http://www.grants.gov/web/grants/form-instructions.html.

4.2. Content and Form of Application Submission. Submissions for this BAA will be conducted in two phases. Phase I is for receipt of pre-application white papers. Phase II is for receipt of invited proposal applications. Invitation to the Phase II proposal submission will be based on the evaluation results of the Phase I pre-application white paper.

4.2.1. The predominance of efforts, including all submissions to the thrust areas and some submissions to topics posted in Attachment 1, as noted within the topic, **must be** coordinated with the relevant technical point of contact (POC) for the appropriate thrust area prior to submission of a pre-application white paper; an e-mail for the DTRA technical POCs for Thrust Areas 1-7 are provided in Section 7. Applicants should note that Thrust Areas 1-5 are not currently accepting abstracts for pre-coordination. Coordination of research ideas and efforts must be accomplished via these email addresses, except in cases where a topic specifically states that pre-coordination is not required, and includes submission of an abstract (recommend less than 250 words) of the proposed project/effort or a paragraph description of the proposed project/effort to the email address in Section 7 and a reply email from the relevant email address in Section 7 with the disposition to the applicant. Pre-coordination may not be accomplished with email addresses other than those listed in Section 7. DTRA will not review white papers without prior coordination. Please note that attachments to e-mails may not be reviewed.

Applicants should note that there is extremely limited funding available for the general thrust areas. Pre-application white papers will only be accepted from the coordinated abstracts under very limited circumstances.

Topics may be posted in Attachment 1 of this announcement that may not require pre-coordination of an abstract. Please review the topics carefully.

4.2.2. Pre-application white papers and invited proposals **must be** submitted electronically

using www.grants.gov and the corresponding application packages linked with this BAA on www.grants.gov (under the "Packages" tab). All applications, including all supporting documents, must be submitted in the English language.

Applicants are responsible for ensuring compliant and final submission of their Phase I pre-application white paper and Phase II invited proposal application. Note that this also applies to applicants using third party systems to submit application packages and attachments. Any submission that does not conform to the requirements outlined in the BAA and in the invitation for proposal may not be reviewed or considered further at the discretion of DTRA.

4.2.3. DTRA will not review any of the following:

- Pre-application white papers that are not pre-coordinated as required
- Pre-application white papers and proposals that are not submitted in the English language.
- Pre-application white papers that are submitted to topics that have been previously closed via an amendment to the BAA.
- Application packages and proposals for Phase II submissions that were not invited.

Exceptions WILL NOT be made under any circumstances.

4.2.4. Phase I Pre-Application White Paper Submission and Content. Each pre-application white paper must address only one thrust area or topic. Each pre-application white paper must use the corresponding thrust area or topic application package.

Each Phase I application package contains the following forms:

Form	Attachment	Action
SF-424 (R&R) Application for Federal Assistance Form	Up to four (4) page white paper	Enter the appropriate information in data fields

Table 3: Phase I Pre-Application White Paper Package Chart.

Each Phase I application package contains the SF 424 (R&R) Application for Federal Assistance. To be considered a complete package, an up to four (4) page white paper is required to be uploaded as an attachment to the SF 424 (R&R).

DTRA-specific instructions for completing the SF 424 (R&R) Application for Federal Assistance are below, general application instructions can be found on www.grants.gov:

- Block 1 – Type of Submission. Applicants should indicate the Phase I submission is a "Pre-Application."
- Block 2.1 – Applicant Identifier. Not applicable.
- Block 3 – Date Received by State. Not applicable.
- Block 3.1 – State Application Identifier. Not applicable.
- Block 5 – Applicant Information. You must provide a Business Office Point of Contact (BPOC) with an e-mail address.
- Block 19 – Authorized Representative. The "signature of AOR" is not an actual signature and is automatically completed upon submission of the electronic application package. Hard copies or email attachments of applications will not be accepted.

- Block 20 – Pre-application. Must be used to attach an up to four (4) page white paper. The white paper itself should provide sufficient information on the research being proposed (e.g., the hypothesis, theories, concepts, approaches, data measurements and analysis, etc.) to allow for an assessment by a technical expert.

Any pages submitted for the white paper that exceed the limit of four pages will not be read or evaluated. A page is defined as 8 ½ x 11 inches, single-spaced, with one-inch margins in type not smaller than 12 point Times New Roman font. The white paper must be provided in portrait layout.

At minimum, the white paper should address the following:

- A project abstract, which should be concise (less than 250 words), provide a summary of the proposed work, and demonstrate relevance to the topic being addressed. The abstract should not contain any proprietary data or markings.
- Potential scientific impact to provide greater knowledge or understanding of the fundamental aspects of phenomena and of observable facts, including how the research contributes to the C-WMD science needs outlined in the thrust area or topic.
- The impact of the research on C-WMD science must be clearly delineated.
- Cost estimate by year and total dollars required to accomplish the research as presented in the white paper (no details or breakout of costs is required).
- Potential team and management plan, including details on student involvement.
- Multidisciplinary white papers should carefully detail each of the institutions/departments involved and the contribution that will be made by each of the investigators.
- Do NOT include corporate or personnel qualifications, past experience, or any supplemental information with the white paper. References may be included within the 4-page limit at the discretion of the applicant; however, extensive references are not required.
- Thrust Area 6 pre-application white papers must also include a description of the extent and duration of the relationship/collaboration between the universities/institutes/entities and/or scientists.
- The thrust area or the topic should be included as a header on the white paper attachment and referenced in the text of the white paper.

4.2.5. Phase I Pre-Application White Paper Re-Submission and Content. On a limited basis a second pre-application white paper may be submitted without pre-coordination of an abstract. These re-submissions will be based on the review of the original pre-application white paper and will be allowed when changes to the project scope, technical approach, and/or cost are envisioned for any potential full proposals. Revised pre-application white papers must conform to the standards for the pre-application white papers detailed in Section 4.2.4.

All submissions should be made with the appropriate Phase I application package which contains the following form:

Form	Attachment	Action
SF-424 (R&R) Application for Federal Assistance Form	Up to four (4) page white paper	Enter the appropriate information in data fields

Table 4: Phase I Pre-Application White Paper Package Chart.

Each Phase I application package contains the SF 424 (R&R) Application for Federal Assistance. To be considered a complete package, an up to four (4) page white paper is required to be uploaded as an attachment to the SF 424 (R&R).

The DTRA-specific instructions for completing the SF 424 (R&R) Application for Federal Assistance are the same as for the original pre-application white paper submission except for the following:

- Block 1 – Type of Submission. Applicants should indicate the Phase I re-submission is a "Changed/Corrected Application."
- Block 4c – Previous Grants.gov Tracking ID. Enter the Phase I Grant ID for the original submission.

At minimum, the revised white paper should address the issues and questions detailed in the debrief summary.

4.2.6. Phase II - Invited Proposal Submission and Content. Each proposal must address only the thrust area or topic for which it was invited. The application package corresponding to the thrust area or topic of interest should be used for submission of invited full proposals.

Each Phase II application package contains the following forms and attachments:

Form	Attachment	Action
SF-424 (R&R) Application for Federal Assistance Form	*N/A*	Enter the appropriate information in data fields
RR Budget Form	Budget Justification for entire performance period	Attach to Section K in budget period one
RR Sub-award Budget Attachment(s) Form (*if applicable*)	Individual sub-award budgets	Attach a separate budget with justification for each sub-award
Research & Related Senior/Key Person Profile Form	PI Biographical Sketch	Attach to Biographical Sketch field
	PI Current/Pending Support	Attach to Current & Pending Support field
	Key Personnel Biographical Sketches	Attach to Biographical Sketch field for each senior/key person
	Key Personnel Current/Pending Support	Attach to Current & Pending Support field for each senior/key person
RR Personal Data Form	*N/A*	Enter the appropriate information in data fields
Research & Related Other Project Information Form	Publically Releasable Proposal Summary/ Abstract	Attach to Block 7 Project Summary/ Abstract

	Project Narrative/Technical Proposal	Attach to Block 8 Project Narrative
Disclosure of Lobbying Activities (SF-LLL) *(if applicable)*	*N/A*	Enter the appropriate information
Attachments Form	Attachment 1 – SOW	Upload as Attachment 1
	Attachment 2 – Quad Chart	Upload as Attachment 2
	Attachment 3 – Supporting Documentation (Thrust Area 6 submissions only)	Upload as Attachment 3

Table 5: Phase II Proposal Package Forms and Attachments.

DTRA reserves the right to consider incomplete application packages and required attachments and to request any missing information via email. Should the applicant fail to provide all the requested information either as part of the www.grants.gov submission or in response to email requests from DTRA, at their discretion, DTRA may not consider the proposal further.

SF 424 (R&R) Application for Federal Assistance: DTRA-specific instructions for completing the SF 424 (R&R) are below. General application instructions can be found on www.grants.gov:

Block 1 – Type of Submission. Applicants should indicate the Phase II submission is an "Application."

Block 2.1 – Applicant Identifier. Not applicable.

Block 3 – Date Received by State. Not applicable.

Block 3.1 – State Application Identifier. Not applicable.

Block 4b – Agency Routing Identifier. Enter the corresponding Phase I Grant ID. If resubmissions were involved, enter the Grant ID for the last submission.

Block 5 – Applicant Information. You must provide a Business Office Point of Contact (BPOC) with an e-mail address.

Block 17 – Regarding Disclosure of Funding Sources. By checking "I Agree" you agree to abide by the following statement: "By signing this application, I certify the proposing entity is in compliance with Section 223(a) of the William M. (Mac) Thornberry National Defense Authorization Act for Fiscal Year 2021 which requires that: (a) the PI and other key personnel certify that the current and pending support provided on the proposal is current, accurate and complete; (B) agree to update such disclosure at the request of the agency prior to the award of support and at any subsequent time the agency determines appropriate during the term of the award; and (c) the PI and other key personnel have been made aware of the requirements under Section 223(a)(1) of this Act. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties. (U.S. code, Title 18, Section 1001)."

Block 19 – Authorized Representative. The "signature of AOR" is not an actual signature and is automatically completed upon submission of the electronic application package.

RR Budget Form: The Research and Related Budget Form provided as part of the application package for the Phase II submission should be filled out in its entirety for each project year proposed. Applicants are responsible for ensuring appropriate, approved rates are used in their budget forms. When notified of selection applicants will be requested to provide their current

rate agreement and the rate agreement of their subcontractor(s), if applicable. Applicants should note that in accordance with 32 CFR 22.205(b), grants shall not provide for the payment of fee or profit to the recipient. Applicants should also carefully review Section 4.5.4 to appropriately evaluate inclusion of Value Added Tax (VAT) or other taxes for assistance awards.

Applicants should plan and budget for travel to accommodate the two meetings outlined below:

- National Conferences/Workshops/Symposia: Applicants are strongly encouraged to attend a nationally recognized conference, workshop, or symposium in the field of research each calendar year (1 at minimum). Research should be presented as soon as adequate data are available to support posters and presentations. Conferences/workshops/symposia should be attended by the PI and students supporting the research, as appropriate.
- Annual Technical Review: Applicants should plan to attend an annual technical program review meeting. For planning purposes the review will be for five days and will be held in Northern Virginia.

Budget Justification: Applicants are required to submit a budget justification. The budget justification should be prepared as outlined in the instructions for the Research and Related Budget and uploaded as an attachment to Section K "Budget Justification" of the Research and Related Budget Form. The budget justification does not have a page limit, but should include sufficiently detailed information for meaningful evaluation. In addition, the budget justification must specifically address subaward costs and type to include the portion of work to be subawarded with a supporting rationale. The budget justification should include a discussion of how the subawardee(s) cost was determined to be fair and reasonable. The budget justification must specifically address VAT and other taxes in accordance with Section 4.5.4.

RR Subaward Budget Attachment(s) Form (if applicable): Detailed cost estimates are required for each proposed subaward. The cost estimate for the subawards should include sufficiently detailed information for meaningful evaluation, including labor rates and indirect cost rates.

Research and Related Senior/Key Person Profile Form (Expanded): The Research and Related Senior/Key Person Profile Form (Expanded) should be completed in its entirety for each of the PIs and Co-PIs on the project. The inclusion of additional personnel is at the discretion of the PI. The Degree Type and Degree Year fields will be used by DoD as the source for career information to assess the success rates of women. In addition to the required fields on the form, applicants should complete these two fields for all individuals that are identified as senior or key persons. For Thrust Area 6 submissions, the PI (and Co-PIs) in the region-of-interest should be included as key personnel.

A biographical sketch is required for each PI and Co-PI on the project. DTRA does not have a preference for the format of the biographical sketch; however, it should be limited to 1 page per person. The biographical sketch should be uploaded as an attachment to the corresponding field on the Research and Related Senior/Key Person Profile Form.

Additionally, a statement of current and pending support must be provided for each of the key personnel (e.g., PI and Co-PI) on the project. This statement must include the following items and requires disclosure of all grants and contracts through which each of the key personnel is currently receiving or may potentially receive financial support:

- A list of all current projects the individual is working on, in addition to any future support the

individual has applied to receive, regardless of the source.

- Title and objectives of the other research projects.
- The percentage per year to be devoted to the other projects.
- The total amount of support the individual is receiving in connection to each of the other research projects or will receive if other proposals are awarded.
- Name and address of the agencies and/or other parties supporting the other research projects.
- Period of performance for the other research projects.

Applicants should note that in accordance with the instructions for completion of the SF 424, checking of Block 17 is required. Further, applicants should note that by checking block 17 and submitting an application package, you agree to abide by the following statement: "By signing this application, I certify the proposing entity is in compliance with Section 223(a) of the William M. (Mac) Thornberry National Defense Authorization Act for Fiscal Year 2021 which requires that: (a) the PI and other key personnel certify that the current and pending support provided on the proposal is current, accurate and complete; (B) agree to update such disclosure at the request of the agency prior to the award of support and at any subsequent time the agency determines appropriate during the term of the award; and (c) the PI and other key personnel have been made aware of the requirements under Section 223(a)(1) of this Act. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties. (U.S. code, Title 18, Section 1001)."

RR Personal Data Form: This form will be used by DoD as the source of demographic information, such as gender, race, ethnicity, and disability information for the PI and Co-PI(s). Each application must include this form with the name fields of the PI and any Co-PI(s) completed; however, provision of the demographic information in the form is voluntary. The demographic information, if provided, will be used for statistical purposes only and will not be made available to merit reviewers. Applicants who do not wish to provide some or all of the information should check or select the "Do not wish to provide" option.

Research and Related Other Project Information Form:

Block 7 – Project Summary/Abstract. To fulfill the requirements of Section 8123 of the Defense Appropriations Act, which states: "The Secretary of Defense shall post grant awards on a public Web site in a searchable format," DTRA will collect and post via the Defense Technical Information Center (DTIC) basic information about all awards made under this BAA. The information posted will include the abstract submitted to Block 7 of this form.

The uploaded project abstract should be less than one page and provide a summary of the proposed work and demonstrate relevance to the topic being addressed. Most importantly, the abstract **must be** written such that the general public may easily understand the potential scientific contribution and the impact of the research. The header of this uploaded document must contain the following statement:

> *"This publically releasable abstract is provided to DTRA for use in fulfillment of Section 8123 of the Defense Appropriations Act and future versions of the same."*

The abstract absolutely must not contain any proprietary data or markings.

Block 8 – Project Narrative (Technical Proposal). The uploaded technical proposal must not exceed 20 pages (including references). If the proposal exceeds 20 pages, only the first 20 pages will be reviewed. A page is defined as 8 ½ x 11 inches, single-spaced, with one-inch margins in type not smaller than 12 point Times New Roman font. The technical proposal must be provided in portrait layout.

The project narrative (technical proposal) must include the following components:

- ***Abstract.*** Should be a technical project abstract that is distinct from the Project Summary/Abstract that is attached to Block 7.
- ***Scope.***
- ***Objective.*** A clear and concise objective of the proposed project.
- ***Background.*** Provide the necessary technical and scientific background to support the scientific and/or technical merit of the proposed project.
- ***Programmatics.*** Describe your organization's management plan for the proposed project; list supporting and collaborating centers, and the roles/responsibilities of each identified academic and/or industrial subcontractor supporting the project. Authors of multidisciplinary proposals must take great care to clearly outline the impact to C-WMD science that is to be gained from the investment and justify the scientific contribution from each investigator.

  Thrust Area 6 narratives must also describe of the extent and duration of the relationship/collaboration between the universities/institutes/entities and/or scientists. Teams with pre-existing collaborative research relationships and those which propose to establish new collaborations will be considered, provided teams can supply documentation to demonstrate that an operational framework exists to support the proposed work. Please see Attachment 3 below for information on the submission of this documentation.
- ***Relevance.*** Describe the relevance of the proposed project in terms of advancing the state of the science and the anticipated scientific impact on capabilities to potentially reduce, eliminate, counter, provide greater knowledge or understanding of the threat, and mitigate the effects of WMD fundamental aspects of phenomena and of observable facts.
- ***Credentials.*** Describe the PI's qualifications and the organization's qualifications to perform the proposed work. Summarize the credentials of the primary performing center, and supporting academic and industrial partners to perform the work. Describe specific examples of equipment and/or facilities available to perform the proposed work. Focus on information directly relevant to the proposed work.
- ***Work to be Performed.*** Provide details of the work to be performed by task and subtask. Tasks must be grouped by project year; base and option years should be clearly labeled. Additional details that are required include the following:
  - ***Sample Repository.*** Thrust Area 6 narratives must also clearly identify how the applicant plans to maintain samples collected during the proposed research effort, along with relevant metadata, for at least 12 months after the project end date. Note that annual sample repository information must be submitted using a DTRA-specified format that will be made available to awardees.
  - ***Protection of Human Subjects.*** For full discussion, see Section 6.2.2. If the proposed

work involves human subjects or the use of human anatomical substances (e.g., biospecimens, blood, tissue, cell lines), either living or post-mortem, applicants are required to: a) justify and b) outline the use, and c) include the source of the human subjects, human biospecimens and/or human data involved in the research. The DTRA Research Oversight Board (ROB) will provide ongoing oversight throughout the duration of the effort to ensure proper approvals are in place. Further information will be required if the proposal is selected for award.

- ***Animal Use.*** For full discussion, see Section 6.2.3. If the proposed work involves the use of animals, applicants are required to: a) justify and b) include detailed information on the use of animals, and c) include the location(s) of where the animal work is to be performed. The DTRA Research Oversight Board (ROB) will provide ongoing oversight throughout the duration of the effort to ensure proper approvals are in place. Further information will be required if the proposal is selected for award.

- ***Performance Schedule.*** Provide a table of tasks and sub-tasks and the duration of performance of each in a Gantt or other suitably formatted chart.
- ***References.*** List any relevant documents referenced.

Disclosure of Lobbying Activities (SF-LLL) Form: The Disclosure of Lobbying Activities Standard Form-LLL, if applicable, should be completed.

Attachments Form: The attachments form should be used to include the following three items with the application:

Attachment 1 – SOW. The SOW does not have a page limit, but should be approximately 3-5 pages in length for incorporation into an award document. The SOW should not contain any proprietary data or markings. Pages should be numbered and the initial page should have a date (document date) shown under the title (the title of the SOW should match that of the proposal).

The proposed SOW must accurately describe the research to be performed. The proposed SOW must also contain a summary description of the technical methodology as well as the task description, but not in so much detail as to make the SOW inflexible. The SOW format/guidance is as follows:

- ***Objective:*** Brief overview of the specialty area. Describe why the research is being pursued and what knowledge is being sought.
- ***Scope:*** Include a statement of what the SOW covers including the research area to be investigated, objectives/goals, and major milestones and schedule for the effort.
- ***Background:*** The applicant must identify appropriate documents, including publications that are applicable to the research to be performed. This section includes any information, explanations, or constraints that are necessary in order to understand the hypothesis and scientific impact on capabilities needed to reduce, eliminate, and counter the threat, and also mitigate the effects of WMD. It may also include previously performed relevant research and preliminary data.
- ***Tasks/Scientific Goals:*** This section contains the detailed description of tasks which represent the research to be performed that are contractually binding. Thus, this portion of the SOW should be developed in an orderly progression and presented in sufficient detail to

establish the methodology and feasibility of accomplishing the overall program goals. The work effort should be segregated by performance period for all tasks to be performed and anticipated milestones realized in that year (e.g., Year 1, Year 2, etc., should be detailed separately). Identify the major tasks in separately numbered sub-paragraphs. Each major task should delineate, by subtask, the research to be performed by year and number each task using the decimal system (e.g., 4.1, 4.1.1, 4.1.1.1, 4.2, etc.). The sequence of performance of tasks and achievement of milestones must be presented by project year and task in the same sequence as in the Project Narrative/Technical Proposal. The SOW must contain every task to be accomplished to include a detailed schedule.

- The tasks must be definite, realistic, and clearly stated. Use "the awardee shall" whenever the work statement expresses a provision that is binding. Use "should" or "may" whenever it is necessary to express a declaration of purpose. Use active voice in describing work to be performed. Do not use acronyms or abbreviations without spelling out acronyms and abbreviations at the first use; place the abbreviation in parenthesis immediately following a spelled-out phrase. If presentations/meetings are identified in your schedule, include the following statement in your SOW: "Conduct presentations/meetings at times and places specified in the grant schedule."
- ***Deliverables:*** Thrust Area 6 **requires** several additional items be included in the SOW. These items are as follows:
    - Submission of annual sample repository information using a DTRA-specified format.
    - Access to all samples collected and data generated during the course of the project, up to and including at least 12 months after the project end date.

Attachment 2 – Quad Chart. The quad chart must be presented on one (1) page. The quad chart must not contain any proprietary data or markings. The quad chart must be provided in landscape layout. The quad chart should be uploaded as "Attachment 2" of the Attachments Form.

Attachment 3 – Supporting Documentation. For Thrust Area 6 proposals ONLY. Thrust Area 6 narratives must also describe an operational framework to support the proposed work. This includes, but is not limited to the following: the extent and duration of the relationship/collaboration between the universities/institutes/entities and/or scientists. Teams with pre-existing collaborative research relationships and those which propose to establish new collaborations will be considered, provided teams can supply documentation to demonstrate that an operational framework exists to support the proposed work. Each of the following should be concatenated into a single document, in the order specified:

- Specific identification of foreign Principal Investigators (PIs) and number of/job title for other members of the foreign research team.
- Detailed description of the relationship between the proposed research project and current research efforts at the foreign institution.
- Description of facilities and any other evidence of suitability of foreign collaborators and sites. In the event that the foreign research component will involve human or other vertebrate animal use, appropriate facilities compliance and certifications documents must be provided. Refer to Section 6.2.2 and Section 6.2.3 for specific information on required

approvals and documentation.

- Foreign PI letter of collaboration describing, at minimum, the suitability of the proposed work with respect to ongoing research efforts at the foreign institution, merit of the proposed collaboration, and the expected mutual benefits.

Protocol Risk Assessment Tool (PRAT). For Thrust Area 6 proposals ONLY. Applicants will be provided a copy of the PRAT file following their invitation to submit a Phase II full proposal and complete it in its entirety for **each** foreign institution participating in the project. Additional instructions for completing the PRAT may be found within the file. The completed PRAT file(s) should be emailed as a Portable Document File (PDF) format to HDTRA1-FRCWMD-A@mail.mil within two (2) weeks of the full proposal submission. **DO NOT** attempt to attach the PRAT(s) to the www.grants.gov submission.

4.2.7. Phase II - Additional Information Requests by DTRA. A revised proposal may be requested based on the review of the original proposal. Revised proposals will be requested when changes to the project scope, technical approach, and/or cost are required before the proposal could be further considered for an award. Applicants whose proposals are of interest to DTRA may be contacted to provide additional information or to make requested revisions prior to the final decision on funding. This request for further information may include revised budgets or budget explanations, revised SOWs, and other information, as applicable, to the proposed award. Additional instructions may be provided in the request for a revised proposal. Applicants who are not responsive to Government requests for information in a timely manner, defined as meeting Government deadlines established and communicated with the request and not making satisfactory updates as requested, may be removed from award consideration. Applicants may also be removed from award consideration if the applicant and the Government fail to negotiate mutually agreeable terms within a reasonable period of time.

Re-submissions should be made with the appropriate Phase II application package for the thrust area or topic of interest and should be completed in accordance with the instructions provided in the notification email.

The DTRA-specific instructions for completing a proposal re-submission are the same as for the original submission, except the SF 424 (R&R) Application for Federal Assistance should be marked as follows:

- Block 1 – Type of Submission. Applicants should indicate the Phase II submission is a "Changed/Corrected Application."
- Block 4b – Agency Routing Identifier. Enter the corresponding Phase I Grant ID.
- Block 4c – Previous Grants.gov Tracking ID. Enter the Phase II Grant ID for the original Phase II submission.

4.2.8. File Format. Documents should be uploaded as a Portable Document File (PDF) format. Perform a virus check before uploading any files to www.grants.gov as part of your application package. If a virus is detected, it may cause rejection of the file.

Do not lock or encrypt any files you upload to www.grants.gov as part of your application package. Movie and sound file attachments will not be accepted.

4.2.9. All submissions must be completely UNRESTRICTED and UNCLASSIFIED;

submissions must not contain Controlled Unclassified Information (CUI), other Proprietary information or export controlled information or be marked as such.

4.2.10. Confirmed Proposal Expiration Date. Applicants requesting contracts must provide written confirmation that holds the proposal, to include proposed costs, firm for 180 days after the submission due date, as included in the invitation to submit a full proposal. This information must be included in the text of the technical proposal.

4.2.11. Withdrawal of Proposals. Proposals may be withdrawn by written notice received at any time before award. Withdrawals are effective upon receipt of notice by the Grants/Contracting Officer via the administrative e-mail address listed in Section 7.

4.3. Submission Dates and Times.

Coordination of abstracts may be accomplished at any time that this BAA is in effect, unless otherwise stated as part of a specific topic. Once an applicant has been notified that a pre-application white paper is welcomed, the white paper should be submitted within 60 days. If the white paper is not submitted within 60 days, DTRA reserves the right to require the applicant to re-initiate the process with another abstract coordination.

Pre-application white papers may be submitted anytime that this BAA is in effect (as long as it occurs within the 60 day window following pre-coordination of the abstract), unless otherwise stated as part of a specific topic. Pre-application white papers may be evaluated at any time after submission and invitations for full proposal submission may occur any time after pre-application white paper evaluation. Note that proposal invitations may be limited to available program funds.

The due date for the Phase II invited proposal submissions will be provided in the letter of invitation. The applicant will not be allowed less than 45 days to prepare a full proposal submission; there is no penalty for early submissions. An extension for submission of the Phase II proposal submission may be requested by emailing the administrative email address in Section 7 prior to the deadline for the proposal submission. Full proposals may be evaluated at any time after submission.

Applicants are responsible for submitting all materials to www.grants.gov. When sending electronic files, the applicant should allow for potential delays in file transfer from the originator's computer server to the www.grants.gov website/computer server, as well as the delay associated with the www.grants.gov validation of applications, which may be up to 48 hours. Applicants are encouraged to submit their proposals early to avoid issues with file transfers, rejection of applications by www.grants.gov, and delays due to high website demand.

Acceptable evidence to establish the time of receipt at the Government office includes documentary and electronic evidence of receipt maintained by DTRA. Applicants should also print, and maintain for their records, the electronic receipt following submission of a proposal to www.grants.gov.

Applicants should note that DTRA uses a system that pulls applications from www.grants.gov en masse, but this system does not mark applications as "retrieved" on www.grants.gov. As a result, when applicants check the status on www.grants.gov the applications will always look like they have not been retrieved by DTRA. Should you require confirmation of receipt by the Agency, you may request such via the administrative email address provided in Section 7. Note that such requests will generally be treated with low priority by the Agency.

Please note 15 U.S.C. 260a establishes daylight saving time as the standard time during the daylight saving period.

If the application package and required attachments are submitted to www.grants.gov after the exact time and date specified in this announcement or in any written communications provided by DTRA, the application may be considered "late" and may not be reviewed.

If an emergency or unanticipated event interrupts normal Government processes so that proposals cannot be submitted to www.grants.gov by the exact time specified by DTRA correspondence, the time specified for receipt of applications will be deemed to be extended to the same time of day specified in the BAA or in the letter of invitation on the first work day on which normal Government processes resume.

4.4. Intergovernmental Review. Not Applicable.

4.5. Other Submission Requirements.

4.5.1. Organizations must have an active System for Award Management (SAM) registration, and Grants.gov account to apply for grants. Creating a Grants.gov account can be completed online in minutes, but SAM registrations may take additional time. Therefore, an organization's registration should be done in sufficient time to ensure it does not impact the entity's ability to meet required application submission deadlines.

All organizations applying online through Grants.gov must register with the SAM and will receive a unique entity identifier (UEI) number. Failure to register with SAM will prevent your organization from applying through Grants.gov. SAM registration must be renewed annually. For more detailed instructions for registering with SAM, refer to: https://www.grants.gov/web/grants/applicants/organization-registration/step-2-register-with-sam.html. Additional information may be found on Grants.gov here: https://www.grants.gov/web/grants/applicants/organization-registration.html

4.5.2. Compliance with Appendix A to 32 CFR 28. All awards require certifications of compliance with Appendix A to 32 CFR 28 regarding lobbying. Proposers are certifying compliance with this regulation by submitting the invited proposal. It is not necessary to include the certification text with your invited proposal. If applicable, proposers should submit the Disclosure of Lobbying Activities (SF-LLL) Form in accordance with Section 4.2.6.

4.5.3. Marking Guidance for Pre-Application White Paper and Invited Proposal and Disclosure of Proprietary Information other than to the Government. The pre-application white papers and invited proposals submitted in response to this BAA may contain technical and other data that the applicant does not want disclosed to the public or used by the Government for any purpose other than application evaluation. Public release of information in any pre-application white paper and invited proposal submitted will be subject to existing statutory and regulatory requirements.

If proprietary information which constitutes a trade secret, proprietary commercial or financial information, confidential personal information, or data affecting national security, is provided by an applicant in a pre-application white paper and/or invited proposal, it will be treated in confidence, to the extent permitted by law, provided that the following legend is included on the front page of the pre-application white paper and/or invited proposal:

“For any purpose other than to evaluate the pre-application white paper and/or proposal, this data

shall not be disclosed outside the Government and shall not be duplicated, used, or disclosed in whole or in part, provided that if an award is made to the applicant as a result of or in connection with the submission of this data, the Government shall have the right to duplicate, use or disclose the data to the extent provided in the agreement. This restriction does not limit the right of the Government to use information contained in the data if it is obtained from another source without restriction. The data subject to this restriction is contained in page(s) _____ of this pre-application white paper and/or proposal."

Any other legend may be unacceptable to the Government and may constitute grounds for removing the pre-application white paper and/or invited proposal from further consideration without assuming any liability for inadvertent disclosure.

The Government will limit dissemination of properly marked information to within official channels. In addition, the pages indicated as restricted must be marked with the following legend:

> *"Use or disclosure of the pre-application white paper and/or proposal data on lines specifically identified by asterisk (*) are subject to the restriction on the front page of this pre-application white paper and/or proposal."*

The Government assumes no liability for disclosure or use of unmarked data and may use or disclose such data for any purpose.

In the event that properly marked data contained in a pre-application white paper and/or invited proposal submitted in response to this BAA is requested pursuant to the Freedom of Information Act (FOIA), 5 U.S.C. § 552, the applicant will be advised of such request and prior to such release of information, will be requested to expeditiously submit to DTRA a detailed listing of all information in the pre-application white paper and/or invited proposal which the applicant believes to be exempt from disclosure under the Act. Such action and cooperation on the part of the applicant will ensure that any information released by DTRA pursuant to the Act is properly identified.

By submission of a pre-application white paper and/or invited proposal, the applicant understands that proprietary information may be disclosed outside the Government for the sole purpose of technical evaluation. DTRA will obtain a non-disclosure agreement from the evaluator that proprietary information in the pre-application white paper and/or invited proposal will only be used for evaluation purposes and will not be further disclosed or utilized.

4.5.4. VAT and Other Taxes in Assistance Awards. Prior to proposal submission, the applicant will require any supplier of goods or services to assess and verify potential VAT, excise duties, and other tax implications to avoid the imposition of such charges with respect to the goods and/or services in question to the maximum extent possible.

In instances where the supplier of goods or services is exempt from the VAT, excise duties, or other taxes or is entitled to claim reimbursement thereof, the taxes must not be included in the proposed cost of the award.

In instances where the supplier of goods or services is not exempt from the VAT, excise duties, or other taxes or is not entitled to claim reimbursement thereof, the applicant must itemize the VAT and/or other taxes in the proposal. Further, applicants are advised that prior to the award of any grant or cooperative agreement, DTRA and the recipient will mutually agree upon the use of DTRA funds for the VAT, excise duties, or other taxes, and project activities may be revised

accordingly. All applicants may include costs in their proposal to pay for VAT costs associated with lodging, meals, and transportation for travel.

4.6. Applicants that Propose Use of Contracts or OTAs.

4.6.1. Recommended Procurement Instrument and Pricing Arrangement. Applicants that propose use of contracts or OTAs must provide a summary of their recommended procurement instrument and pricing arrangement as part of the Phase II proposal. However, the Government reserves the right to negotiate and award the types of instruments determined most appropriate under the circumstances. It is anticipated that most instruments will be grants.

4.6.2. Representations and Certifications. Representations and Certifications must be completed at the time of Phase II submission. The applicant must complete the annual representations and certifications electronically via the System for Award Management (SAM) website at https://www.sam.gov/portal/SAM/#1#1. After reviewing their information, the applicant verifies by submission of the application that the representations and certifications currently posted electronically have been entered or updated within the last 12 months.

4.6.3. Organization Conflict of Interest Advisory. Certain post-employment restrictions on former federal officers and employees may exist, including special Government employees (including but not limited to 18 U.S.C § 207, the Procurement Integrity Act, 41 U.S.C. § 2101 *et.seq*). If a prospective applicant believes that a conflict of interest exists, the situation should be raised to the DTRA Contract/Grant Officer before time and effort are expended in preparing a proposal. All applicants and proposed sub-contractors must therefore affirmatively state whether they are providing scientific, engineering and technical assistance (SETA), advisory and assistance services (A&AS) or similar support, through an active contract or subcontract, to any DoD technical office to include, but not limited to, the Joint Program Executive Office (JPEO), the Office of the Assistant Secretary of Defense for Nuclear, Chemical, and Biological Defense Programs (ASD-NCB), or the Office of the Special Assistant for Chemical and Biological Defense and Chemical Demilitarization Programs (OSA (CBD&CDP)). This information must be included in Technical Proposal of the Phase II full submission. All affirmations must state which office(s) the applicant(s) supports, and identify the prime contract number. Affirmations must be furnished at the time of Phase II full proposal submission. All facts relevant to the existence or potential existence of organizational conflicts of interest, including but not limited to those arising out of activities with the above-referenced organizations, must be disclosed. The disclosure must include a description of the action the applicant has taken or proposes to take to avoid, neutralize, or mitigate such conflict.

4.6.4. Contracts with Subcontracts. Any applicant, other than small businesses, submitting a proposal that exceeds $750,000.00 must submit a subcontracting plan in accordance with FAR 19.704(a) (1) and (2). This information must be included in Technical Proposal of the Phase II full submission. The plan format is outlined in FAR 19.704. Pursuant to Section 8(d) of the Small Business Act (15 U.S.C. § 637(d)), it is the policy of the Government to enable small business and small disadvantaged business concerns to be considered fairly as subcontractors to contractors performing work or rendering services as prime contractors or subcontractors under Government contracts, and to assure that prime contractors and subcontractors carry out this policy.

4.6.5. Limitations on OTAs. Applicants are advised that an Other Transaction for Research Agreement (10 U.S. Code § 2371) or an Other Transaction for Prototype Agreement (10 U.S.

Code § 2371b) will only be awarded if the use of a standard contract or CA is not feasible or appropriate. Applicants are advised that an OTA may only be awarded if there is:

a. At least one nontraditional defense contractor participating to a significant extent in the prototype project, or

b. All significant participants in the transaction other than the Federal Government are small businesses or nontraditional defense contractors; or

c. At least one-third of the total cost of the prototype project is to be paid out of funds provided by the parties to the transaction other than the Federal Government. The cost share should generally consist of labor, materials, equipment, and facilities costs (including allocable indirect costs).

d. Exceptional circumstances justify the use of a transaction that provides for innovative business arrangements or structures that would not be feasible or appropriate under a procurement contract.

e. Although use of one of these options is required to use an Other Transaction for Prototype agreement as the procurement vehicle, no single option is encouraged or desired over the others.

NOTE: For purposes of determining whether or not a participant may be classified as a nontraditional defense contractor or a small business and whether or not such participation is determined to be participating to a significant extent in the prototype project, the following definitions are applicable:

a. "Nontraditional defense contractor" means an entity that is not currently performing or has not performed, for at least the one-year period preceding this solicitation, any of the following for the Department of Defense: any contract or subcontract that is subject to full coverage under the cost accounting standards prescribed pursuant to section 26 of the Office of Federal Procurement Policy Act (41 USCS §§ 1501 et seq.) and the regulations implementing such section; or any other contract in excess of $500,000 under which the contractor is required to submit certified cost or pricing data under section 2306a of this title (10 USCS § 2306a).b. "Small business" means a small business concern as defined under Section 3 of the Small Business Act (15 U.S.C. § 632).

"Participating to a significant extent in the prototype project" means that the nontraditional defense contractor or small business is supplying a new key technology or product, is accomplishing a significant amount of the effort wherein the role played is more than a nominal or token role in the research effort, or in some other way plays a significant part in causing a material reduction in the cost or schedule of the effort or an increase in performance of the prototype in question. NOTE: Applicants are cautioned that if they are classified as a traditional defense contractor, and propose the use of an Other Transaction for Prototype Agreement, the Government will require submittal of both a cost proposal under the guidelines of the FAR/DFARS, and a cost proposal under the proposed Other Transaction for Prototype Agreement, so that an evaluation may be made with respect to the cost tradeoffs applicable under both situations. The Government reserves the right to negotiate either a FAR based procurement contract, or Other Transaction for

Prototype Agreement as it deems is warranted under the circumstances.

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5. APPLICATION REVIEW INFORMATION

5.1. Evaluation Criteria. The four evaluation criteria to be used for responses received to this BAA are as follows:

1. Scientific and Technical Merit. The objective of this criterion is to assess the extent to which the applicant presents ideas that are innovative and/or unique with the potential for high payoff in the science area and details a comprehensive technical approach based on sound scientific principles. Innovation will be judged contextually against the white paper's/proposal's scope, goals, and setting. To the extent possible, the technical risks, including those of biosafety and security, to accomplish the research or project should be identified with appropriate mitigation/management details.

   For Thrust Area 6 white papers/proposals, innovation will also be considered with respect to partner country capabilities.

2. Value to Mission Goals. The objective of this criterion is to assess the extent to which the applicant demonstrates an understanding of the C-WMD research or mission challenges and the contribution to the C-WMD research or mission needs of that thrust area/topic. White papers/proposals must detail research or a project that is responsive to the thrust area/topic as presented in this solicitation. This criterion also addresses the benefit of the proposed effort on enabling knowledge, technology, or capabilities over current methods and/or practices and on the transition potential that is appropriate to the proposed effort. Applicants must also demonstrate an impact of the proposed effort on the institution's ability to perform research relevant to reducing the global WMD threat; and/or to train, through the proposed effort, students and/or partner scientists in science, technology, engineering and/or mathematics.

   Thrust Area 6 white papers/proposals must demonstrate an understanding of the CBEP priorities and mission. As such, the degree to which the proposed collaborations may lead to long-term partner country self-sufficiency and sustainment of the jointly developed capabilities will be considered.

3. Capability of the Personnel and Facilities to Perform the Proposed Effort. The objective of this criterion is to assess the extent to which the applicant's team has the requisite expertise, skills and resources necessary to perform the proposed program. This includes an assessment of the team's management construct, key personnel, facilities and past technical experience in conducting similar efforts of the proposed scope. Applicants must demonstrate that their team has the necessary background and experience to perform this project. Facilities should be detailed with discussion of any unique capabilities pertinent to the research. Subcontractors may include Government facilities or Agencies; however the unique expertise or specialized facilities provided through their inclusion must be clearly presented and the validity of the proposer-Governmental relationship must be clearly documented.

4. Cost Realism Evaluation. The objective of this criterion is to establish that the proposed costs are reasonable, realistic, and justified for the technical approach offered and to assess the applicant's practical understanding of the scope of the proposed effort.

5.2. Review and Selection Process. The pre-application white paper and proposal selection

process will be conducted based upon a technical review as described in the DoDGARs (32 CFR 22.315(c)) and includes the use of non-Government peer-reviewers.

Each pre-application white paper and invited proposal submitted to a general TA will be reviewed on a rolling basis; topic-based submissions will be reviewed as a batch following receipt deadlines. All applications will be reviewed based on the merit and relevance of the specific pre-application white paper/proposal as it relates to the DTRA program, rather than against other pre-application white papers/proposals for research in the same general area.

Pre-application white paper (Phase I) evaluation will be based on the two (2) equally weighted criteria of (1) Technical/Scientific Merit and (2) Value to Mission Goals. The criteria will be scored as Outstanding (O), Good (G), Acceptable (A), Marginal (M) or Unacceptable (U). Any criterion scored as "Unacceptable (U)" will render the pre-application white paper "Not Selectable," and the pre-application white paper will not be considered further.

The full proposal evaluation will be based on the four criteria listed above. Of these, the first two (2) criteria of (1) Technical/Scientific Merit and (2) Value to Mission Goals are equally weighted and more important than the third criterion of (3) Capability of the Personnel and Facilities to Perform the Proposed Effort. These first three criteria will be scored Outstanding (O), Good (G), Acceptable (A), Marginal (M) or Unacceptable (U). The fourth criterion of Cost Realism will be scored as either Acceptable (A) or Unacceptable (U). Any criterion scored as "Unacceptable (U)" will render the proposal "Not Selectable," and the proposal will not be considered further.

Other factors that may be considered are duplication with other research, program balance, past performance and budget limitations. Prior to award, the Government reserves the right to perform a review of past performance. Sources that may be used for past performance review may include the Past Performance Information Retrieval System (PPIRS) and the Federal Awardee Performance and Integrity Information System (FAPIIS). The Government will also evaluate the impact of any proposed limitations to the use of intellectual property (e.g. asserted technical data/computer software restrictions or patents) during the selection and/or negotiation process, and may request additional information from the applicant, as may be necessary, to evaluate the applicant's assertions. Accordingly, proposals may be selected for funding which are not reviewed as highly as others, which are of higher risk and/or which may be of a higher cost.

The Government reserves the right to select all, some, or none of the proposals, or any part of any proposal received in response to this BAA and to make awards without discussions with applicants; however, the Government reserves the right to conduct discussions if determined necessary.

5.3. DTRA anticipates that the total Federal share of awards made under this announcement will be greater than the simplified acquisition threshold over the period of performance (see §200.88 Simplified Acquisition Threshold). Therefore, in accordance with Appendix I to 2 CFR Part 200, Section E.3, this section serves to inform applicant:

i. That DTRA, prior to making a Federal award with a total amount of Federal share greater than the simplified acquisition threshold, is required to review and consider any information about the applicant that is in the designated integrity and performance system accessible through SAM (currently Federal Awardee Performance and Integrity

Information System (FAPIIS)) (see 41 U.S.C. 2313);

ii. That an applicant, at its option, may review information in the designated integrity and performance systems accessible through SAM and comment on any information about itself that a Federal awarding agency previously entered and is currently in the designated integrity and performance system accessible through SAM;

iii. That DTRA will consider any comments by the applicant, in addition to the other information in the designated integrity and performance system, in making a judgment about the applicant's integrity, business ethics, and record of performance under Federal awards when completing the review of risk posed by applicants as described in §200.205 Federal awarding agency review of risk posed by applicants.

iv. For awards that exceed $500,000 over the period of performance, DTRA will employ the additional post-award reporting requirements reflected in Appendix XII—Award Term and Condition for Recipient Integrity and Performance Matters of 2 CFR 200.

5.4. Technical and Administrative Support by Non-Government Personnel. It is the intent of DTRA to use both Government and non-Government personnel to assist with the review and administration of submittals for this BAA. All pre-application white papers and invited proposals may be reviewed by subject matter experts, including, but not limited to, peer reviewers from across the academic and industrial community, as applicable to the research proposed.

Further, participation in this BAA requires DTRA support contractors to have access to pre-application white paper and invited proposal information including information that may be considered proprietary or otherwise marked with restrictive legends Each contract contains organizational conflict of interest provisions and/or includes contractual requirements for non-disclosure of proprietary contractor information or data/software marked with restrictive legends. The applicant, by submitting a white paper or proposal, is deemed to have consented to the disclosure of its information to the aforementioned contractors under the conditions and limitations described herein.

All individuals—including subject matter experts and support contractors—having access to any proprietary data must certify that they will not disclose any information pertaining to this BAA including any submittal, the identity of any submitters, or any other information relevant to this BAA. All applicants to this BAA consent to the disclosure of their information under these conditions.

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6. AWARD ADMINISTRATION INFORMATION

6.1. Award Notices. Applicants will be notified regarding the status of their applications (invitation/non-invitation for full proposals, re-submission of white papers, selection/non-selection for award, etc.) via e-mail to the BPOC listed in Block 5 of the SF-424 and the PI listed in Block 14 of the SF-424 provided at the time of submission. A debrief summary will be provided as part of all notification e-mails.

A notice of selection should not be construed as an obligation on the part of the Government; only duly authorized procurement personnel may commit resources, this will be done by issuing a grant or contract document to the selected applicant. Also, this notification must not be used as

a basis for accruing costs to the Government prior to award. Selected applicants are not authorized to begin work, as any award is subject to successful negotiations (if determined necessary by DTRA) between the DTRA contracting division and the selected organization, and to the availability of funds.

All notifications will be made from notification@dtrasubmission.net. **E-mails to this e-mail address will not be answered or forwarded.**

Applicants must be aware that it is their responsibility to ensure: (1) correct e-mail addresses are provided at the time of submission, (2) this e-mail notification reaches the intended recipient(s), and (3) the e-mail is not blocked by the use of 'spam blocker' software or other means that the recipient's Internet Service Provider may have implemented as a means to block the receipt of certain e-mail messages.

If for any reason there is a delivery failure of these e-mail notices, DTRA will not further attempt to contact the applicants.

6.2. Administrative and National Policy Requirements. All awards require certifications of compliance with national policy requirements. Statutes and Government-wide regulations require some certifications to be submitted at the time of proposal submission. See Section 4.5.2 and Section 4.6.2 for the certification(s) required at the time of submission.

This BAA focuses on fundamental research in a DoD contractual context, which was defined in Section 1.2 of this BAA. Per DoD policy[1], "…products of fundamental research are to remain unrestricted to the maximum extent possible." Furthermore, "The DoD will place no other restrictions on the conduct or reporting of unclassified fundamental research, except as otherwise required by statue [sic], regulation, or Executive Order." As such, fundamental research is normally exempt from controls under the International Traffic in Arms Regulation (ITAR) (22 CFR Parts 120-130) and/or the Department of Commerce regarding the Export Administration Regulations (15 CFR Parts 730-774), but the DoD rule recognizes that there are "rare" situations where export-controlled information or technology may be used in fundamental research that may require a license(s) or restrictions on products.

6.2.1. Export Control Notification. Applicants are responsible for ensuring compliance with any export control laws and regulations that may be applicable to the export of and foreign access to their proposed research. Applicants may consult with the Department of State with any questions regarding the International Traffic in Arms Regulation (ITAR) (22 CFR Parts 120-130) and/or the Department of Commerce regarding the Export Administration Regulations (15 CFR Parts 730-774). Please note that the prime awardee is responsible for monitoring ITAR compliance of all subawardees.

6.2.2. Protection of Human Subjects. If the proposed work involves human subjects or the use of human anatomical substances (e.g., biospecimens, blood, tissue, cell lines), either living or post-mortem, applicants are required to: a) justify and b) outline the use, and c) include the source of the human subjects, human biospecimens and/or human data involved in the research,

[1] Under Secretary of Defense for Acquisition, Technology and Logistics Memorandum, SUBJECT: Contracted Fundamental Research, dated 26 Jun 2008

hereafter referred to as "research."

The DTRA Research Oversight Board (ROB) will provide ongoing oversight throughout the duration of the effort to ensure proper approvals are in place. Further information will be required if the proposal is selected for award. Further information will be required if the proposal is selected for award.

DTRA PMs responsible for the research are required to complete and submit Section A of the DTRA Form 156, available through the DTRA1 Forms Library, to the DTRA Research Oversight Board (ROB) through the ROB Central Mailbox, dtra.belvoir.rd.mbx.research-oversight-board@mail.mil.

Through an Agreement with DTRA and the U.S. Army Medical Research Development Command, Office of Human and Animal Research Oversight(MRDC OHARO), OHARO must review and approve all DTRA funded or supported research prior to the start of the proposed work. This review requirement is in addition to the DTRA ROB review. Therefore, along with the DTRA Form 156, the DTRA PM/STM must complete and submit the MRDC OHARO form titled "USAMRDC_ORP_Proposal Submission_Form" to the DTRA ROB for review of the proposed work. These forms are available through the ROB DTRA1 Sharepoint site, https://dtra1portal.unet.dtra.mil/RD/ROB/default.aspx. Allow up to four months, from date award is submitted to the DTRA ROB, for regulatory review and approval processes. Applicants are to build the review time into their project schedules.

All work under any award made under this BAA involving research must be conducted in accordance with 32 CFR 219, 10 U.S.C. § 980, and DoD Instruction (DoDI) 3216.02, DTRA Instruction (DTRAI) 3216.01, and, as applicable, 21 CFR parts 11, 50, 56, GCP, the International Council for Harmonization (ICH) as well as other applicable federal and state regulations. Contracts must include DFARS clause 252.235-7004 and DTRA Clause 252.223-9002. Other funding vehicles (e.g., grant, OTA) must include similar language. Non-compliance with any provision of this clause may result in withholding of payments under the contract pursuant to the terms and conditions. The Government shall not be responsible for any costs incurred for research involving human subjects prior to protocol approval by the MRDC OHRO and ROB.

It is the responsibility of the PM to ensure performers are cognizant of and abide by the additional restrictions and limitations imposed by the DoD regarding research involving human subjects and human anatomical substances, specifically in regards to vulnerable populations (32 CFR 219 modifications to subparts B-D of 45 CFR 46), recruitment of military research subjects (32 CFR 219), and surrogate consent (10 U.S.C. § 980).

Through the Component Management Plan (CMP), reviewed and approved by USD(R&E), the DTRAI 3216.01 establishes the DTRA Human Research Protection Program (HRPP), and sets forth the policies, defines the applicable terms, and delineates the procedures necessary to ensure DTRA compliance with federal and DoD regulations and legislation governing human subject research, and is managed by the DTRA ROB. The regulations mandate that all DoD activities, components, and agencies protect the rights and welfare of human subjects in DoD funded or supported research, development, test and evaluation, and related activities.

The DTRAI 3216.01 requires that research involving human subjects or human anatomical substances may not begin or continue until the DTRA ROB and MRDC OHRO have reviewed

and approved the proposed work. The requirement to comply with the regulations applies to new starts and continuing research for the life of the project, until closure. The completion of a research project requires closure document (e.g., IRB Final Review submission) submitted to the DTRA ROB and/or the MRDC OHRO.

A study is considered to involve human research subjects if: 1) there is interaction with the subject (even simply talking to the subject qualifies; no needles are required); and 2) if the study involves collection and/or analysis of personal/private information about an individual, or if material used in the study contains links to such information.

A study is considered to use human anatomical substances if it involves human biospecimens such as peripheral blood mononuclear cells, primary cells, blood, saliva, tissue, etc. Commercially available sources (e.g., a vendor, medical facility's discarded materials, research collaborators, biobanks, repositories) of human anatomical substances require review. This includes cadaveric specimens and substances.

Commercially available cell lines are exempt from this definition and do not require review (note: commercially available embryonic cell lines are not exempt, and must be reviewed).

Approval to begin research or to subcontract under the proposed protocol will be provided in writing from the MRDC OHRO and the DTRA ROB Executive Secretary (ES) or Program Manager, in absence of the ROB ES. Both the contractor and the Government must maintain a copy of this approval. Any proposed modifications or amendments to the approved research must be submitted to the DTRA ROB and/or the MRDC OHRO for review and approval. Examples of modifications or amendments to the approved work that would require a new review of the project include, but are not limited to:

- a change of the Principal Investigator (PI);
- a change or addition of an institution (note: review and approval of institution is required),
- elimination or alteration of the informed consent process,
- a change in the human subjects study population (e.g., adding children, active duty, etc.) has regulatory implications
- changes in duration or intensity of exposure to some stimulus or agent;
- changes in the information requested of volunteers, or changes to the use of specimens or data collected;
- changes in perceived or measured risks or benefits to volunteers that require changes to the study,
- a change in the IRB of record;
- a change that could potentially increase risk to human subjects
- significant change in study design (i.e., would prompt significant additional scientific review).

Research pursuant to such modifications or amendments must not be initiated without IRB and OHRO approval except when necessary to eliminate apparent and immediate hazards to the subject(s). All unanticipated problems involving risk to subjects or others (UPIRTSOs),

suspensions, clinical holds (voluntary or involuntary), or terminations of the research by the IRB or regulatory agencies, the institution, the sponsor, or any instances of serious or continuing noncompliance with the federal regulation or IRB requirements, must be promptly reported to the DTRA ROB and/or MRDC OHRO.

Greater than minimal risk research projects lasting more than one year require IRB and OHRO review at least every 365 days, or more frequently as required by the responsible IRB. ROB review and approval is required annually from the date of Section A of the DTRA Form 156, through recertification of the DTRA Form 156. The awardee must provide documentation of continued IRB review of protocols for MRDC OHRO review and approval. Research must not continue without renewed OHRO and ROB approval unless necessary to eliminate apparent and immediate hazards to the subject(s).

6.2.3. Animal Use. If the proposed work involves the use of animals, applicants are required to: a) justify and b) include detailed information on the use of animals, and c) include the location(s) of where the animal work is to be performed. The DTRA Research Oversight Board (ROB) will provide ongoing oversight throughout the duration of the effort to ensure proper approvals are in place. . Further information will be required if the proposal is selected for award.

DTRA PMs responsible for the research are required to complete and submit Section A of the DTRA Form 156, available through the DTRA1 Forms Library, to the DTRA Research Oversight Board (ROB) through the ROB Central Mailbox, dtra.belvoir.rd.mbx.research-oversight-board@mail.mil.

Through an Agreement with DTRA, the Animal Care and Use Review Office (ACURO), a component of the USAMRDC Office of Human and Animal Research Oversight (MRDC OHARO) must review and approve all DTRA funded or supported research involving animal use prior to the start of the proposed work. This review requirement is in addition to the DTRA ROB review. Therefore, along with the DTRA Form 156, the DTRA PM must complete and submit the MRDC OHARO form titled "USAMRDC_ORP_Proposal Submission_Form" to the DTRA ROB for review of the proposed work. This form is available through the ROB DTRA1 Sharepoint site, https://dtra1portal.unet.dtra.mil/RD/ROB/default.aspx. Allow up to four months, from date award is submitted to the DTRA ROB, for regulatory review and approval processes. Applicants are to build the review time into their project schedules.

All work under any award made under this BAA involving the use of animals must be conducted in accordance with DoD Instruction (DoDI) 3216.01, DTRA Instruction (DTRAI) 3216.01, and Army Regulation (AR) 40-33. Provisions include rules on animal acquisition, transport, care, handling, and use in: (i) 9 CFR parts 1-4, Department of Agriculture rules that implement the Laboratory Animal Welfare Action of 1966 (U.S.C. 2131-2156); and (ii) the "Guide for the Care and Use of Laboratory Animals," National Institutes of Health Publication No. 86-23. Contracts must include DFARS Clause 252.235-7002 and DTRA Clause 252.235-9001. Other funding vehicles (e.g., grant, OTA) must include similar language. Non-compliance with any provision of this clause may result in withholding of payments under the contract pursuant to the terms and conditions. The Government shall not be responsible for any costs incurred for research involving animal use prior to protocol approval by the MRDC ACURO and ROB. It is the responsibility of the PM to ensure performers are cognizant of and abide by the additional restrictions and limitations imposed by the DoD regarding animal-use research.

The DTRAI 3216.01 requires that research using animals not begin or continue until the DTRA

ROB and MRDC ACURO have reviewed and approved the proposed work.

Through the DTRA Component Animal Use Management Plan (CAUMP), reviewed and approved by the USD(R&E), the DTRAI 3216.01 establishes the DTRA Animal Use Oversight Program (AUOP), and sets forth the policies, defines the applicable terms, and delineates the procedures necessary to ensure DTRA compliance with federal and DoD regulations and legislation governing research involving animal use, and is managed by the DTRA ROB. The regulations mandate that all DoD activities, components, and agencies protect the care and welfare of animals in DoD funded or supported research, development, test and evaluation and training, and related activities. The requirement to comply with the regulations applies to new starts and continuing research for the life of the project, until closure. The completion of a research project requires closure document (e.g., IACUC Final Review submission) submitted to the DTRA ROB and/or the MRDC ACURO.

The DoD definition of animal is "any living or dead vertebrate animal, including birds, cold blooded animals, rats of the genus rattus and mice of the genus mus." "Dead" is defined as animals killed for the direct purpose of conducing RDT&E or training.

Approval to begin research or to subcontract under the proposed protocol will be provided in writing from the MRDC ACURO and the DTRA ROB Executive (ES) Secretary or the ROB PM, in the absence of the ROB ES. Both the awardee and the Government must maintain a copy of this approval. Any proposed modifications or amendments to the approved research must be submitted to the DTRA ROB and/or the MRDC ACURO for review and approval. Examples of modifications or amendments to the approved protocol that would require a new review of the project include, but are not limited to:

- a change of the Principal Investigator (PI),
- a change or addition of an institution (note: review and approval of institutions is required),
- a change in the duration or intensity of exposure to a stimulus or agent,
- a change in the animal model and/or numbers of animals used,
- a change in the IACUC of record, or
- a significant change to in study design (i.e., would prompt significant additional scientific review).

Research pursuant to such modifications or amendments must not be initiated without IACUC and ACURO approvals.

6.2.4. Biological Defense Research Program (BDRP) Requirements: BioSurety and Select Agent Use.

Proposals must specify what Select Agent work will be conducted at the applicant's facility and what Select Agent work will be performed in other facilities. Proposals also must provide the source of the Select Agent(s), any appropriate registration information for the facilities, and specify the Laboratory Bio-safety Level. All Select Agent work is subject to verification of information and certifications. Further information may be required if the proposal is successful.

For those institutions in which PI's are conducting research with Bio-safety Levels 3 and 4 material, a Facility Safety Plan must be prepared and made available during the project award

phase in accordance with 32 CFR 626.18. For grants awarded to foreign institutions, you must follow either local or U.S. laws (as stated above) depending on which laws provide stronger protection. (DTRA requires that research using Select Agents not begin or continue until DTRA has reviewed and approved the proposed agent use. See URL: https://www.gpo.gov/fdsys/pkg/CFR-2002-title32-vol3/pdf/CFR-2002-title32-vol3-sec626-18.pdf for a copy of 32 CFR 626.18, Biological Defense Safety Program.)

For projects that will employ the use of chemical agents, either neat agent or dilute agent, the offeror must provide approved Facility Standard Operating Procedures that conform to Federal, State and local regulations and address the storage, use and disposition of these chemical materials.

6.2.5. Dual-Use Potential. In accordance with National Science Advisory Board for Biosecurity (NSABB) recommendations, DTRA will not support research that, based on current understanding, can reasonably be anticipated to provide knowledge, information, products, or technologies that could be directly misapplied to pose a significant threat with broad potential consequences to public health and safety, agricultural crops and other plants, animals, the environment, materiel, or national security. Research involving select agents and toxins is within scope of the DTRA mission; however, the use of select agents and toxins in certain experimental categories is considered "dual-use research of concern" (DURC) according to U.S. policy. (http://www.phe.gov/s3/dualuse/Documents/us-policy-durc-032812.pdf) Proposals that contain DURC will not be funded. Dual-use potential will be assessed based on application of the following criteria:

- Use of select agents or toxins. This factor evaluates whether the proposed research involves use of one or more select agents or toxins [as identified by the Select Agent Program under Federal Law (7 C.F.R. part 331, 9 C.F.R. part 121, and 42 C.F.R. part 73)] which pose significant risk of deliberate misuse with potential for mass casualties or devastating effects to the economy, critical infrastructure, or public confidence.
- Scope of proposed experiments. This factor evaluates whether the proposed research involves experiments that will produce, aim to produce, or is reasonably anticipated to produce: (a) Enhanced harmful consequences of the agent or toxin; (b) Disruption of immunity or effectiveness of an immunization against the agent or toxin without clinical or agricultural justification; (c) Conferred resistance by the agent or toxin to clinically or agriculturally useful prophylactic or therapeutic interventions against the agent or toxin, or facilitated ability to evade detection methodologies; (d) Increased stability, transmissibility, or dissemination ability of the agent or toxin; (e) Altered host range or tropism of the agent or toxin; (f) Enhanced susceptibility of a host population to the agent or toxin; or (g) Eradicated or extinct select agents or toxins.

6.2.6. Military Recruiting. This is to notify potential applicants that each award under this announcement to an institution of higher education, with exception of any grants awarded to institutions of higher education entirely located outside the United States and/or its territories, must include the following term and condition: "As a condition for receipt of funds available to DoD under this award, the recipient agrees that it is not an institution of higher education (as defined in 32 CFR 216) that has a policy of denying, and that it is not an institution of higher education that effectively prevents, the Secretary of Defense from obtaining the following for military recruiting purposes: (A) entry to campuses or access to students on campuses; or (B)

access to directory information pertaining to students. If the recipient is determined, using procedures in 32 CFR 216 to be such an institution of higher education during the period of performance of this agreement, and therefore to be in breach of this clause, the Government will cease all payments of DoD funds under this agreement and all other DoD grants and CAs, and it may suspend or terminate such grants and agreements unilaterally for material failure to comply with the terms and conditions of award." 32 CFR 216 may be accessed electronically at http://www.ecfr.gov/cgi-bin/text-idx?SID=ee45add5e352854b7089ce420c7fd0a6&mc=true&tpl=/ecfrbrowse/Title32/32cfr216_main_02.tpl . If your institution has been identified under the procedures established by the Secretary of Defense to implement Section 558 of Public Law 103-337, then: (1) no funds available to DoD may be provided to your institution through any grant, including any existing grant; and (2) your institution is not eligible to receive a grant in response to this BAA. This is to notify potential applicants that each award under this announcement to an institution of higher education, with exception of any grants awarded to institutions of higher education entirely located outside the United States and/or its territories, must include the following clause: 32 CFR 22.520 (DoDGARS 22.520), Military Recruiting and Reserve Officer Training Corps Program Access to Institutions of Higher Education.

6.2.7. Combating Trafficking in Persons. The recipient agrees to comply with the trafficking in persons requirement in Section 106(g) of the Trafficking Victims Protection Act of 2000 (TVPA), as amended (22 U.S.C. 7104(g)).

6.2.8. Reporting Subawards and Executive Compensation. The recipient agrees to ensure they have the necessary processes and systems in place to comply with the reporting requirements of the Transparency Act, as defined at 2 CFR 170.320, unless they meet the exception under 2 CFR 170.110(b).

6.2.9. Representation Regarding the Prohibition on Using Funds under Grants and Cooperative Agreements with Entities that Require Certain Internal Confidentiality Agreements. By submission of its proposal or application, the applicant represents that it does not require any of its employees, contractors, or subrecipients seeking to report fraud, waste, or abuse to sign or comply with internal confidentiality agreements or statements prohibiting or otherwise restricting those employees, contractors, or subrecipients from lawfully reporting that waste, fraud, or abuse to a designated investigative or law enforcement representative of a Federal department or agency authorized to receive such information. Note that: (1) the basis for this representation is a prohibition in section 743 of the Financial Services and General Government Appropriations Act, 2015 (Division E of the Consolidated and Further Continuing Appropriations Act, 2015, Pub. L. 113-235) and any successor provision of law on making funds available through grants and cooperative agreements to entities with certain internal confidentiality agreements or statements; and (2) section 743 states that it does not contravene requirements applicable to Standard Form 312, Form 4414, or any other form issued by a Federal department or agency governing the nondisclosure of classified information.

6.2.10. Prohibition on Covered Telecommunications Equipment or Services. Section 889 of the National Defense Authorization Act (NDAA) for Fiscal Year (FY) 2019 (Public Law 115-232) prohibits the head of an executive agency from obligating or expending loan or grant funds to procure or obtain, extend, or renew a contract to procure or obtain, or enter into a contract (or extend or renew a contract) to procure or obtain the equipment, services, or systems prohibited systems as identified in section 889 of the NDAA for FY 2019.

(a) In accordance with 2 CFR 200.216 and 200.471, a recipient and subrecipient are prohibited from obligating or expending grant funds to:

1. Procure or obtain;
2. Extend or renew a contract to procure or obtain; or
3. Enter into a contract (or extend or renew a contract) to procure or obtain equipment, services, or systems that use covered telecommunications equipment or services as a substantial or essential component of any system, or as critical technology as part of any system. Covered telecommunications equipment is telecommunications equipment produced by Huawei Technologies Company or ZTE Corporation (or any subsidiary or affiliate of such entities).
    - For the purpose of public safety, security of government facilities, physical security surveillance of critical infrastructure, and other national security purposes, video surveillance and telecommunications equipment produced by Hytera Communications Corporation, Hangzhou Hikvision Digital Technology Company, or Dahua Technology Company (or any subsidiary or affiliate of such entities);
    - Telecommunications or video surveillance services provided by such entities or using such equipment; or
    - Telecommunications or video surveillance equipment or services produced or provided by an entity that the Secretary of Defense, in consultation with the Director of the National Intelligence or the Director of the Federal Bureau of Investigation, reasonably believes to be an entity owned or controlled by, or otherwise connected to, the government of a covered foreign country.

(b) In implementing the prohibition under Public Law 115-232, section 889, subsection (f), paragraph (1), heads of executive agencies administering loan, grant, or subsidy programs shall prioritize available funding and technical support to assist affected businesses, institutions and organizations as is reasonably necessary for those affected entities to transition from covered communications equipment and services, to procure replacement equipment and services, and to ensure that communications service to users and customers is sustained.

(c) See Public Law 115-232, section 889 for additional information.

COVERED FOREIGN COUNTRY means the People's Republic of China.

6.3. Reporting. General requirements are provided below; however, each awardee should check the award agreement and its contract data requirements list (CDRLs) and/or terms and conditions to determine the requirements for that specific award.

6.3.1. Annual Reports. Annual Reports will be due no later than 1 July of each year. Awards effective after 31 January will not require an Annual Report until 1 July of the following year. The Annual Report is *not* a cumulative report.

6.3.2. Final Technical Reports. A comprehensive final technical report is required prior to the end of an effort, due on the date specified in CDRLs and/or the terms and conditions of the award document. The purpose of the Final Report is to document the results of the effort. The Final Report *is* a cumulative report.

The final report will always be sent to the Defense Technical Information Center (DTIC) and reports may be available to the public through the National Technical Information Service (NTIS).

6.3.3. Financial Reports. Federal Financial Reports (SF-425) are due no later than 1 July of each year. Grants effective after 31 January will not require a Federal Financial Report until 1 July of the following year.

6.3.4. Foreign Travel Reports. Within thirty (30) days after returning to the United States from foreign travel, the PI may be required to submit an acceptable trip report summarizing the highlights of the trip. For grants, contracts, or OTAs awarded to institutions entirely located outside the United States and/or its territories, this is not required.

6.4. After-the-Award Requirements for *Grants*. Closeout, subsequent adjustments, continuing responsibilities, and collection of amounts due are subject to requirements found in 32 CFR 32.71 – 73 (Institutions of Higher Education, Hospitals, and Other Non-Profit Organizations) and 32 CFR 34.61 – 63 (For-Profit Organizations).

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7. AGENCY CONTACTS

Administrative Correspondence and Questions	HDTRA1-FRCWMD-A@mail.mil
Thrust Area 1: *Science of WMD Sensing and Recognition*	HDTRA1-FRCWMD-TA1@mail.mil
Thrust Area 2: *Network Sciences*	HDTRA1-FRCWMD-TA2@mail.mil
Thrust Area 3: *Science for Protection*	HDTRA1-FRCWMD-TA3@mail.mil
Thrust Area 4: *Science to Defeat WMD*	HDTRA1-FRCWMD-TA4@mail.mil
Thrust Area 5: *Science to Secure WMD*	HDTRA1-FRCWMD-TA5@mail.mil
Thrust Area 6: *Cooperative Counter WMD Research with Global Partners*	HDTRA1-FRCWMD-TA6@mail.mil
Thrust Area 7: *Fundamental Science for Chemical and Biological Defense*	HDTRA1-FRCWMD-TA7@mail.mil

Table 6: Agency Contacts.

7.1. Questions regarding administrative content of this BAA must be addressed to the administrative e-mail address listed above. Applicants should include the relevant thrust area email address.

7.2. Questions regarding technical content of this BAA must be referred to the thrust area email listed above.

DTRA will not release employee personal contact information.

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8. OTHER INFORMATION

Topics from previous periods may or may not be repeated. DTRA will not provide additional information regarding the posting of future topics, including dates for posting, the potential for a topic to be repeated in out years, the potential for similar topics to be posted, and/or topic details in advance of issuance of an amended BAA.

The Post Doc-Topic B spans the technical areas covered by Thrust Areas 1-7 and is ***NO longer accepting pre-application white paper submission. Submissions to the general thrust area descriptions for Thrust Areas 1-7 are closed; abstracts and pre-application white papers submitted to the general descriptions for Thrust Areas 1-7 will NOT be reviewed.

DTRA anticipates that the award(s) made under Post Doc-Topic B will be contracts. Pre-application white papers and proposals submitted to Post Doc-Topic B must have a single lead organization and single submission for the pre-application white paper and the invited proposal. Awards will be made by a single award to the lead institution. Subawards, including all grants and/or contracts, are the responsibility of the award recipient; exceptions will not be made.

Post Doc-Topic B: Postdoctoral Scholars Program (Thrusts 1-7)

*****PRE-APPLICATION WHITE PAPERS FOR THIS TOPIC ARE NO LONGER BEING ACCEPTED.**

Background: The Defense Threat Reduction Agency (DTRA) Postdoctoral Scholars Program started more than 20 years ago and has a proven track-record of success—measured by program participation by excellent professionals and follow-on career decisions. The future of the Postdoctoral Scholars Program remains consistent with the initial program goals of fostering and strengthening long-term strategic partnerships with the scientific community while leveraging the best and the brightest professionals to address critical science and technology challenges of interest to DTRA. This topic seeks a contracted partner(s) to continue the Postdoctoral Program at DTRA.

The benefits to the contracted partner(s) are that of enhanced institutional ability to effectively combat the threat posed by WMD through strengthened relationships with DTRA as well as workforce development. The benefit for the individual program participants is also tremendous. Each Postdoctoral Scholar is exposed to a breadth of information and gains knowledge of current capabilities, critical national security challenges, and the business of government—areas of skill development that are not readily available through many other early career opportunities.

Impact: The overall purpose of this programmatic initiative is to provide advanced research support, technical expertise, and execution capabilities in scientific, technical, and engineering disciplines relevant to the DTRA mission, specifically those that directly enhance the institutional ability to effectively combat the threat posed by WMD.

Objective: To find a contracted partner(s) organization that is capable of providing up to eight Postdoctoral Scholars (as an initial estimate of the program). The overall program contract period of performance will be 60 months (5 years).

Postdoctoral Scholars will possess doctoral degree credentials. They will be American citizens, capable of obtaining security clearances at the Secret level; clearances at higher levels will be considered on a case-by-case basis. They will exhibit such academic, research, and/or professional credentials as to demonstrate a disciplinary “state of the art” focus, flexibility, and innovation in methodology and approach, which will ultimately enhance the mission capabilities of DTRA.

Requirements and needs for the provision and assignment of such Postdoctoral Scholars, as may be selected to participate under this program, will be defined and prioritized by Agency

leadership, as deemed appropriate. The successful applicant(s) to this topic will provide an appropriate process for the provision of suitable candidates with advanced educational credentials and capabilities responsive to the stated requirements and needs.

Science, technology, engineering, and mathematical skills particularly critical and highly desirable to the enhancement of the Agency mission include: nuclear and radiation physics; weapons engineering; structural, electrical, and mechanical engineering; broad-based nano-technological engineering and applications; weapons effects and system response technologies; physics, chemistry, and biological sciences related to detection, characterization, and destruction of WMD materials; medical and pharmaceutical sciences; information technology, modeling, technical editing and publication, data visualization, data science, and advanced computational sciences; social, adversarial, and behavioral modeling, science, and analysis. This is not an exclusive listing, and DTRA reserves the right to amend this skill list as mission requirements warrant.

Postdoctoral Scholars will be assigned to DTRA functions, missions, and projects according to their respective specialties and at such places and locations determined to be in the best interests of the Agency (taking into practicable account individual career and professional needs). These locations may include DTRA facilities, national and/or DoD laboratories, other government facilities, military facilities, and contractor sites. They will serve as independent technical advisors and professional subject matter experts (SME) on scientific, technical, and engineering issues related to the execution of the DTRA mission.

Each Postdoctoral Scholar will be assigned a DTRA Mentor to guide, lead, and ensure the optimal utilization of each participant, ensuring that all assigned Postdoctoral Scholars' duties maximize individual intellectual contributions to the overall enhancement of the Agency's capabilities. The Postdoctoral Scholars will report to these individuals on a regular basis to provide informal status reports, to present feedback, and to obtain guidance on current and future activities. Institutionally provided online library services is an important aspect to the success of the Postdoctoral Scholar.

To ensure the goals of the program are met to the maximum extent possible, as well as minimize disruption on academic and professional careers, a minimum commitment of one calendar year of effort per Scholar appointment is required. Two additional one calendar year extensions may be made available as determined by the DTRA manager and on the desire of the Postdoctoral Scholar to extend.

Postdoctoral Scholars will be expected to perform some travel (both local and non-local) approximately once a month during their assignment to DTRA to maintain their technical proficiency. This travel will include, but is not limited to, national conferences, symposia, workshops, interfacing with their home organizations, and site visits for outreach. Travel may also include, but is not required, one or more international conferences.

Thrust Area 7 has ten (10) topics —Topics M1-M10 —detailed below. Submissions to the general thrust area descriptions for this thrust area in accordance with the requirements detailed in this BAA are also welcome.

- If NOT submitting to one of the specific topic numbers detailed below, use one of the **Thrust Area NO TOPIC** application packages
- If you ARE submitting to one of the specific topic numbers detailed below, use the applicable **Basic Research-Thrust Area 7-Topic M1 to M10** application package

Great care must be taken to use the appropriate application package on www.grants.gov, as the package selection dictates how each submission will be reviewed:

*****BASIC RESEARCH TOPICS M1-M10*****

In accordance with Section 4.2.1, the requirement for abstract pre-coordination is waived for Topics M1-M10; these topics do NOT require pre-coordination of an abstract prior to the submission of pre-application white papers. All other pre-coordination requirements remain in effect.

The pre-application white paper deadline for Topics M1-M10 is 3 February 2023. **PRE-APPLICATION WHITE PAPERS FOR THESE TOPICS MUST BE SUBMITTED BY 11:59 PM (MIDNIGHT) EST ON 3 February 2023.** White papers submitted to Topics M1-M10 may not be considered if they are received after this deadline.

Topics M1-M10 are interested in research projects that span from those that focus on exploratory aspects of a unique problem or approaches to those that involve a comprehensive program with interdisciplinary areas. Consistent across all proposals should be the focus on innovative research with the potential for high impact to C-WMD science.

The following topics are Basic Research topics, and proposals should not be solely written with or marketed to a DoD centric application; the offerer should also present a description of the broader implications of their work to our Nation and the whole of society.

DTRA anticipates that the predominance of awards made under Topics MI-M10 will be grants. Pre-application white papers and proposals submitted to Topics M1-M10 must have a single lead organization and single submission for the pre-application white paper and the invited proposal. Awards will be made by a single award to the lead institution. Sub-awards, including all grants and/or contracts, are the responsibility of the award recipient; exceptions will not be made.

Thrust Area 7, Topic M1: Host Response to Emerging Viral Threats: Discovery of Common Mechanisms for Therapeutic Intervention

Award Amounts for this topic are anticipated to be up to $500,000 per year (total dollar value = direct and indirect costs). In all cases, the proposed award value should be clearly substantiated by the scope of the effort. Further guidance on scope and cost may be provided in each full proposal invitation.

The preferred award structure for this topic is a base period of two (2) years with up to three (3) additional years as possible options. However, pre-application white papers and proposals that outline scope and effort for only the base period and do not propose options are also acceptable. Pre-application white papers and proposals that outline scope and effort for different base period and option combinations may also be considered; however, note that pre-application white

papers and proposals that outline scope and effort that exceed a total of five (5) years will not be considered.

Background: Direct acting antivirals deployed during the COVID-19 pandemic have helped mitigate the effects of the viral infection when administered early in the course of disease; however, the disease processes targeted and induced by SARS-COV2 have been persistent and have contributed to long periods of morbidity in several subsets of the population. The COVID-19 pandemic highlights the need for additional therapeutic strategies, such as host-targeted therapeutics, that are effective in mitigating or reversing symptoms caused by viral infection.

This solicitation seeks research to understand host processes and mechanisms involved in viral infection, replication and disease progression for members of viral families Filoviridae (e.g. Sudan, Marburg, Ebola), Hantaviridae (e.g. Hantaan), Arenaviridae (e.g. Lassa, Machupo), and/or Togaviridae (e.g. VEEV, EEV), which can leveraged to identify common host targets for broad-spectrum (i.e., multi-pathogen) therapeutic intervention.

Impact: If successful, this effort will result in the discovery and validation of host processes and mechanisms common among/within viral families that can be targeted to develop broad-spectrum, host-directed therapeutic medical countermeasures for emerging threats.

Objective: Pre-application white papers and proposals will describe technical approaches to characterize and identify host processes and mechanisms common among/within viral families of interest to the ChemBio Defense Program that are involved in the propagation of viral pathogens and/or are linked to the progression of disease in an infected patient. The approach proposed must include methods for confirming the role of identified targets in viral infection or pathogenesis in more than one orthogonal assay. Experimental methods may include cell cultures, micro-physiological systems, *ex-vivo* systems, and small animal models of disease. The data collected must be documented and formatted such that it can be used by potential collaborators for future development of therapeutic medical countermeasures.

References:

1. Geraghty RJ, Aliota MT, Bonnac LF (2021) Broad-Spectrum Antiviral Strategies and Nucleoside Analogues, Viruses, 13(4):667. doi: 10.3390/v13040667.
2. Lu L, Su S, Haitao Yang H, Shibo Jiang S. (2021) Antivirals with common targets against highly pathogenic viruses, Cell 184(6):1604-1620.
3. García-Cárceles J, Caballero E, Carmen Gil C, Martínez A,(2022) Kinase Inhibitors as Underexplored Antiviral Agents J. Med Chem 65(2):935-954

Thrust Area 7, Topic M2: Exploration of Consumer Non-invasive Brain-Computer Interface (BCI) Technologies for Chemical and Biological Defense (CBD) Applications

Award Amounts for this topic are anticipated to be up to $500,000 per year (total dollar value = direct and indirect costs). In all cases, the proposed award value should be clearly substantiated by the scope of the effort. Further guidance on scope and cost may be provided in each full proposal invitation. Award amounts for this topic are anticipated to be commensurate with the

proposed work involved in exploring consumer non-invasive brain-computer interface for CBD applications, evaluating materials, and other aspects of this program outlined in the metrics below. It is anticipated that teams with varying expertise are required to meet the metrics outlined below.

The preferred award structure for this topic is a base period of one (1) years with up to four (4) additional years as possible options. However, pre-application white papers and proposals that outline scope and effort for only the base period and do not propose options are also acceptable. Pre-application white papers and proposals that outline scope and effort for different base period and option combinations may also be considered; however, note that pre-application white papers and proposals that outline scope and effort that exceed a total of five (5) years will not be considered.

Note: For this topic, awardees will be required to grant the US Government a licensing agreement to all software and or hardware in perpetuity for all Chemical and Biological Defense Program (CBDP) uses.

Background: The battlespace is a complex environment with the six fundamental variables of combat represented by the military acronym METT-TC (Mission, Enemy, Troops, Terrain and weather, Time available, and Civilian considerations). For CBRN operators, the operation variables are further complicated by the threat of operating in contaminated environments. To protect our Warfighters, Mission Oriented Protective Posture (MOPP) is the standard protective gear used when operating in contaminated environments. However, it is known that wearing MOPP gear can cause physical and mental stress as well as reduction in mobility, sensory awareness, attention, alertness, and both gross and fine dexterity [1-3]. The situational uncertainties, physical limitations, and the associated risks can impact awareness of the situation while operating in such a complex and dynamic environment. Yet, effective decision making is built off of situational awareness and the ability to recognize the essence of a given problem and the creative ability to devise a practical solution. These abilities are the products of experience, education, and intelligence [4]. Therefore, the Joint Science and Technology Office Digital Battlespace Management Division (JSTO CBI) is seeking methods that can allow easier access to information while operating to enable comprehensive decision making, and promote the inheritance of knowledge and experience.

JSTO CBI has identified brain-computer interface and the related neurotechnologies as the potential solution. The human brain, formed by nearly 100 billion neurons and 100 trillion connections, is one of the greatest mysteries in science. With the dedication of neuroscientists, it is now common knowledge that these neurons and connections work to process and transmit electrophysiological signals. Jacque Vidal asked in his publication Toward Direct Brain-computer Communication nearly half a century ago [5], “Can these observable electrical brain signals be put to work as carriers of information in man-computer communication or for the purpose of controlling such external apparatus as prosthetic devices or spaceships?”. In this topic, JSTO CBI would like to ask a similar question—can these observable electrical brain

signals be put to use in order to serve our Warfighters in Chem/Bio defense missions and tasks.

A brain-computer interface (BCI) is a communication system that allows human interactions with surroundings without the involvement of peripheral nerves and muscles. Such interaction is achieved by leveraging control signals generated from electroencephalographic activity [6]. BCI can serve to record data from the brain for further decoding to transform the data into meaningful outputs for purposes such as control of external devices (e.g., robotic limb) [7]. Because of the possible external device control, BCI applications in medical fields for restoration purposes are often seen. However, the advancements in neuroscience and BCI is shining light on other applications. This expansion toward other applications is evident with an unprecedented amount of corporate investments in neurotechnologies, such as Nissan's brain-to-vehicle [8], Valve Software's focus on BCI for virtual reality (VR) interaction [9], Facebook's optical BCI investment [10], and Neuralink's focus of bringing neurotechnology to the mass market. Although nascent, this trend is expanding toward applications other than restoration of physical functions.

The combination of BCI, Team Awareness Kit (TAK), and heads-up display technologies are potential tools that can provide enhanced situational awareness to the Warfighters while operating in highly dynamic and hazardous environment as an alternative to commonly seen hand gesture, eye-tracking, or voice controls, allowing Warfighters to have hands on tasks, eyes on target, and minimal risk of exposing their locations. It is desired to explore whether consumer non-invasive BCI can be exploited for human interest detection (HID) [11] and decision making [12, 13] to support CBD situational awareness and mission readiness. JSTO CBI is seeking to investigate the possibility of leveraging consumer non-invasive BCI for human interest detection (HID) in combination with artificial intelligence (AI) or machine learning (ML) models to help reduce cognitive load by reducing amount of information to be projected on head-up displays (HUD). It is also of interest to explore if and how the detected interest can be leveraged to enhance or facilitate decision making. Additionally, we seek to answer the question whether consumer non-invasive BCI can assist understanding the key responses that lead to successful completion of tasks in hazardous and dynamic environments.

Impact: The research in this topic seeks to explore the potential and possibilities of leveraging signals from consumer non-invasive BCI for detecting human interest toward the applications of HUD content control, CBD knowledge inheritance, and decision making. The results of the studies are envisioned to reduce cognitive load and enhance decision making when operating in high-stress, dynamic, and hazardous environments as well as to promote the preservation of critical CBD operational knowledge.

Objective: Pre-application white papers and proposals should describe the development and demonstration of the potential to leverage signals from consumer non-invasive BCI to detect human interest for the purposes of HUD content control to reduce cognitive load, enhance decision making, and preserve and extract critical knowledge or responses from experienced

CBD operators.

Applicants should consider methods that leverage consumer non-invasive BCI and related neurotechnologies for the above mentioned 3 areas. Proposed work focusing on selecting any number of the above listed areas will be considered. Proposed work should employ a phased approach with the following recommended stages of applications:

- Demonstrate capabilities in controlled indoor settings to allow explorations and baselining
- Demonstrate capabilities in controlled outdoor settings to explore influences from outdoor environment
- Demonstrate capabilities in a stressed outdoor environment that simulates a dynamically changing battlefield
- Demonstrate capabilities in a stressed outdoor environment with tasks to simulate the level of complexity to carry out a mission in dynamically changing battlefield

The order and stages of milestones are open to modifications that best support the proposed studies. The number of subjects to be involved in phases should be considered. Applicant should consider IRB and HRPO processes when proposing the research schedule.

Research areas may include, but are not limited to:

- Exploration of applicability of one or multiple consumer non-invasive BCIs for signal extraction
- Exploration of different modalities of consumer non-invasive BCI (e.g. EEG-based, in-ear BCI)
- Development of necessary AI/ML algorithms or models to achieve the goals of HID for HUD content control, achieve enhanced decision making, or preserve critical CDB knowledge

Proposed work may leverage a combination of multiple consumer non-invasive BCI devices. It should be noted that minimum modifications to consumer non-invasive BCI devices are acceptable. However, this topic is not aimed at hardware development and should focus on making use of signals available from consumer non-invasive BCI devices. Applicants are encouraged to consider leveraging TAK variants when reasonable.

It is anticipated that this topic will require teams of researchers with different expertise in brain-computer interface, neurotechnologies, AI/ML, computer science modeling, and extended reality. A justification of the budget will need to be provided to supplement the costs proposed for this effort.

References:

1. FM 3-4. NBC Protection. Department of Army. US Marine Corps. Washington D.C. 29 May 1992.
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4. U.S. Marine Corps (1997). MCDP 1: Warfighting. PCN 142 000006 00
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6. Nicolas-Alonso, L. F. and Gomez-Gil, J. (2012). Brain Computer Interfaces, a Review. Sensors, 12, 1211-1279. https://doi.org/10.3390%2Fs120201211
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8. *Brain-to-Vehicle*. Nissan Motor Coporation https://www.nissan-global.com/EN/INNOVATION/TECHNOLOGY/ARCHIVE/B2V/
9. Hayden, S., (2021), *Valve, OpenBCI & Tobii to Launch VR Brain-computer Interface "Galea" in Early 2022*. Road to VR. https://www.roadtovr.com/valve-openbci-immersive-vr-games/
10. BCI milestone: New research from UCSF with support from Facebook shows the potential of brain-computer interfaces for restoring speech communication. (2021). Facebook Technology. https://tech.fb.com/bcimilestone-new-research-from-ucsf-with-support-from-facebook-shows-the-potential-of-brain-computerinterfaces-for-restoring-speech-communication/
11. Solon, A. J. et. al., (2018). Collaborative Brain-Computer Interface for Human Interest Detection in Complex and Dynamic Settings. 2018 IEEE International Conference on Systems, Man, and Cybernetics. pp. 970-975, doi: 10.1109/SMC.2018.00172.
12. Bhattacharyya, S., Valeriani, D., Cinel, C. et al. Anytime collaborative brain–computer interfaces for enhancing perceptual group decision-making. Sci Rep 11, 17008 (2021). https://doi.org/10.1038/s41598-021-96434-0
13. Poli R, Valeriani D, Cinel C. Collaborative brain-computer interface for aiding decision-making. PLoS One. 2014 Jul 29;9(7):e102693. doi: 10.1371/journal.pone.0102693. MID: 25072739; PMCID: PMC4114490.

Thrust Area 7, Topic M3: Leveraging quantum effects to improve diagnostic and detection capabilities for threat agents

Award Amounts for this topic are anticipated to be up to $500,000 per year (total dollar value = direct and indirect costs). In all cases, the proposed award value should be clearly substantiated by the scope of the effort. Further guidance on scope and cost may be provided in each full proposal invitation. Award amounts for this topic are anticipated to be commensurate with proposed research seeking to characterize, control, or exploit quantum mechanical effects and properties involved in biological interactions for the purpose of improving detection and diagnostic tools. It is anticipated that teams with varying expertise are required to meet the metrics outlined below.

The preferred award structure for this topic is a base period of two (2) years with up to three (3) additional years as possible options. However, pre-application white papers and proposals that outline scope and effort for only the base period and do not propose options are also acceptable.

Pre-application white papers and proposals that outline scope and effort for different base period and option combinations may also be considered; however, note that pre-application white papers and proposals that outline scope and effort that exceed a total of five (5) years will not be considered.

Background: Current diagnostic and detection technologies are largely based on our understanding of biological processes at the macromolecular scale. In order to develop transformative solutions that improve our ability to identify threats and approach the ultimate limit of single cell and single molecule detection in handheld assays, we must seek to understand and leverage knowledge of the processes that occur in the quantum realm. There are numerous ways quantum mechanics can be exploited to improve portable, handheld diagnostic and detection assays including leveraging knowledge gained through quantum biological processes, exploitation of quantum light properties, and the use of quantum materials that interact with biological molecules.

The burgeoning field of Quantum biology seeks to achieve a better understanding of quantum mechanics in biological processes. It involves the study of the influence of non-trivial quantum phenomena which can be explained by reducing the biological process to fundamental physics. Quantum measurements in noisy biological environments pose a formidable challenge, but work in this field has already begun leveraging higher resolution tools and even synthetic biology methodologies to elucidate biological activities that involve quantum effects such as tunneling, coherence, and entanglement. Is it possible that knowledge gained from this work could be leveraged to increased signal amplification or reaction speeds for biological assays?

Diagnostic and detection technologies could also be improved by exploiting the quantum properties of light. Many biological assays, such as optical microscopy, cellular histology, and fluorescence immunoassays use classical light for detection/readout. In contrast to classical light, quantum light can have unique properties, such as temporal/spatial coherence or entanglement that can provide unique advantages, including increased sensitivity, lower light dose, superior spatial resolution, or wider and novel spectral windows of observation. Recent work has brought together quantum physics and biology to show that quantum light can be used to track enzyme reactions in real time. This made it possible to use low illumination without disrupting the enzymes, with the potential to achieve a better sensitivity. This type of work is an important step toward development of quantum sensors for biomedical applications.

Another potential area of exploration is the use of quantum materials to improve sensitivity of assay read out. Here, we define quantum materials as systems where key material properties (such as discrete and quantized fluorescence emission or altered/enhanced electrical conductivity properties) stem from quantum effects such as quantum confinement. This confinement may come in zero dimensional materials (e.g. quantum dots or vacancy centers), 1D materials (such as carbon nanotubes or nanowires) or 2D quantum materials such as graphene. The small and reduced dimension of these materials generally makes them sensitive to their local nano-environments, which have the potential to be used as ultra-sensitive reporters of chem/bio agents and their effects on cellular and human health.

This topic seeks to leverage quantum mechanics to gain better understanding of biological processes and to exploit quantum mechanical effects or properties to develop transformative

solutions for the diagnosis of disease and detection of threat agents.

Impact: This topic encompasses broad topics related to applying quantum mechanics and quantum tools and materials, as well as exploitation of quantum effects to improve diagnostic and detection capabilities for chemical/biological threats. Successful efforts from this topic will provide novel solutions for improving the sensitivity or specificity of diagnostic and detection capabilities.

Objective: Proposals for this topic should address the broad objective of using quantum mechanics to improve diagnostic and detection capabilities. Quantum material proposals for this call should focus on novel materials and synthesis methods, therefore, proposals including established commercial off-the-shelf (COTS) materials will not be considered. Examples of specific research questions that may be addressed include but are not limited to the following:

- What quantum effects play a non-trivial role in biological processes and how can these effects be exploited to improve point of care diagnostic and detection capabilities?
- Can quantum mechanical effects or properties be used to enhance the sensitivity, resolution, or specificity of biological assays in areas important to biological health or chemical/biological agent detection?
- Can quantum mechanical effects or properties be used to extend bio-imaging and sensing to important novel spectral windows difficult to obtain via classical methods?
- Can quantum mechanical effects or properties be used to extend the observation period or increase the spatial resolution of visualizing host-pathogen or host-agent interactions and interventions?
- Can quantum mechanical effects or properties be used to reduce the cost or SWAP (Size, Weight, and Power) requirements for standard biological assays, chemical/biological agent detection, or imaging methods?
- Are there key biological molecule and quantum materials interactions of relevance to chemical and biological agent interactions that could be shaped/manipulated/detected because of quantum interactions?
- Can new quantum materials be developed and exploited to measure parameters of interest to biological health in response to chemical/biological agents?
- Can new quantum materials be developed for chem/bio detection and sensing, measuring key biomolecular properties on spatial or temporal scales un-obtainable via classical methods?

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Thrust Area 7, Topic M4: Let's Get Moving: Surface Agitation and Self-propellant Materials in Liquid Films for Decontamination

Award Amounts for this topic are anticipated to be up to $500,000 per year (total dollar value = direct and indirect costs). Award Amounts for this topic are anticipated to be indicative of the amount of work involved to comprehensively address the objectives of this program outlined below. It is anticipated based on this topic that there will be teams with varying expertise that is required. In all cases, the proposed award value should be clearly substantiated by the scope of the effort. Further guidance on scope and cost may be provided in each full proposal invitation.

The preferred award structure for this topic is a base period of two (2) years with up to three (3) additional years as possible options. However, pre-application white papers and proposals that outline scope and effort for only the base period and do not propose options are also acceptable. Pre-application white papers and proposals that outline scope and effort for different base period and option combinations may also be considered; however, note that pre-application white papers and proposals that outline scope and effort that exceed a total of five (5) years will not be considered.

Background: Decontamination and protection rely on diffusion of toxic chemicals (for example GD, HD, and VX) to active/catalytic sites that will then detoxify those materials. Although highly efficient towards toxic chemical decomposition these active/catalytic materials have the rate limiting step of being controlled by diffusion of the toxic chemical to active sites. Having a reactive or catalytic decontamination technology with the capacity to be "mobile" could impact the ability for the decontaminant to improve efficacy with respect to time (increasing the amount of analyte decontaminated in a specific time) and/or allow for migration of the decontaminant into small spaces where the current dominant mechanism is reliant on chemical diffusion out of these small spaces such as rivets or metal joints on vehicles.

Current research in micromotors, active droplets, small scale/micro-swimmers, and propelled metal organic frameworks (as examples) have explored these "mobile" materials for toxic chemical remediation, detection and neutralization, improved mixing to reduce increase reaction efficiency and also including wastewater remediation of various chemical and biological contaminants. Researchers have also studied mechanisms of particulate self-propellancy and interactions with other particles (e.g. swarm behaviors) and different geometries and chemistries to introduce additional functionalities for detection, monitoring, and decontamination. These technologies have the potential to allow for self-propellancy of the decontamination technology such that the decontaminant could maneuver across surfaces and/or into complex features to allow for self-propellant behavior to/from contaminated areas or surfaces increasing rates of decontamination and getting into complex features where current diffusion based mechanisms

are dominant.

This topic seeks to develop a fundamental understanding of how to develop and integrate components for both self-propellant and decomposition of toxic chemicals (surrogates, simulants or model systems) into one material. Initial studies can be initially demonstrated in solution, with the ultimate goal to minimize the solvent to a liquid film on a surface. This topic also seeks self-propellant behavior through utilizing internal or external stimuli (chemical reactions, attraction/repellency, enzymatic, biologically inspired, light, pH, magnetism, electrostatic, ultrasound, predator/prey relationships or other mechanism) without requiring mechanical stirring or other direct intervention.

Impact: If successful the methods and materials developed during this effort could be developed into a new decontamination technology or integrated with existing platforms to increase rates of surface decontamination and increased diffusion/decontamination rates in areas that are difficult to decontaminate.

Objective: This topic seeks to develop a fundamental understanding of how to develop and integrate components for both self-propellant and decomposition of toxic chemicals (surrogates, simulants or model systems) into one material capable of neutralizing toxic chemicals. Proposals should describe the approach for investigation of the components and integration of these materials for eventual use in or on surfaces or in complex features that will be self-propellant with internal or external additives or other triggers (e.g. chemicals, catalytically propelled, light, pH, magnetic particles, Marangoni effect, thermal, concentration gradients, other particle attraction/repulsion interactions) and reactive or catalytic towards toxic chemicals decomposition/destruction. Proposal designs should include the following:

- Base Period Goals:
    - Proof-of-concept (iterative design, development, fabrication, and characterization) of an active/self-propellant material or self-propellant phase (micromotor, active droplet, etc.) and a catalytic or reactive component for decomposition of surrogates/simulants or a model system for one or more toxic chemicals.
    - Initial studies of mechanisms and kinetics of decomposition with these materials in the model system or for simulants/surrogates including determination of efficacy of static versus self-propellant version of the material in liquid films
    - Initial studies of multiple materials in close proximity (e.g. aggregation potential)
    - Preliminary fundamental studies of self-propellancy and development/use of methods to measure self-propellancy of these materials in liquid films
    - Demonstration of relative efficacy for simulants of static and self-propellant version of the offerors concept and demonstration of self-propellancy of the initial designs in liquid films to adjudicate potential for future work
- Option Period(s) Goals
    - Year 3: Refined studies of mechanisms and kinetics of decomposition with these materials in the model system or for simulants/surrogates ; continued advancement of decontamination rates and improved kinetics of diffusion to/from

these integrated materials on surfaces; continued study of efficacy rates relative to static materials

- Year 4: Determine stability (mechanical, chemical); formal study of behavior of multiple self-propellant materials in close proximity; determination of initial ability for use on actual surface materials or in complex features
- Year 5: Determination of ability to maneuver, traverse longer distances and encounter other materials; determination of efficacy of reaction of simulant/surrogates and efficacy relative to time, determination of chemical decomposition rates relative to static material using DMMP, DIMP, CEES or other simulants.

The selected providers must provide appropriate model systems/reactions for CB defense applications showing promise for compatibility with potential protection and hazard mitigation technologies.

Proposals should focus on one or more toxic chemicals and associated model compounds. It is expected that the awardees will work only with simulant/surrogates and/or model compounds as part of this research. Actual chemical agents should be considered but not proposed as part of this work.

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Thrust Area 7, Topic M5: Medical Countermeasures Against the Aging of Acetylcholinesterase Complexed with Organophosphate Agents

Award Amounts for this topic are anticipated to be up to $500,000 per year (total dollar value = direct and indirect costs). In all cases, the proposed award value should be clearly substantiated by the scope of the effort. Further guidance on scope and cost may be provided in each full proposal invitation. Award amounts for this topic are anticipated to be commensurate with the proposed work involved in developing novel in vitro models. It is anticipated that teams of collaborating organizations with varying expertise maybe required to meet the metrics outlined below.

The preferred award structure for this topic is a base period of three (3) years with up to two (2) additional years as possible options. However, pre-application white papers and proposals that outline scope and effort for only the base period and do not propose options are also acceptable. Pre-application white papers and proposals that outline scope and effort for different base period and option combinations may also be considered; however, note that pre-application white papers and proposals that outline scope and effort that exceed a total of five (5) years will not be considered.

Background: Organophosphate agents (OP) continue to be a threat to the Armed Forces and

agricultural workers, and there are no broad spectrum medical countermeasures for treatment of OP intoxication. The management of OP exposure is further compounded by the phenomenon of "aging," during which the OP-ChE conjugate is dealkylated, and the enzyme inhibition becomes irreversible. Once aging has occurred, existing medical countermeasures become ineffective. The rate of onset of aging is variable **across** OP agents, but can occur fast enough to severely limit treatment options post-exposure.

The structural basis of the aging process for OP-ChE conjugate has been investigated, and protein structural effects seem to be critical to the irreversibility of aging. As early as 2008, X ray diffraction and other analysis have shown that the aging process involves agent O-dealkylation resulting in a negatively charged oxygen "salt bridge" to a proximal histidine in AChE, as well as conformational changes. These insights have not, however led, to progress against the problem of aging.

Since then, there are multiple lines of evidence that the aging phenomenon both is dependent on particular enzyme structural features in order to occur, and imposes structural effects on the enzyme that prevent reactivation. Specifically, aging can be inhibited by both mutations to the AChE and interactions with other molecules that affect protein structural dynamics. On the other hand it is shown that aged OP-AChE conjugate has increased rigidity compared to the native enzyme, which is thought to prevent countermeasure access to the active site and interaction to restore function.

Separately the science of structural biology has advanced significantly since the time of these findings. There are now theoretical and analytical tools for structural analysis that have not yet been fully investigated for their relevance to this problem. Therefore, the intent of this topic is to support novel approaches using emerging structural tools and insights to defeat the problem of OP-AChE aging and to expand the utility of existing OP countermeasures.

Impact: Successful execution of this effort will establish the foundation for novel therapeutic approaches using the prevention of OP-AChE aging as an adjunct or alternative to the standard of care, reactivation of AChE from OP inhibition. As a result, the utility of existing countermeasures will be expanded to a larger therapeutic window after exposure, and exposures to fast-aging OPs that were previously not treatable in practical terms will become more available to medical intervention.

Objective: This program seeks to leverage the known structural aspects of the OP-AChE aging process and the emerging technologies in the prediction, study, and control of protein structure to establish a basis for preventing OP-AChE aging.

Pre-application white papers and proposals should describe the development and demonstration of novel insight into the role of protein structure changes in the OP-AChE aging process as well as approaches to modulating structural effects to prevent or reverse OP-AChE aging.

- Considerations for a responsive proposal can include the following:
- The investigation of a structural basis for restoring catalytic function to aged OP-AChE conjugate.
- The investigation of a structural basis for preventing formation of aged OP-AChE conjugate.
- The targeting of OPs, e.g. with small molecules, to prevent them from being able to age with AChE.

- Mechanistic understanding of any of the above processes.
- Ensuring the mechanisms developed are broadly applicable to a spectrum of OP agents.
- Use of AI/ML and/or microphysiological systems (MPS) (e.g. organ-on-a-chip or human-on-a-chip) to establish and characterize rational approaches to these solutions.
- Integrating the issue of physiological relevance early in the development of these medical solutions, to support their ability to eventually transition to human use.

References:

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Thrust Area 7, Topic M6: In Vitro Model Development of Alphavirus Infection

Award Amounts for this topic are anticipated to be up to $500,000 per year (total dollar value = direct and indirect costs). In all cases, the proposed award value should be clearly substantiated by the scope of the effort. Further guidance on scope and cost may be provided in each full proposal invitation. Award amounts for this topic are anticipated to be commensurate with the proposed work involved in developing novel in vitro models. It is anticipated that teams of collaborating organizations with varying expertise maybe required to meet the metrics outlined below.

The preferred award structure for this topic is a base period of three (3) years with up to two (2) additional years as possible options. However, pre-application white papers and proposals that outline scope and effort for only the base period and do not propose options are also acceptable. Pre-application white papers and proposals that outline scope and effort for different base period and option combinations may also be considered; however, note that pre-application white papers and proposals that outline scope and effort that exceed a total of five (5) years will not be considered.

Background: Neuroinflammation is present in nearly all pathological conditions in the central nervous system (CNS) by either being the primary cause of the condition or as a response to the disruption of homeostasis following disease progression. Microglia and astrocytes become

activated following insult or injury to the CNS. The resulting crosstalk between neurons, astrocytes, and microglia has been shown to play a significant role in the observed neuroinflammatory response including dysregulation of endothelial signaling pathways.

For alphaviruses the pathology surrounding brain inflammation is a significant factor of the disease and is therefore a target for medical countermeasure development. For example, Severe Combined Immunodeficient (SCID) mice survive longer than immune-competent mice suggesting that it is the inflammation that is the major contributor to morbidity, rather than any immune deficiency.

Alphaviruses poorly elicit an innate immune response in cell culture which leads to mischaracterization of in vivo tropisms. In addition, current cell culture models are limited in their ability to observe the effects of membrane-bound or cell proximity-dependent mechanisms and such innate responses. Recent advances in the manipulation of individual cell types and culture systems offers new possibilities to develop cell, tissue, and 3-D models to better represent animal model systems.

Impact: The research explored in this topic seeks to develop new neurotropic cell and tissue based models that better mimic the natural response to infection. Few current neuroinflammatory models are able to capture the important interplay between neurons, astrocytes, and microglia. Thus, there is a need for new, multicellular culture systems that are capable of modeling the impact of crosstalk between different cells in the CNS. This could result in an enhanced understanding of the fundamental regulators of the CNS, increase target identification, better prediction and screening for promising medical countermeasures.

Objective: Pre-application white papers and proposals should describe how their proposal would be used in drug discovery.

Research areas may include, but are not limited to:

- Neurovascular tissues and alphaviruses (VEEV/EEEV/WEEV)
- Characterization of molecular pathways and/or mechanisms of action for alphavirus-induced inflammation.
- Identify common molecular pathways and/or mechanisms of action for alphavirus-induced host response that may be suggestive of novel targets for therapeutic intervention.
- Scaffold based techniques such as hydrogel-based support, polymeric hard material-based support, hydrophilic glass fiber, and organoids
- Scaffold free hanging drop microplates, magnetic levitation, and spheroid microplates with ultra-low attachment coating
- The development of CNS organoids, spheroids, 3D printed microfluidics, methods of improving imaging, automation of liquid handling, and other innovative technologies.

It is anticipated that this topic will require teams of researchers with different expertise in modeling, synthesis and characterization of these materials. A justification of the budget will

need to be provided to supplement the costs proposed for this effort.

Pre-application white papers and proposals may focus on incremental and high risk attempts to develop realistic in vitro models.

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Thrust Area 7, Topic M7: Correlating Multi-omics Changes with Measurable Physiological Responses

Award amounts for this topic are anticipated to be commensurate with the proposed work involved in elucidating the underlying principles for multi-omics and other biomarkers in response to chemical and biological (CB) agent exposure and the ability to use them to quantify human physiological responses. It is anticipated that a multi-disciplinary team is required to meet the metrics outlined below.

The preferred award structure for this topic is a base period of one (1) year with up to two (2) additional years as possible options. However, pre-application white papers and proposals that outline scope and effort for only the base period and do not propose options are also acceptable. Pre-application white papers and proposals that outline scope and effort for different base period and option combinations may also be considered; however, note that pre-application white papers and proposals that outline scope and effort that exceed a total of three (3) years will not be considered.

Background: Infectious disease pathogenesis is often first recognized at the onset of symptoms in an infected individual. A cascade of chemical mediators and cellular pathways trigger the inflammatory response to infection and cause measureable physiological symptoms, such as increased temperature and a change in cardiac rhythms, which are felt by the individual as having a fever and feeling generally unwell.[i] Throughout this response to infection, changes in specific biomarkers have been characterized, to include total white blood cell count (WBC), C-reactive protein (CRP), and procalcitonin (PCT).

Beyond vital signs and biomarkers, multi-omics (transcriptomic, proteomic, and metabolomics) have been increasingly studied to advance the molecular understanding of host-pathogen interactions during viral and bacterial infections. Specifically, multi-omic approaches have been

used to characterize virulence, pathogenicity, and metabolic pathway regulation after infection.[ii]

Similarly, exposure to chemical agents has been shown to cause substantial omics changes, notably significant downregulation of multiple proteins. A proteomic-based study examined the impact of the never agent soman on brain tissue in small animals. The soman-induced proteomic changes impacted key pathways for cell inflammation, metabolism, neurodegeneration, and cell death, among others, and demonstrated that persistent proteomic changes in the brain can cause multiple neurological effects.[iii]

While research on both physiological effects and multi-omics data related to CB agent exposure continue to advance alongside one another, there is limited evidence correlating the two research areas. This topic seeks to determine the feasibility of correlating multi-omics from the course of infection or chemical exposure with specific physiological responses such as *changes* in heart rate, temperature, respiratory rate, and blood pressure.

Impact: DTRA continues to invest in leveraging data collected from wearable devices to provide early warning of CB threat agent exposure[iv], as well as in advancing research on understanding multi-omics effects of CB exposure to aid in improved diagnostics, therapeutics, and medical intervention to minimize warfighter casualties. However, physiological data collected from wearable devices is limited to more common infections, such as influenza and COVID, and does not cover the full breadth of CB threats. By answering fundamental questions on the feasibility of correlating multi-omics data with the physiological response to infection/exposure, it may be possible to extend the current state-of-the-art for wearables-based early warning to encompass additional threat agents. Organ-on-a-chip (OOC) technology provides an opportunity to explore multi-omics changes across a wide array of tissue types in response to different CB threats, which may allow for data collected from multi-omics OOC studies to be leveraged to improve predictive wearable based algorithms specific to CB agents.

Objective: Pre-application white papers and proposals should describe an approach for inferring changes in, and quantifying, if possible, specific physiological responses (e.g., vital signs) as they relate to biomarkers and multi-omics data collected during biological pathogenesis or chemical agent exposure. Applicants should propose access to datasets that include **both** physiological and multi-omics data for the same patient during course of infection or chemical exposure, and may consider leveraging additional Sponsor-provided datasets.

Applicants should demonstrate expertise in the areas below and propose methodology to assess the following:

- Commercial-off-the-shelf (COTS) wearable-based data analysis
    - Understand changes in the physiological response after exposure to CB agents.
    - Propose methodology to compare COTS wearable data with varying temporal resolutions, varying devices, and varying features.
    - Hypothesize key feature importance and probability for correlation to host response to infection.
- Multi-omics data analysis
    - Demonstrate experience with analyzing multi-omics data (e.g., proteomics,

metabalomics, transcriptomics, epigenomics) from blood, serum, interstitial fluid, saliva, or breath for biomarkers in response to disease/infection or exposure to chemical agents
    - Propose methodology for identifying key markers and/or specific cellular pathways following infection or chemical exposures and the intended approach for analyzing the impact of multi-omics changes on physiological response.
- Machine Learning (ML) approaches for analyzing disparate datasets
    - Propose intended ML approaches and demonstrate experience in selecting ideal approaches depending on dataset parameters and intended algorithm development.
- Hypothesis on correlating multi-omics and other biomarkers to vital sign data
    - Propose methodology and anticipated capability to leverage multi-omics data to qualitatively correlate it with changes in physiological responses (e.g., temperature, heart rate).
    - If feasible, expand the qualitative correlation between multi-omics data and the physiological responses to explore if changes in vital signs can be quantified (e.g., actual temperature or heart rate) based on multi-omics data alone.
    - Hypothesize the granularity of any correlation and methodology to assess this.

Final output of this topic should be a report on the feasibility of correlating multi-omics data with the physiological response (e.g., changes in vital signs) to a CB threat exposure. If a qualitative or quantitative correlation can be demonstrated, the methodology and underlying data analysis to support the correlation is an expected deliverable.

Data collections are not a part of this topic, and data proposed must already exist. Responses should discuss availability of or access to in-vivo datasets of omics and vital sign data, and/or expect to be able to Sponsor-provided datasets which include in-vivo omics data and vital sign data for all individuals.

It is anticipated that this topic will require teams of researchers with expertise spanning data analytics, ML and algorithm development, physiology, CB threat agents, and immunology / molecular cell biology. A justification of the budget will need to be provided to supplement the costs proposed for this effort.

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Thrust Area 7, Topic M8: Project MAGNETO, lowering SWaP for Microsensors

Award Amounts for this topic are anticipated to be up to $500,000 per year (total dollar value = direct and indirect costs). In all cases, the proposed award value should be clearly substantiated by the scope of the effort. Further guidance on scope and cost may be provided in each full proposal invitation. Award amounts for this topic are anticipated to be commensurate with the proposed work involved in elucidating the underlying principles for multiferroic materials, evaluating materials, integrating materials into a sensor prototype, computational models to understand the properties of proposed materials, and other aspects of this program outlined in the metrics below. It is anticipated that teams with varying expertise are required to meet the metrics outlined below.

The preferred award structure for this topic is a base period of three (3) years with up to two (2) additional years as possible options. However, pre-application white papers and proposals that outline scope and effort for only the base period and do not propose options are also acceptable. Pre-application white papers and proposals that outline scope and effort for different base period and option combinations may also be considered; however, note that pre-application white papers and proposals that outline scope and effort that exceed a total of five (5) years will not be considered.

Background: A variety of sensors are being researched and developed to detect chemical and biological (CB) threats in ambient conditions. Recently, there has been a paradigm shift in the field towards developing lower size, weight, and power (SWaP) microsensors to detect CB threats. As sensors are being studied to possibly use for detection applications of CB threats, there is a need to address how to smartly store the data gathered from the sensor. Employing a data storage capability that enables magnetoelectrically-coupled multiferroic materials to write electrically (faster, low power) and read magnetically (nondestructive), this integration into a sensor could provide a means to advance innovation into this area, resulting in a smaller bit size and reducing the applied external fields required within the circuity of the sensor. By having a lower demand on power, this will also help reduce the battery power needed to operate the sensor. Collectively, this could lower SWaP to advance microsensor development, enabling technologies that can operate longer in the battlefield due to lower power consumption and smarter data storage. This would equip the Warfighter with state-of-the-art innovative microsensors to support their mission and increase their situational awareness.

However, the challenge is to find single-phase materials exhibiting multiferroic properties, i.e. more than one type of ferroic property, at room temperature or composites merging ferroelectric and ferromagnetic materials. A major step toward making viable magnetoelectric technology would be the ability to alternate ferroelectric states with relatively minor voltages. The discovery of more magnetoelectrically-coupled materials or composites would help in the potential development of such sensors and modernizing technology.

This topic seeks to develop a fundamental understanding of multiferroic materials or composites (characterizing bulk materials and thin films), computational models to investigate the structural, magnetic, and electrical properties of materials and to compare to experimental studies, and

integration of the magnetoelectrically-coupled materials or composites into a sensor prototype. This topic also seeks to evaluate these materials integrated into a sensor prototype during year 5, and if milestones are achieved, this technology will be transitioned into a 6.2 program, supporting the Unconventional Detection Modalities Thrust Area listed in Division CBA's portfolio. This project also seeks to potentially develop a microsensor prototype to detect CWAs, emerging threats, and/or simulants of CWAs, and utilize data storage capacity to collect measurements. Measurements collected by the sensor can be AC impedance, infrared, Raman, photovoltaics, but not limited to.

While the ultimate intention is to integrate these materials into suit-based technologies that detect CB threats when exposed to low levels of emerging threats, CWAs, and/or simulants, the basic research should be focused solely on the magnetoelectrically-coupled multiferroic materials or composites to use as data storage for potential optimization and development of the next generation of microsensors.

Impact: The research explored in this topic seeks to develop a fundamental understanding of multiferroic materials (characterizing bulk materials and thin films), computational models to investigate the structural, magnetic, and electrical properties of materials and to compare to experimental studies, and integration of the magnetoelectrically-coupled materials or composites into a sensor prototype. This could be integrated into suit technology for microsensors that could be used to detect agents and other toxic emerging threats, mitigating risk for operational deployment and increasing the Warfighter's situational awareness. Thus, this technology can help unburden the Warfighter and promote integrated layer defense to support the mission.

Objective: Pre-application white papers and proposals should describe the research, development, and demonstration of the technology. Applicants should keep in mind the technology must be operationally relevant to support the Warfighter's mission and ability to tolerate varying environmental conditions in the battlefield.

Applicants should propose magnetoelectrically-coupled multiferroic materials or composites merging ferroelectric and ferromagnetic materials that will be able to have adequate activity to meet metrics including:

- Multiferroic properties –
    - More than one ferroic property: ferromagnetism, ferroelectricity, ferroelasticity, ferrotoroidicity
        - Antiferromagnetism and weak ferroelectricity will also be considered
    - Minimally magnetoelectric materials
- Characterization profile of the structural, magnetic, and electrical properties –
    - X-ray diffraction for crystal structure identification, phase verification, and Rietveld structure refinement
    - Neutron diffraction for crystal structure and magnetic structure identification as well as determining the atomic positions and bond lengths in the unit cell
    - Zero-field and field cooled susceptibility curves and hysteresis loops to provide information about the magnetism
    - Electrical polarization hysteresis loop to monitor the current density and

ferroelectricity
    - Dielectric constant as a function of temperature to provide insights toward the ferroelectric behavior and magnetoelectric coupling
- Fabrication of thin films –
    - Characterization profile of the structural, magnetic, and electrical properties
    - Investigation of films for data storage capability
- Computational models to help understand properties of materials
- Integration of materials into a sensor prototype as an optional year –
    - Demonstration that sensor can operate for at least an hour (threshold) and up to 12 hours (objective) after powered on.
        - Maintain ability to detect emerging threats, CWAs, and/or simulants for 1 to 12 hours but not necessarily continuously monitoring of agents or simulants
        - Ability to operate in varying environmental conditions, such as low and high relative humidity conditions, low and room temperature conditions, and salty, sandy, and smoky conditions
        - Demonstration of threshold metrics (above) within base period (3 years from award) or prove significant progress towards those goals
    - Require little or no external resources required to operate
- Live warfare agent testing should be strongly considered but not limited to as part of this effort or proposed work.

Ultimately, these materials should be cost-realistic and deployment in varying environmental conditions should be strongly considered.
Research areas may include, but are not limited to:

- Determination (theoretical and experimental) of magnetoelectrically-coupled materials or composites that work in ambient conditions, using external fields, that are capable of storing the data over time to allow for measurements to continue for 1 to 12 hrs.
    - Experimental synthesis and characterization of the materials (bulk materials and thin films) and measurements at the laboratory scale
    - Applicants are encouraged to submit magnetoelectrically-coupled materials or composites for data storage capability and will function at ambient conditions.
    - Applicants are encouraged to evaluate technologies including the potential for detection capability of emerging threats, CWAs, and/or simulants (as vapors or aerosols).
    - Applicants should propose determination of environmental conditions (e.g., light, temperature, relative humidity, salty, sandy, smoky), preferably ambient conditions, for operational relevance of technology.
    - Applicants should propose evaluation of emerging threats, CWAs, and/or simulants (as vapors or aerosols) with these magnetoelectrically-coupled materials or composites.

It is anticipated that this topic will require teams of researchers with different expertise in modeling, synthesis, and characterization of these materials (bulk materials and thin films). A justification of the budget will need to be provided to supplement the costs proposed for this

effort.

Pre-application white papers and proposals should focus on a fundamental understanding of research, development, integration, and demonstration of magnetoelectrically-coupled multiferroic materials or composites to use as data storage and integrate these materials into a sensor prototype aimed to detect emerging threats, chemical warfare agent, and/or simulants for potential use with Warfighter suit of technologies.

References:

1. Eerenstrein, W.; Mathur, N.D.; Scott, J.F. Nature 2006, 442, 759-765.
2. Zvezdin, A.K.; Logginov, A.S.; Meshkov, G.A.; Pyatakov, A.P. Bulletin of the Russian Academy of Sciences: Physics 2007, 71, 1561-1562.
3. Spaldin, N.A.; Fiebig, M. Science 2005, 309, 391-392.
4. Du, Y.; Cheng, Z.X.; Dou, S.X.; Wang, X.L.; Zhao, H.Y.; Kimura, H. Applied Physics Letters 2010, 97, 122502.
5. Azuma, M.; Kanda, H.; Belik, A.A.; Shimakawa, Y.; Takano, M. Journal of Magnetism and Magnetic Materials 200

Thrust Area 7, Topic M9: "Next" Next-Generation Material(s) for Chemical and Biological (CB) Protection and Decontamination

Award Amounts for this topic are anticipated to be up to $200,000 (total dollar value = direct and indirect costs) for a maximum of one (1) year effort.

The preferred award structure for this topic is a base period of a maximum of one (1) year with no option years. Phase I white papers and proposals that outline scope and effort for periods beyond the base period will not be considered.

Award Amounts for this topic are anticipated to be indicative of the amount of work involved in demonstrating feasibility of highly innovative basic research areas that may serve as the basis for advancement of new concepts for chemical and biological protection and decontamination, and other aspects of this program outlined below.

Background: DTRA/JSTO's recent basic research investments relevant to chemical and biological (CB) protection and decontamination have identified materials that demonstrate adsorption, reactivity, selective permeability or a combination thereof. Examples include metal-organic frameworks, metal oxide nanoparticles, reactive polymers, and biomimetic and bioengineered systems. While studies with these material classes continue to evolve, new foundational materials research and discoveries are essential to shape future technological capabilities for the Warfighter.

In order to realize new materials with extraordinary function, the exploration of novel scientific opportunities is desired. These studies should seek to identify scientific breakthroughs that may begin to address technical or logistical challenges associated with materials utilized in current

protection and hazard mitigation technologies. For example, conventional adsorbent materials function by means of both physical adsorption and chemisorption; however the lack of selectivity and reactivity limits the chemical protection that can be attained. Elastomers provide excellent barrier properties but at the trade-off of reduced tactility and moisture vapor transport. Polyfluoroalkyl (PFAS)-based coatings provide a high level of surface repellency, but due to environmental and health impacts, efforts are shifting towards non-PFAS material alternatives. Decontaminants have proven to reduce hazard levels, but materials that can turnover agent efficiently and are compatible with complex surfaces are needed.

Impact: This topic will foster innovative ideas to advance the scientific state-of-the-art, with potential of identifying creative material solutions to modernize CB filtration, decontamination, and protective suit technologies.

Objective: This topic seeks short-term, fundamental research investigations focused on proof-of-concept studies and collection of preliminary data of highly innovative research initiatives in support of the "*next"* next-generation material(s) and concepts for CB protection and decontamination. Example research areas *include but are not limited to:*

- Catalysis and reaction engineering to generate concepts that demonstrate rapid, efficient turnover
- Polymer and soft materials to generate concepts that demonstrate stretchable, repellent barriers
- Hybrid or multifunctional materials to generate concepts that demonstrate a combination of adsorptive, catalytic, and/or repellent properties
- Interface and colloid science to generate concepts that demonstrate the ability to enhance reaction rates, diffusion, transport, and/or mechanical properties
- Computational approaches that provide a new means of screening novel materials, developing quantitative structure-activity relationships and other models, predicting the properties of materials, and performing other materials-related studies

Additional considerations:

- Research must be completed within 12 months of award of the agreement.
- No capital equipment may be purchased under this award.
- Due to the relatively small dollar amount and short-term nature of this award, applicants are encouraged to maximize the benefit derived from this funding by prioritizing labor and employing other cost-saving measures in support of the effort. In particular, applicants are strongly encouraged to contribute as a cost-share or significantly reduce the indirect costs associated with the proposed effort.
- The Phase II project narrative (technical proposal) should reflect the level of work to be performed within 12 months, and emphasize the key tasks leading to proof of idea. Due to the short-term nature of this award, the Phase II project narrative (technical proposal) should not exceed 10 pages (including references), and does NOT require the following components/attachments: programmatics, performance schedule, and quad chart. For budgeting purposes, there is no travel requirement for an annual technical review.
- Under this reward, reporting requirements are reduced to a final technical report only.

References:

1. Mondloch, J.E.; Katz,M.J.; Isley, W.C.; Ghosh, P.; Liao,P.; Bury,W.; Wagner, G.W.; Hall, M.G.; DeCoste, J.B.; Peterson, G.W.; Snurr, R.Q.; Cramer, C.J.; Hupp, J.T.; Farha, O.K. Destruction of chemical warfare agents using metal–organic frameworks. *Nat. Mater.* **2015,** *14*, 512-516.
2. Lasseuguette, E.; Malpass-Evans, R.; Casalini, S.; McKeown, N.B.; and Ferrari, M. Optimization of the fabrication of amidoxime modified PIM-1 electrospun fibres for use as breathable and reactive materials. *Polymer* **2021** *213*, 123205.
3. Jung, D.; Su, S.; Syed, Z.H.; Atilgan, A.; Wang, X.; Sha, F.; Lei, Y.; Gianneschi, N.C.; Islamoglu, T.; Farha, O.K. A Catalytically Accessible Polyoxometalate in a Porous Fiber for Degradation of a Mustard Gas Simulant. *ACS Appl. Mater. Interfaces* **2022**, *14*, 14 16687-16693.
4. Tu,Y.; Samineni, L.; Ren, T.; Schantz, A.B.; Song, W.; Sharma, S.; Kumar; M. Prospective applications of nanometer-scale pore size biomimetic and bioinspired membranes. *J. Membr. Sci.* **2021**, *620*, 118968.
5. McEntee, M.; Gordon, W.O.; Balboa, A.; Delia, D.J.; Pitman, C.L.; Pennington, A.M.; Rolison, D.R.; Pietron, J.J.; DeSario, P.A. Mesoporous Copper Nanoparticle/TiO2 Aerogels for Room-Temperature Hydrolytic Decomposition of the Chemical Warfare Simulant Dimethyl Methylphosphonate. *ACS Appl. Nano Mater.* 2020 *3* (4), 3503-3512.
6. Li, T.; Tsyshevsky,R.;McEntee, M.; Durke, E.M.; Karwacki, C.; Rodriguez, E.E.; Kuklja, M.M. Titania Nanomaterials for Sarin Decomposition: Understanding Fundamentals. *ACS Appl. Nano Mater.* 2022**,** *5* (5), 6659-6670.
7. S.1605-117th Congress (2021-2022): National Defense Authorization Act for Fiscal Year 2022, Section 347. (2021, December 27). https://www.congress.gov/bill/117th-congress/senate-bill/1605/text

Thrust Area 7, Topic M10: Leveraging Protein Structure Insights and Allosteric Effectors of MCM Targets

Award Amounts for this topic are anticipated to be up to $500,000 per year (total dollar value = direct and indirect costs). Award amounts for this topic are anticipated to be indicative of the amount of work involved to comprehensively address the objectives of this program outlined below. It is anticipated based on this topic that there will be teams with varying expertise that is required.

The preferred award structure for this topic is a base period of three (3) years with up to two (2) additional years as possible options with options being dependent on meeting benchmarks established in the proposal and statement of work. Phase I white papers and proposals that outline scope and effort for only the base period and do not propose options are acceptable, but note if

option(s) are not proposed they will not be granted at a later date. Note that efforts that propose exceeding a total of five (5) years will not be considered.

Background: Development of safe and effective pre-exposure prophylactic or post-exposure therapeutic medical countermeasures (MCMs) to counter the adverse effects of CWAs (including blister agents, nerve agents, pharmaceutical based agents and other emerging chemical threats) is necessary to protect the warfighter and maintain Force Lethality in CWA-contested environments.

The full complement of protein receptors, metabolic pathways, and tissue distribution of receptor targets of chemicals of concern are not well understood. The improved understanding of the identity, abundance, and tissue-specific distribution of receptors in humans and animal models affected by various chemicals of concern will provide a significantly improved profile to facilitate enhanced medical countermeasure development and identify receptors that are impacted by more than one class of existing and emerging chemical threats.

In order to develop more effective and broadly acting medical countermeasures there is a need to identify host-related biomarkers and biochemical pathways that overlap amongst various threat exposures.

There is also a need to implement a state-of-the-art computational approach, including artificial-intelligence (AI) and machine-learning (ML) based predictive methods that will prioritize specific protein targets for further experimental validation. Use of experimental and/or predicted protein structures to discover novel receptors for CWAs (including blister agents, nerve agents, pharmaceutical based agents and other emerging chemical threats) will help to determine the basis for potential new CWA -protein interactions that can include active sites, ligand binding sites and/ or allosteric sites.

Impact: This approach can leverage protein structure prediction capabilities to identify novel protein targets that are most likely to be functionally impacted by CWA interactions. There are breakthroughs happening in the understanding of protein structure, through both analytical methods and AI- and ML-based predictive methods.

An in-depth understanding of the nature of the molecular recognition/interaction is also of great importance in facilitating the discovery, design, and development of new MCMs.

This approach also enables prioritization of the most biologically meaningful proteins for further study and development of MCMs.

Objective: CWAs continue to be a threat to the warfighter, against which there are no broad spectrum medical countermeasures.

Identification of overlapping biomarkers and biochemical pathways that are affected by exposure to various types of chemical agent threats can help with development of broad-spectrum, potentially cross-toxidromic, medical countermeasures.

The proposal should include one or several of the following approaches, as applicable:

- Enable discovery and quantification of both known and novel (e.g., off-target) receptors of CWAs to allow for development of novel MCMs for broad-spectrum prophylactic and/or therapeutic interventions.

- Use of multi-omic approaches and other pathway analysis to identify nodes of convergence for identifying targets for cross-toxidromic MCM development.
- Use of AI/ML and/or microphysiological systems (MPS) (e.g. organ-on-a-chip or human-on-a-chip) to identify MCMs that can be used to treat multiple chemical toxidromes, allowing for broad-spectrum chemical agent MCMs to enable a suite of MCMs that addresses CWAs (including blister agents, nerve agents, pharmaceutical based agents and other emerging chemical threats).
- Leverage novel protein structure prediction capabilities to identify novel protein targets that are likely to be impacted by CWA interactions to help prioritize specific protein targets for further experimental validation.
- Identify common molecular pathways and/or mechanisms of action for adverse effects resulting from exposure to a broad range of chemicals (e.g., blister agents, organophosphorus compounds, opioids, non-opioid sedatives and other emerging chemical threats) in order to identify new areas for broad-spectrum, cross-toxidromic pre-exposure prophylactic and/or post-exposure therapeutic interventions.
- Include mapping of receptors and enzymes across various CWA-relevant laboratory animal models to allow for more relevant animal model selection in medical countermeasure development.

The CWAs (including blister agents, nerve agents, pharmaceutical based agents and other emerging chemical threats) of interest may include, but are not limited to, organophosphorus compounds, opioids and non-opioid sedatives.

References:

1. Convention on the Prohibition of the Development, Production, Stockpiling and use of Chemical Weapons and Destruction, Technical Secretariat of Organization for Prohibition of Chemical Weapons, The Hague, accessible through internet. 1997, https://www.opcw.org/about-us/history.
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3. Prentiss AM. Chemicals in warfare. New York: McGraw-Hill Book Company; 1937. p. 579
4. May LT, Leach K, Sexton PM, Christopoulos A (2007). "Allosteric modulation of G protein-coupled receptors". Annual Review of Pharmacology and Toxicology. 47: 1–51.
5. Jianlin Cheng, Allison N Tegge, Pierre Baldi (2008). “Machine learning methods for protein structure prediction”. IEEE Reviews in Biomedical Engineering (Volume: 1): 41-49
6. Sheela Kolluri, Jianchang Lin, Rachael Liu, Yanwei Zhang, and Wenwen Zhang (2022). “Machine Learning and Artificial Intelligence in Pharmaceutical Research and Development: a Review”. AAPS J. 24(1): 19.
7. Abdel-Magid AF (2015). "Allosteric modulators: an emerging concept in drug discovery". ACS Medicinal Chemistry Letters. 6 (2): 104–7.

ATTACHMENT 2: INTELLECTUAL PROPERTY

(Applies to FAR Contracts & OTAs)

Applicants must describe any limitations on the use of any intellectual property (patents, inventions, trade secrets, copyrights, trademarks, technical data or computer software) that will impact the offeror's performance of the contract or impact the Government's subsequent use of any deliverable under the contract. In particular, the applicant must describe the intellectual property in sufficient detail and describe the limitations on its use (potential patent licenses required by the Government, data assertions of the offeror or any subcontractor, etc.) and describe how the Government can accomplish the stated objectives of this BAA with the limitations described or proposed by the offeror.

Patents. Applicants must list any known patents, patent applications, or inventions which the offeror may be required to license in order to perform the work described in the Applicant's proposal, or which the Government may be required to license to make or use the deliverables of

the contract should the Applicant's proposal be selected for award. For any patent, patent application or invention listed, the Offeror must provide the invention title, a summary of the invention, patent number, patent application publication number, or provisional patent application number, and indicate whether the offeror is the patent or invention owner. If a patent or invention is in-licensed by the offeror, identify the licensor.

If any listed patent, patent application or invention is owned or licensed by the applicant, the applicant must provide a statement, in writing, confirming that it either owns or possesses the appropriate licensing rights to patent, patent application or invention to perform the work described in the proposal and/or to grant the Government a license to make or use the deliverables for this program. If any listed patent, patent application or invention is not owned or licensed by the applicant, then the applicant must explain how it will obtain a license, how the Government may obtain a license and/or whether the offeror plans to obtain these rights on behalf of the Government.

Be advised that no patent, patent application, or invention disclosure will be accepted if identified in the Data Rights Assertion list. **The list of patents, patent applications, and inventions of this section must be a separate list from the Data Rights Assertion list.**

Government rights in any technology that might be invented or reduced to practice under the contract are addressed in the patent rights clause to be included in the contract.

Data Rights. Applications submitted in response to this BAA shall identify, to the extent known at the time an offer is submitted to the Government, the technical , the technical data, or computer software that the Offeror, its subcontractors or suppliers, or potential subcontractors or suppliers assert should be furnished to the Government with restrictions on use, release, or disclosure, in accordance with DFARS 252.227-7017, Identification and Assertion of Use, Release or Disclosure Restrictions, and DFARS 252.227-7028, Technical Data or Computer Software Previously Delivered to the Government. The applicant's assertions, including the assertions of its subcontractors or suppliers, or potential subcontractors or suppliers, shall be submitted in the following format, dated and signed by an official authorized to contractually obligate the applicant. If the applicant will deliver all technical data and computer software to the Government without restrictions, enter "NONE" in this table under the heading "Technical Data or Computer Software to be Furnished with Restrictions."

Identification and Assertion of Restrictions on the Government's Use, Release, or Disclosure of Technical Data or Computer Software.

The applicant asserts for itself, or the persons identified below, that the Government's rights to use, release, or disclose the following technical data or computer software should be restricted:

Technical Data or Computer Software to be Furnished with Restrictions*	Basis for Assertion**	Asserted Rights Category***	Name of Person Asserting Restrictions****
(LIST)*****	(LIST)	(LIST)	(LIST)

*For technical data (other than computer software documentation) pertaining to items,

components, or processes developed at private expense, identify both the deliverable technical data and each such item, component, or process. For computer software or computer software documentation identify the software or documentation.

**Generally, development at private expense, either exclusively or partially, is the only basis for asserting restrictions. For technical data, other than computer software documentation, development refers to development of the item, component, or process to which the data pertain. The Government's rights in computer software documentation generally may not be restricted. For computer software, development refers to the software. Indicate whether development was accomplished exclusively or partially at private expense. If development was not accomplished at private expense, or for computer software documentation, enter the specific basis for asserting restrictions.

***Enter asserted rights category (e.g., government purpose license rights from a prior contract, rights in SBIR data generated under another contract, limited, restricted, or government purpose rights under this or a prior contract, or specially negotiated licenses).

****Corporation, individual, or other person, as appropriate.

*****Enter "none" when all data or software will be submitted without restrictions.

Date	_____
Printed Name	_____
Printed Title	_____
Signature	_____

Applicants responding to this BAA requesting an Other Transaction or Other Transaction for Prototype shall specifically identify any asserted restrictions on the Government's use of intellectual property contemplated under those award instruments. For this purpose, applicants must propose specific Intellectual Property terms and conditions and a data deliverable list. Further, the applicants must explain why an Other Transaction is necessary and, in particular, how the intellectual property terms and conditions proposed will meet the objectives of this BAA.

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[i] El-Radhi, A. Sahib (2019). Pathogenesis of Fever. *PubMedCentral* *<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7122269/>*

[ii] Cristea, Ileana M. (2017). Proteomics and integrative omic approaches for understanding host-pathogen interactions and infectious diseases. *Molecular Systems Biology.* *<https://www.embopress.org/doi/full/10.15252/msb.20167062>.*

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[iv] McFarlane, Daniel C. (2022). Real-time infection prediction with wearable physiological monitoring and AI to aid military workforce readiness during COVID-19. *Nature Scientific.* *<https://www.nature.com/articles/s41598-022-07764-6>*

From: (b) (6)
To: (b) (6) v
Cc: Eric Laing; (b) (6)
Subject: Re: Internal USU review deadline for WAB-Net 2 proposal?
Date: Wednesday, February 8, 2023 5:12:05 PM
Attachments: WABNet2-whitepaper_7Sep2022.pdf
FRBAA14-24-Amendment 17.pdf

Thank you, (b) (6)! We'll let you know if we have any questions.

I've attached our White Paper as well as the grant guidelines (the Broad Agency Announcement). Please let me know if you need any additional information.

Best,

(b) (6)

On Wed, Feb 8, 2023 at 3:29 PM (b) (6) wrote:

> Hello (b) (6),
>
> Attached is the budget. If it is OK - we will proceed with the rest of the documentation.
>
> Can you also send me a copy of the proposal/guidelines? It is needed for the review process.
>
> Thanks much .... (b) (6)
>
> On Tue, Feb 7, 2023 at 7:54 AM (b) (6) > wrote:
>
> > Thanks for letting me know, (b) (6)!
> >
> > (b) (6)

(b) (6)

On Mon, Feb 6, 2023 at 5:01 PM (b) (6) > wrote:

Hello (b) (6),

We do but it does not involve paperwork needed from EcoHealth.

Thanks much .... (b) (6)

On Fri, Feb 3, 2023 at 1:20 PM (b) (6) wrote:

Hi (b) (6),

I was just curious if USU has an internal review deadline for our WAB-Net 2 proposal that I should be aware of? Is there a certain date by which you need to submit all of USU's components of the proposal? Please let me know, and happy Friday!

Best,

(b) (6)

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(b) (6) [redacted]

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From: Laing, Eric (b) (6)
To: (b) (6)
Subject: Re: Noam/Eric"s Serology NIH R01 grant submission status
Date: Friday, February 3, 2023 5:08:57 PM
Attachments: Vertebrate Animals (b) (6) .doc
serostats-research-strategy-v2_CURRENT-(b) (6) .docx

Hi (b) (6),

Attached are very likely my final edits. Feel free to reach out. I added the vertebrate section language (it kicked down 2 blank pages) and also uploaded a doc to the folder "Other Documents."

I think it's there.

(b) (6)

[redacted]

On Fri, Feb 3, 2023 at 11:15 AM Laing, Eric (b) (6) wrote:

> Sorry - I sent the wrong .doc
>
> (b) (6)
>
> [redacted]
>
> On Fri, Feb 3, 2023 at 11:07 AM (b) (6) > wrote:
>
> > Thanks! Did we have a biosketch or are we expecting it?
> > --
> >
> > (b) (6)
> >
> > [redacted]

(b) (6) [redacted]

On Fri, Feb 3, 2023 at 10:47 AM Laing, Eric (b) (6) [redacted] wrote:

> Hey - here are (b) (6) [redacted]s docs
>
> (b) (6) [redacted]
>
> On Fri, Feb 3, 2023 at 10:34 AM (b) (6) [redacted] wrote:
>
> > Great, working on admin stuff and other meetings this morning, but I've put this one up in the shared folder. Will let you know when I'm back on it addressing the things you flagged for me.
> > --
> >
> > (b) (6) [redacted]
> >
> > On Fri, Feb 3, 2023 at 12:26 AM Laing, Eric (b) (6) [redacted] wrote:
> >
> > > Revisions attached.
> > >
> > > Need to tweak Summary Abstract.

Student seminar from 1-2pm tomorrow but I'm free besides that. Just need to add in Vertebrate section.

Let's touch base about any figures tomorrow?

(b) (6)

On Thu, Feb 2, 2023 at 7:25 PM (b) (6) > wrote:

Hi all,

Just sending a state-of-the submission of the serology grant with USU as we barrel to the end of the week and the Monday 5PM deadline.

Grant materials are in this folder: (b) (5)

This includes a document tracker of development and upload status in the base folder. Note that several documents are all consolidated together in the research folder.

About 1/3 of documents and forms are filled out and uploaded in ASSIST (Application 1344425) . The plan is to spend Friday finishing the rest, including filling in the budget and writing the justification, pending any last-minute input from (b) (6). There is one outstanding administrative item (b) (6) is tracking down for our vertebrate animal testing justification document, and a letter of support we are expecting. These should be done tomorrow, too.

The core research strategy is near-done, but (b) (6) and I have decided to give ourselves a bit of breathing room to revise, taking a break to re-read then uploading and submitting from our end on Sunday. If all goes according to plan, (b) (6) can give organizational approval Monday morning.

I am on a flight to Calfornia 6:30 AM on Monday morning and will pay for internet, but I will assume I'm incapable of doing anything. Should anything not go right with final submission, or an administrative document not arrive until Monday. (b) (6) on my team has full edit capabilities for the proposal ASSIST. (b) (6) please be on standby to liaise with (b) (6) if something needs to be fixed.

Thank you all for your help!

(b) (6)

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[redacted]

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From: (b) (6)
To: (b) (6) Eric Laing; (b) (6)
Cc: (b) (6)
Subject: Grant submitted!
Date: Friday, February 3, 2023 4:55:00 PM
Attachments: NIH R01_Liberia_Research_Final_Feb 2023.pdf

We did it!
Great job everyone! Thanks for all of your input, I think it came together nicely. Special thanks to (b) (6) and (b) (6) for all the hard work you did to keep us on track and make sure the proposal came together on time. Attached is the final version, as submitted.

I'll be interested to see what the reviewers think. I requested that it go to the TVZ study section which handles One Health and disease ecology type proposals. I believe it's a new study section, but it's really a good fit and I know several of the members.

Have a great weekend, everyone!

Cheers,
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From: (b) (6)
To: (b) (6)
Cc: Laing, Eric; (b) (6)
Subject: Re: Complete draft 7 for rapid review
Date: Thursday, February 2, 2023 10:38:25 PM
Attachments: Liberia R01 Technical proposal d7_clean_(b) (6).docx

Well done – see attached for minor edits and suggestions.

I know we're tight on space but a figure triangulating seroprevalence in humans, PCR positivity in bats, and host/behavior/environmental risk factors might be helpful to tie the three aims together.

Thanks for everyones' work on this!!

(b)

_____

(b) (6)

From: (b) (6)
Date: Thursday, February 2, 2023 at 5:48 PM
To: (b) (6)
Cc: Laing, Eric (b) (6)
Subject: Re: Complete draft 7 for rapid review

Still…

That is important. If there is smoke the is fire and agree important to say that it is present and understudied.

Thanks

(b) (6)

From: (b) (6)
Date: Thursday, February 2, 2023 at 5:45 PM
To: (b) (6)
Cc: Laing, Eric (b) (6)
Subject: Re: Complete draft 7 for rapid review

Antibodies....

Our work is done :)

On Thu, Feb 2, 2023 at 5:43 PM (b) (6) wrote:

> You found a henipavirus in bats in west Africa?
>
> If so, what I said:

(b) (6) [redacted]

Sent from my iPhone

On Feb 2, 2023, at 5:07 PM, Laing, Eric (b) (6) [redacted] wrote:

I think I get why you're sticking with "Nipah-like henipavirus," but it seems odd to me. Is every henipavirus, "Nipah-like." Why not just call out Ghana virus, or shrew-associated Langya virus that causes acute febrile illness in humans.

The manuscript write-up was EBOV focused but when my student rant the multiplex we included henipaviruses. This prelim you should use that would address the henipavirus concern. I'd drop Figure 5 and use this

<image.png>

(b) (6)
[redacted]

On Thu, Feb 2, 2023 at 4:33 PM (b) (6) [redacted] wrote:

> Hi (b) (6),
>
> See attached. Looks solid.
>
> Main issues to consider:
>
> The conflating of filoviruses and henipaviruses makes me nervous (as (b) (6) [redacted] often says). We have good rationale for expecting filoviruses in bats and humans. For henipaviruses, not so much. This could be shifted to a strength if acknowledged not just at the end but earlier and make clear that this is an opportunity to look for these viruses given the bat species and limited ability to detect encephalitis in Liberia – we could detect the tinderbox for a future outbreak.
>
> I do think in the limitations section we need to address more explicitly that not finding henipavirus exposure in bats or people is possible and is ok in that this is an important negative finding given the potential. I am concerned reviewers will be attracted to henipavirus and unclear if their reaction will be sweet or sour.
>
> Less of an issue is the seroincidence. I am of two minds about this. We can double down and take our chances that reviewers won't ding us for placing bets on a 24 month follow-up period by going the route selected. Alternatively, we could acknowledge that incidence is less certain but that we hypothesize is not uncommon. Given hours left to submit, doubling down makes sense.

For Aim 3, I agree could be clearer about putative factors for infection/exposure. It is not just behavior but also structural (literally and figuratively). Environment plays a role too as does super poverty among the impoverished. Could list hypotheses regarding risks including men>women, hunters vs non, having domestic animals vs not, less sturdy housing vs more, etc.

Let us know if you need anything else.

Thanks for leading the charge.

(b) (6)

From: (b) (6)
Date: Thursday, February 2, 2023 at 2:31 PM
To: (b) (6)
Eric Laing (b) (6)
Cc: (b) (6)
Subject: Complete draft 7 for rapid review

Hi all,
I've compiled a near final draft, incorporating the text you each provided and then cutting it down to fit within the 13 page (1 page specific aims and 12 page research strategy) limit. Thank you all for excellent contributions to this. I'm sure it's not perfect, but I think it's solid.

If you're able to go through it today and check it for overall readability and accuracy where you have preliminary data, that would be fantastic. Please do use track changes and change the file name to add your initials when you edit, and please do not add too much text, as there's not much space at the end.

I do think Aim 3 might be a little thin, so if (b) (6) could look through that and see if you think it's missing anything, there's space for a few extra lines there.

Please return your edits to me by 7pm, if possible, so I can finalize the draft. Our plan is to submit at noon tomorrow and deal with any errors that may pop up.

While you're reviewing this, I'll work on the summary and narrative, and other peripheral pieces.

Thanks again. We're in the home stretch!

Cheers,

(b) (6)

[redacted]

<Liberia R01 Technical proposal d7_clean(b) (6) .docx>

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From: (b) (6)
To: (b) (6) Eric Laing; (b) (6)
Cc: (b) (6)
Subject: RE: Complete draft 7 for rapid review
Date: Thursday, February 2, 2023 5:04:58 PM
Attachments: Liberia R01 Technical proposal d7_clean_(b) (6).docx

---

Couple of very minor comments,

I agree with (b) (6) on the henipa's as you are coupling a clear direct public health threat (Filos's – EBOV and MARV) with a history of large outbreaks, with a group of viruses for which there is no direct evidence of causing any morbidity and mortality in Africa. It makes complete sense to do exactly what we are proposing, but i think it would be stronger to argue that the risk of henipa's is currently unknown. Particularly tying this back to the human data, you know that most filo's will have direct zoonotic potential, for anything you find in the bats that will remain clear unless you can isolate said virus from a human case.

Other than that, cool proposal, great team!

(b) (6)

From: (b) (6)
Sent: Thursday, February 2, 2023 2:33 PM
To: (b) (6)
Eric Laing (b) (6)
Cc: (b) (6)
Subject: [EXTERNAL] Re: Complete draft 7 for rapid review

Hi (b) (6),

See attached. Looks solid.

Main issues to consider:

The conflating of filoviruses and henipaviruses makes me nervous (as (b) (6) often says). We have good rationale for expecting filoviruses in bats and humans. For henipaviruses, not so much. This could be shifted to a strength if acknowledged not just at the end but earlier and make clear that this is an opportunity to look for these viruses given the bat species and limited ability to detect encephalitis in Liberia – we could detect the tinderbox for a future outbreak.

I do think in the limitations section we need to address more explicitly that not finding henipavirus exposure in bats or people is possible and is ok in that this is an important negative finding given the potential. I am concerned reviewers will be attracted to henipavirus and unclear if their reaction will be sweet or sour.

Less of an issue is the seroincidence. I am of two minds about this. We can double down and take our chances that reviewers won't ding us for placing bets on a 24 month follow-up period by going the route selected. Alternatively, we could acknowledge that incidence is less certain but that we hypothesize is not uncommon. Given hours left to submit, doubling down makes sense.

For Aim 3, I agree could be clearer about putative factors for infection/exposure. It is not just behavior but also structural (literally and figuratively). Environment plays a role too as does super poverty among the impoverished. Could list hypotheses regarding risks including men>women, hunters vs non, having domestic animals vs not, less sturdy housing vs more, etc.

Let us know if you need anything else.

Thanks for leading the charge.

[redacted]

From: [redacted]
Date: Thursday, February 2, 2023 at 2:31 PM
To: [redacted] Eric Laing [redacted]
Cc: [redacted]
Subject: Complete draft 7 for rapid review

Hi all,
I've compiled a near final draft, incorporating the text you each provided and then cutting it down to fit within the 13 page (1 page specific aims and 12 page research strategy) limit. Thank you all for excellent contributions to this. I'm sure it's not perfect, but I think it's solid.

If you're able to go through it today and check it for overall readability and accuracy where you have preliminary data, that would be fantastic. Please do use track changes and change the file name to add your initials when you edit, and please do not add too much text, as

there's not much space at the end.

I do think Aim 3 might be a little thin, so if (b) (6) could look through that and see if you think it's missing anything, there's space for a few extra lines there.

Please return your edits to me by 7pm, if possible, so I can finalize the draft. Our plan is to submit at noon tomorrow and deal with any errors that may pop up.

While you're reviewing this, I'll work on the summary and narrative, and other peripheral pieces.

Thanks again. We're in the home stretch!

Cheers,

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From: (b) (6)
To: (b) (6)
Cc: (b) (6); Eric Laing; (b) (6)
Subject: Re: Complete draft 7 for rapid review
Date: Thursday, February 2, 2023 4:23:11 PM
Attachments: Liberia R01 Technical proposal d7_clear(b) (6).docx

---

(b) (6),

Minor edits and tidying throughout + a few comments. Happy to go through v8 tomorrow morning (if you would like me to) to conduct any needed cleanup that slips through (you will see the sort of thing I am talking about when you open the document).

---(b) (6)

On Thu, Feb 2, 2023 at 3:02 PM (b) (6) wrote:

> On it. Will review and send back shortly. Thanks
>
> (b) (6)
>
> ---
>
> **From:** (b) (6) >
> **Date:** Thursday, February 2, 2023 at 2:31 PM
> **To:** (b) (6)
> Eric Laing (b) (6)
> **Cc:** (b) (6)
> **Subject:** Complete draft 7 for rapid review
>
> Hi all,
>
> I've compiled a near final draft, incorporating the text you each provided and then cutting it down to fit within the 13 page (1 page specific aims and 12 page research strategy) limit. Thank you all for excellent contributions to this. I'm sure it's not perfect, but I think it's solid.
>
> If you're able to go through it today and check it for overall readability and accuracy where you have preliminary data, that would be fantastic. Please do use track changes and change the file name to add your initials when you edit, and please do not add too much text, as there's not much space at the end.
>
> I do think Aim 3 might be a little thin, so if (b) (6) could look through

that and see if you think it's missing anything, there's space for a few extra lines there.

Please return your edits to me by 7pm, if possible, so I can finalize the draft. Our plan is to submit at noon tomorrow and deal with any errors that may pop up.

While you're reviewing this, I'll work on the summary and narrative, and other peripheral pieces.

Thanks again. We're in the home stretch!

Cheers,
(b) (6)

[redacted]

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(b) (6) [redacted]

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From: AIMS-DoNotReply@hjf.org
To: (b) (6)
Cc: ERIC LAING; (b) (6)
Subject: Message sent on behalf of (b) (6) : Eric Laing subaward to EcoHealth Alliance
Date: Wednesday, February 1, 2023 4:16:30 PM
Attachments: Biosketch (b) (6) .pdf
Budget Justification pdf
Equipment.pdf
Facilities.pdf
Key Biologicals.pdf
SOW.pdf
RR_Budget_3_0-V3.0 UPDATE.pdf
HJF LETTER OF INTENT_(b) (6) _(PRO4479)-NIH Subaward-2-1-23-(b) (6) _signed.pdf

---

Hello Dr. (b) (6)

On behalf of the Henry M. Jackson Foundation for the Advancement of Military Medicine, Inc., attached are the documents for Dr. (b) (6) , Uniformed Services University of the Health Sciences to collaborate with EcoHealth Alliance in response to PA-20-185 Funding Opportunity. Please let me know if you need additional information and/or you have any questions. Also, please reply to this email to confirm receipt of this proposal submission.

Yours in good health,

(b) (6)
[redacted]

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From: Laing, Eric (b) (6)
To: (b) (6)
Subject: Re: [EXTERNAL] R01_Applied serology methods and statistics
Date: Tuesday, January 31, 2023 9:06:19 PM
Attachments: (b) (6) .doc
image001.png

Biosketch.

(b) (6)

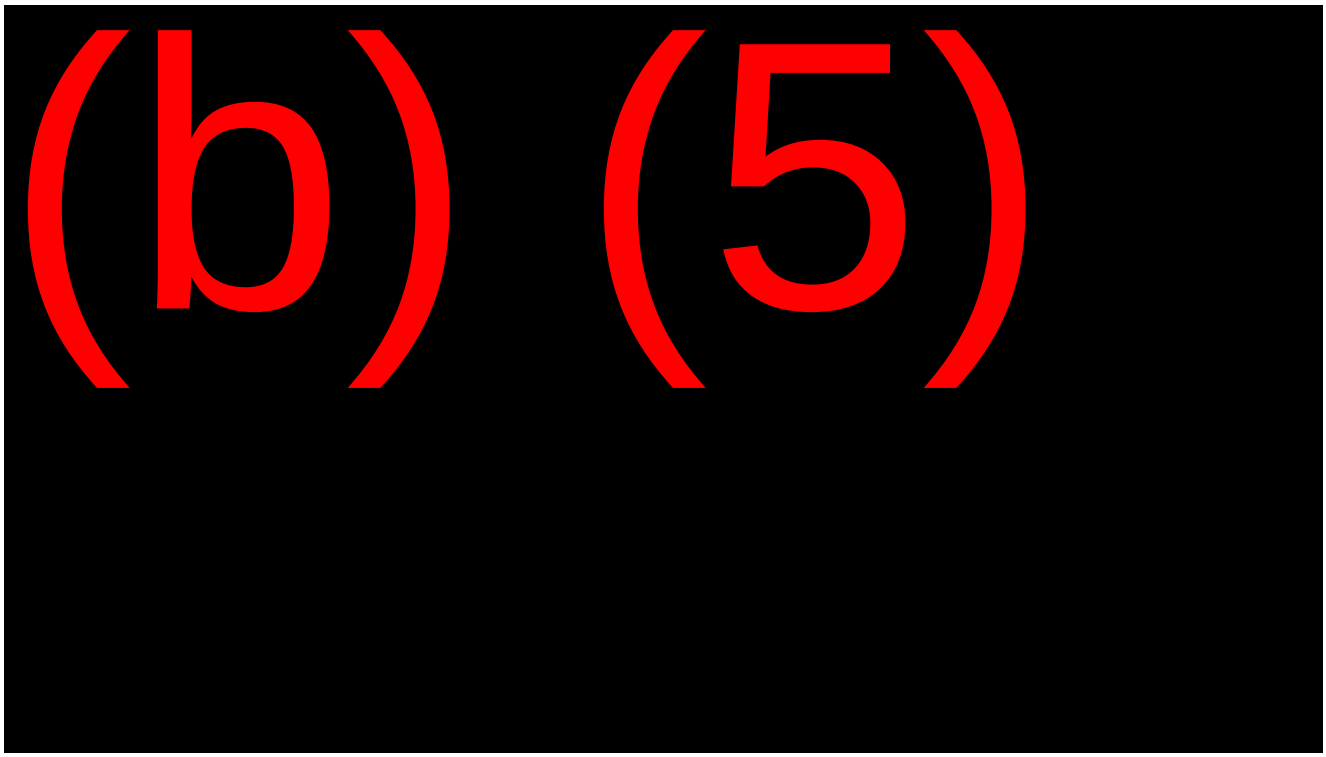
On Tue, Jan 31, 2023 at 12:48 PM (b) (6) wrote

> Thanks, (b) (6) !
> --
>
> (b) (6)
>
> .
>
> On Tue, Jan 31, 2023 at 12:40 PM (b) (6) wrote
>
> > Here it is guys,
> >
> > Cheers,
> >
> > (b) (6)
> >
> > ---
> >
> > **From:** Laing, Eric (b) (6)
> > **Sent:** Tuesday, January 31, 2023 7:40 AM
> > **To:** (b) (6)
> > **Cc:** (b) (6)
> > **Subject:** Re: [EXTERNAL] R01_Applied serology methods and statistics
> >
> > Hi (b) (6) ,
> >
> > Sorry for the late reply to this email and follow-up for R01 Letter of Support. (b) (6) and I are hoping to submit by this Friday. I've drafted a LOS (attached), can you make any edits, add your letterhead, sign and send back to us? You sent (b) (6) a biosketch for the Liberia-focused R01, I can grab that from the email thread if that works for you.
> >
> > Also, there's a good chance my research group will be participating in surveillance in Jordan, Oman, and Republic of Georgia so I'll probably need to increase the breadth of MERS-like CoVs in the multiplex. We're still finalizing the IAA with NCI FNL but I'll prob loop back to you for aliquots of bat sera from Jordan to cross-verify antigen performance.
> >
> > (b) (6)

(b) (6)

On Tue, Jan 10, 2023 at 4 42 PM Laing, Eric (b) (6) wrote

> Hi (b) (6),
>
> Really appreciate your support.
>
> One of my ideas was to write that we could leverage the R24 Bat ID model to verify/validate rabbit-immunizations. So it's awesome to hear that you are already on the same page. (b) (6) even brought this up during a meeting this morning and I think it's an obvious limitation that a reviewer might pick at, if we only propose antigen immunization in rabbits. (b) (6) has also offered to do some bat-infection experiments so I'll write that in and make sure there is money in a budget for her lab to do that as well.
>
> RE CoVs
>
> (b) (6) /my lab pulled back from expressing CoV antigens in-house and have been working with (b) (6) Protein Expression lab at NCI FNLCR. We're in the process of re-establishing an IAA with his group and he's interested in de novo expression of bat CoV antigens. Do you have particular RBD, S1, or spike (S-2P) antigens that you would want for a custom panel? We're going to build out something related to (b) (6) work, and work with (b) (6) and (b) (6) in southeast Asia.
>
> (b) (6)
>
> [redacted]
>
> On Tue, Jan 10, 2023 at 2 44 PM (b) (6) [redacted] wrote
>
> > Sounds great,
> >
> > Looping in my postdoc Dr (b) (6) as well, as she will be doing some bat infection studies soon here at RML and we could share some sera (Artibeus) from these experiments as well.
> >
> > We could also in the future (my colony is still at CSU, but will be moving to RML) do some bat protein immunizations and use those as controls to validate your rabbit antisera.
> >
> > Speaking of which, I have a nice serum set of bats from Jordan (Rhinolophus and Rousettus) for which we detected a variety of corona s in (the darker ones), would like to run these through the corona luminex and see what their serum cross reactivity looks like. I could do it here on the magpix, but could share an aliquot of the sere with you as well.



(b) (6)
[redacted]
[redacted]
[redacted]

From: Laing, Eric (b) (6)
Sent: Tuesday, January 10, 2023 10:10 AM
To: (b) (6)
Subject: [EXTERNAL] R01_Applied serology methods and statistics

Hi (b) (6),

(b) (6) and I are putting together a "methods" focused R01 that leverages ongoing work via a NIH center and DTRA BTRP project with (b) (6). My lab is going to further optimize a 28plex panel of antigens (henipa/filo-focused) that balances two goals: a) discovery of new viruses through serology and b) specific detection of homotypic viruses. We are going to include a ton of protein-immunizations of rabbits to establish cross-reaction matrices for each virus and standard antigenic cartography. (b) (6) is working on novel multidimensional spatial analyzes for Aim 2. In Aim 3 we are proposing to re-test/re-analyze data with the in vitro and in silico tools developed through Aims 1 and 2.

I was wondering whether you would be willing to share with my lab some of the sera collected from bats in the Republic of Congo that has interesting sero-positives for the research being outlined in Aim 1 and 3, and be listed as a collaborator on the project? If so, I would include the restesting of those sera with the optimized panel and downstream analysis in the proposal, and I could provide a draft LOS. The Specific Aims are below:

Specific Aims

1. Create multiplex serological panels optimized for viral discovery

2. Develop novel statistical approaches for identifying and characterize novel virus signals from multiplex data

3. Determine epidemiological patterns of novel virus exposure in human and bat populations.

Kind regards,

(b) (6)

[redacted]

(b) (6)

CAUTION: This email originated from outside of the organization. Do not click links or open attachments unless you recognize the sender and are confident the content is safe.

(b) (5)

(b) (6)

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(b) (6)

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From: (b) (6)
To: (b) (6)
Cc: Laing, Eric
Subject: Re: NIH submission proposed budget
Date: Tuesday, January 31, 2023 1:58:31 PM
Attachments: nih-serostats_BioSketch(b) (6)_TOSUMBIT.docx
nih-serostats_BioSketch(b) (6)_TOREVIEW.docx

Many thanks, (b) (6)!

The NIH biosketch section (C) is a little more expansive and narrative than just the list of four papers, unfortunately. They ask for descriptions of major contributions, each with up to four references as examples. I'm attaching (b) (6)'s and mine for reference. (Sorry! I am always embarrassed for how much of a to-do NIH and NSF make these.)

Would you be able to expand on this? If you just take a few minutes to put in some bullets or pasted text and references to additional papers/topics, we're happy to with stitching it together and formatting!

(b) (6)

[redacted]

On Tue, Jan 31, 2023 at 7:52 AM (b) (6) wrote:

> Afternoon (b) (6)
>
> See the attached documents requested. Let me know if you need anything else.
>
> (b) (6)
>
> On Sat, 28 Jan 2023 at 00:28, (b) (6) wrote:
>
> > Dear (b) (6),
> >
> > Find attached two documents for your review: A letter of support and a budget justification.

- The LoS requires only your review or edits, signature and to be on UP letterhead.
- The budget justification just requires your review. I do note that it describes a 35% fringe benefits rate, as the budget did, but this should be based on the institutional rate. If this should be different, described differently, and/or we should modify the budget to incorporate this into base salary or time, please let me know. Thank you!

Best,
(b) (6)

[redacted]

On Thu, Jan 26, 2023 at 1:50 PM (b) (6) [redacted] wrote:

> Great, (b) (6). I've moved your salary to two lines for the postdoc and lab technician. We will still list you as committing 2 weeks/yr as the institutional lead at no salary.
>
> If we could have the BioSketch and Facilities description by the end of your Tuesday, that would be great.
>
> I'm doing a last pass over all the paperwork requirements to make sure there isn't other info I'll need from any partners. There will be a Letter of Support and Budget Justification, both of which I'll draft before next week for you to review.
>
> (b) (6)
>
> [redacted]

(b) (6)

[redacted]

On Thu, Jan 26, 2023 at 9:47 AM (b) (6) [redacted] wrote:

> Afternoon (b) (6)
>
> Thanks for this. Overall it looks fine. I usually do not claim direct salary, but it can be directed to a postdoc salary, so you can keep it as is or split a technician and a postdoc and have both. I think both will be needed.
>
> The sample costs are fine.
>
> I still owe you a bio sketch and equipment description. When do you need that at the latest?
>
> (b) (6)
> [redacted]
>
> On Thu, 26 Jan 2023 at 02:10, (b) (6) [redacted] wrote:
>
> > Dear (b) (6),
> >
> > Find attached a rough proposed budget for your participation in our serology methods proposal, based on the per-plate costs you provided. My apologies for getting this to you late. We've estimated that we want to re-test up to 6,000 samples from SABRENET and related projects. This is based on current positive hit rates, and including matched negative samples. We propose to do this across years 3 and 4 of the project, after the bulk of our panel modifications and statistical testing take place in years 1 and 2. We include budget for personnel time extending into year 5 of the grant for you and a postdoc/tech to continue to participate in analysis and interpretation.
> >
> > I have made some very rough guesses in the attached to understand the order of magnitude. For instance, I'm unsure if you need to take direct salary and don't know at rate that would be (other PI partners doing testing/re-analysis are taking 2 weeks/yr). Please let us know how this looks and adjust as appropriate.
> >
> > Best,
> > (b) (6)
> > [redacted]
> >
> > [redacted]
> >
> > [redacted]

[redacted]

[redacted]

[redacted].

On Sun, Jan 15, 2023 at 6:17 AM [redacted]> wrote:

> Afternoon
>
> Thank you for the discussion. It will be an interesting project
>
> Attached are some costs for consideration
>
> To run one plate (about 80 sample, considering controls also) costs us about 265 USD labour included. So the total costs will depend on how many samples you want to include and repeats. These costs also assume we are getting coupled beads with antigen from USU, so no costs on that was considered.
>
> I also mention the costs for collecting the samples and costs of a research scientists for consideration.
>
> Let me know if you have questions. [redacted] will send you the breakdown of the samples.
>
> Will send the bio and equipment list by the end of this week.
>
> [redacted]
>
> On Wed, 11 Jan 2023 at 01:19, [redacted] wrote:
>
> > Dear [redacted],
> >
> > Good to speak with you today. I'm writing with the materials for the biosketch and facilities document for the application. Find attached a template and example NIH format biosketch that we'll need for [redacted]. (Further instructions here if needed).
> >
> > We'll also need a Facilities & Equipment document that summarizes resources available at your institution and lab, with a focus on what is needed to run Luminex and associated we will need (this is actually [redacted]'s for a different NIH project), (Instructions)
> >
> > It would be great if we could get these by **Wednesday, Jan 25th.**
> >
> > If we get some preliminary figures on samples by species and costs, we'll get budget info to you ASAP next.

Best,
(b) (6)

[redacted]

This message and attachments are subject to a disclaimer.
Please refer to http://upnet.up.ac.za/services/it/documentation/docs/004167.pdf for full details.

This message and attachments are subject to a disclaimer.
Please refer to http://upnet.up.ac.za/services/it/documentation/docs/004167.pdf for full details.

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From: (b) (6)
To: (b) (6)
Cc: Laing, Eric; (b) (6)
Subject: Re: R01 itemized budgets & justifications!
Date: Monday, January 30, 2023 7:07:01 PM
Attachments: R01_LiberiaEBOV(b) (6)_Facilities_20JAN2023.docx.pdf
R01_LiberiaEBOV(b) (6)_Biosketch_22JAN2023.docx.pdf
RR_Budget_LiberiaR01(b) (6).pdf
R01_LiberiaEBOV(b) (6)_Budget Justification_25JAN2023.docx.pdf

---

Attached!

The only things missing per your instructions are the authentication of key resources and letter of support.

On Mon, Jan 30, 2023 at 4:48 PM (b) (6) wrote:

> Hi, all,
>
> Please send me your itemized budgets & justifications ASAP. I am looking for updated budgets to meet the new (reduced) yearly totals for USU, UNC, and NPHIL.
>
> I need to have enough time to get them approved by EHA and input into NIH ASSIST, so please have them to me by Wednesday morning at the latest.
>
> Please let me know if I can answer questions about yearly budget targets or anything else.
>
> Best,
> (b) (6)
>
> [redacted]

[redacted]

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From: (b) (6)
To: info@creid-network.org
Cc: (b) (6) ; Eric Laing; (b) (6)
Subject: Applying for Pilot Research Program 2023 - EID-SEARCH (Thailand)
Date: Monday, January 30, 2023 11:01:48 AM
Attachments: CREID Pilot Research Program 2023 - Full application_Thailand.pdf
RR_Budget_Form_Thailand.pdf

To whom it may concern,

We are part of EID-SEARCH research group and we would like to submit for the CREID Pilot Research Program 2023.
Please find attached the two documents including;
1. Full application
2. Budget

If you require any further information, please do not hesitate to contact us.

Best regards,
(b) (6)

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From: Laing, Eric (b) (6)
To: (b) (6)
Cc: (b) (6)
Subject: Re: Don't forget to fill and sign your Other Support form
Date: Monday, January 30, 2023 9:45:37 AM
Attachments: (b) (6) Other support_30JAN2023-signed.docx

Nope, not your misunderstanding. I forgot to update this title for this pilot project.

Attached doc with updated language

(b) (6)

[redacted]

On Mon, Jan 30, 2023 at 9:35 AM (b) (6) wrote:

> Hi (b) (6),
>
> In the overlap section *"In my role on this project, titled: Establishment of a Bat Resource for Infectious Disease Research, my group will be providing recombinant antigenic material for multiplex serologies and our expertise in virology and assay development and interpretation..."* is the project title correct, or it's my misunderstanding?
>
> (b) (6)
>
> [redacted]
>
> On Mon, Jan 30, 2023 at 8:56 PM Laing, Eric (b) (6) wrote:
>
> > signature in word doc

(b) (6)

[redacted]

On Mon, Jan 30, 2023 at 7:18 AM Laing, Eric (b) (6) wrote:

(b) (6)

[redacted]

On Mon, Jan 30, 2023 at 7:06 AM (b) (6) wrote:

Please see mine attached.

On Mon, Jan 30, 2023 at 12:53 PM (b) (6) wrote:

(b) (6) - your draft is attached, since you are paid by EID-SEARCH already for 2 months, you can only ask for 10-month salaries on the pilot, we can adjust everything after we get the award. Please double-check and sign it for submission.

For All, EID-SEARCH information is in the template for (b) (6).

Dr. (b) (6) - I have your Other Support, but it's in the NSF format, maybe someone can convert it into NIH format with the template?

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From: (b) (6)
To: (b) (6)
Cc: (b) (6) ; Laing, Eric; (b) (6)
Subject: Re: Pilot study 2023
Date: Monday, January 30, 2023 2:12:04 AM
Attachments: Pilot study full_V.5.pdf

Dear All,

Please find attached the full application.
I will edit page no. later.

Thank you
Best regards,
(b) (6)

On Mon, Jan 30, 2023 at 11:39 AM (b) (6) > wrote:

> I think if we cite the MojV, LayV, and GAKV and DARV papers it should be easily justifiable.
>
> See attached.

On Mon, Jan 30, 2023 at 10:40 AM (b) (6) > wrote:

> Languages for animal sample size justification, feel free to edit, (b) (6) .
> (I think we are dropping the 100 bats and doing 200 rodents/shrews now?)
>
> We will target a total of 200 individuals of rodent/shrew for this project, this sample size is estimated based on a realistic detection rate and required sampling efforts from our previous wildlife surveillance work, given the rodent/shrew population at the study site. Previous work of PMVs in wildlife had an average PCR detection prevalence of ~1.5%, sampling 200 individuals will yield 3 positives. We assume the serology prevalence will be higher, so expect about **5 positives for serology surveillance???**. We acknowledge the challenge of identifying positive results, and very limited serology research has been done to provide guidance. However, we will make the best use of previous data and experience to conduct sampling at the selected site where human positives have been identified and based on the potential seasonality at different time points throughout the project period (Ref. S. Wacharapluesadee *et al.*, A longitudinal study of the prevalence of Nipah virus in Pteropus lylei bats in Thailand: evidence for seasonal preference in disease transmission. *Vector borne and zoonotic diseases (Larchmont, N.Y.)* **10**, 183-190 (2010)). We are confident that this targeted sampling and testing strategy offers the best chance to identify positive results and potentially additional strains henipaviruses.
>
> On Mon, Jan 30, 2023 at 10:02 AM Laing, Eric (b) (6) wrote:
>
> > Edits - Deleted objective 3, antigenic cartography is not discussed in Aim 2. Make sure you clean up track changes throughout.

(b) (6)

[redacted]

On Sun, Jan 29, 2023 at 9:12 PM (b) (6) [redacted] > wrote:

Hi (b) (6) [redacted],

We have modified the Specific Aims section, can you please look it over?

(b) (6) [redacted]

On Mon, Jan 30, 2023 at 8:13 AM (b) (6) [redacted] wrote:

Hi All,

1. Would it be possible to strengthen the sample size justification. I know there might not be any sample size calculation because this focuses on developing the method, but there is a statistical review panel, maybe you can briefly talk about this? E.g. given the estimated positive rate of X%, or total population of the community or animals etc.

2. Are you doing questionnaires when collect samples for humans? If so please describe it in the Method part; if not, please remove these languages from the human subject section.

3. It seems you don't plan to enroll children, then please remove these languages about parents/legal guardians in the human subject protection section.

(b) (6) [redacted]

On Jan 30, 2023, at 7:39 AM, Laing, Eric (b) (6) [redacted] wrote:

Hi (b) (6) [redacted],

My edits are attached. Please revise the specific aims page and look at the previously awarded applications when you make those revisions. Everything else looks good. Spend time on the Aims Page.

[redacted]

On Sun, Jan 29, 2023 at 9:58 AM [redacted] wrote:

> Dear All,
>
> Please find attached the word file of the full application for CREID Pilot study Program.
>
> Thank you
> Best regards,
> [redacted]
>
> On Sun, Jan 29, 2023 at 9:21 PM [redacted] wrote:
>
> > Dear [redacted]
> >
> > Thank you very much for your support in the CREID Pilot study.
> > Please find attached the Full application.
> > If you have any comments please let me know.
> >
> > Thank you very much
> > Best Regards,
> > [redacted]

<Pilot study full_V.3 (1)-EDL.docx>

--
[redacted]

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From: (b) (6)
To: Laing, Eric
Cc: (b) (6)
Subject: Re: Pilot study 2023
Date: Saturday, January 28, 2023 7:54:06 AM
Attachments: Research aim, methods_V.3.docx

Dear (b) (6),

Please find attached the edit research aim and method.
If you need more information please let me know.

Thank you,
Best regards,
(b) (6)

On Sat, Jan 28, 2023 at 1:52 PM Laing, Eric (b) (6) wrote:

> Hi (b) (6),
>
> My biosketch and LOS are attached
>
> (b) (6)
>
> On Fri, Jan 27, 2023 at 4:46 PM Laing, Eric (b) (6) wrote:
>
> > Hi (b) (6),
> >
> > Do you guys have a version in which Aim 2 is more developed? You need to focus on that Aim.
> >
> > LOS from Dr. (b) (6) is attached
> >
> > (b) (6)

(b) (6)

On Thu, Jan 26, 2023 at 11:02 PM (b) (6) wrote:

Hi (b) (6),

Attached are

- Vertebrate Animals Section
- Human Subject Research
- Foreign Clearance
- Letter of Collaboration from CREID RC signed by (b) (6)

You need to read through and edit based on the project activities, for example, sample types, enrollment of children or not, age group, and other relevant information.

Please also ensure the format, font, size, line spacing, margin, etc. are consistent and follow the guidance.

Let me know if you have any questions! Send me documents to review anytime.

Best,
(b) (6)

On Thu, Jan 26, 2023 at 10:23 PM (b) (6) > wrote:

Dear (b) (6),

Please find attached the Pilot study including all documents.
If you need further information please let me know.

Thank you very much

Best regards,
(b) (6)

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From: Laing, Eric (b) (6)
To: (b) (6)
Subject: Fwd: New NIH proposal on serology methods
Date: Wednesday, January 25, 2023 10:12:31 PM
Attachments: Biosketch_Dr. (b) (6) _NIH 2023_23-Jan-23.docx
Blood specimens_animal-human_25-Jan-23.xlsx
EIDCC facilities and equipment_26-Jan-23.docx

---

see attachments in the forwarded email

(b) (6)

---------- Forwarded message ---------
From: (b) (6)
Date: Wed, Jan 25, 2023 at 10:08 PM
Subject: Re: New NIH proposal on serology methods
To: Laing, Eric (b) (6)
Cc: (b) (6)

Dear (b) (6),

Attached please find the NIH-biosketch of Dr. (b) (6) , the facilities and equipment, and the excel file containing the number of animal and human sera samples we have at our EIDCC lab.

We would be grateful if you could send us a draft of the letter of support for Dr. (b) (6) to sign.

Let me know if you require any additional information or if there is anything I can help with.

Best regards,
(b) (6)

(b) (6)

On 11 Jan 2023, at 21:44, (b) (6) wrote:

Hi (b) (6),

I have sent the following info and attachments to (b) (6) before, but just resending for your reference.

Attached please find the latest versions of relevant materials I have:

- (b) (6)'s NIH-biosketch - this needs to be updated with the new format (no section D) https://grants.nih.gov/grants/forms/biosketch.htm
- Facilities and equipment
- Other support - this need to be updated with the new format and other grants https://grants.nih.gov/grants/forms/othersupport.htm - *but you will only need this for the Just-In-Time procedure,* ***not*** *now for proposal submission*

Other things you need from (b) (6) are:

- Budget
- Budget justification

And if you need to fill in IRB approval information to use human samples under EID-SEARCH (on the cover page):

- US: Health Medical Lab Institutional Review Board (No. 894ECOH21b) on May 12, 2021
- Thailand: Institute Review Board of the Faculty of Medicine, Chulalongkorn University (No. 211/64) on June 8, 2021

Let me know if anything else I can help with.

Best,
(b) (6)

---------- Forwarded message ---------
From: **Laing, Eric** (b) (6)
Date: Tue, Jan 10, 2023 at 4:54 PM
Subject: Re: New NIH proposal on serology methods
To: (b) (6)
Cc: (b) (6)

Hi (b) (6),

My lab is working on expanding our filo/henipa panel to a 28-plex, most of which should be available for further testing under EID-SEARCH. If this proposal is funded (b) (6) and I would like to make sure that there are additional funds in this R01 proposal to support retesting in your lab.

Which groups of the samples (host species and project name) do you plan to test? Now I have moved to work at the new lab, and it isn't easy to access the old samples (before 2020). But it is possible. For humans and wildlife, how many samples/species have you collected now and what is your projected target for the remainder of the EID-SEARCH POP? What would be really helpful would be a cost estimate for retesting all those samples under this R01 with the optimized in vitro and in silico serologic approaches.

We'd like to list you as a Co-Investigator, which means we'd need a biosketch, LOS, equipment/facilities pages, and that additional budget that we would include in YRS4-5 of this proposal to supplement additional testing. (b) (6) can be involved, just let me know whether salary support is coming from my end as HJF employee or whether he'd be listed as key personnel in your lab directly.

Best regards,
(b) (6)

(b) (6)

On Thu, Jan 5, 2023 at 5:51 AM (b) (6) wrote:

Dear (b) (6),

Thank you for your email and invitation to collaborate on the new proposal. I would love to join and have (b) (6) be responsible for lab work if it is funded. However, I would like to clarify objective 3 on retesting the archived samples. **Which groups of the samples (host species and project name) do you plan to test?** Now I have moved to work at the new lab, and it isn't easy to access the old samples (before 2020). But it is possible.

Best,
(b) (6)

From: (b) (6)
Sent: Thursday, January 5, 2023 4:48 AM
To: (b) (6)
Cc: (b) (6) Laing, Eric (b) (6)
Subject: New NIH proposal on serology methods

Dear (b) (6),

Happy New Yea! I hope this finds you well. I'm writing because I am putting together a new NIH grant proposal at EcoHealth together with (b) (6) at USU that we wanted to include you in. The aim of the grant is to improve both lab and statistical methods for luminex serology. The main elements of the project would be to:

1) Develop statistical methods to simultaneously analyze multiplex serology to

quantify the probability that signals from multiple beads represent antibodies from exposures to different viruses, cross-reactions of viruses in the multiplex panel, or signals of new, previously unknown viruses.
2) Develop discovery-optimized panels for filoviruses and henipaviruses that mix high-specificity and high-sensitivity beads so as to maximize the ability to identify signals of novel viruses.
3) Re-test and re-anlayze results from samples from previous serology surveillance studies that have ambiguous results to identify signals of exposure to previously unknown viruses, and conduct antigenic mapping to estimate where they fall phenotypically in relation to known viruses.

We anticipate sharing all our work across partners over the course of the project (if/when funded). However, we specifically hope to collaborate with you on #3 above. We'd like to use this to look deeper at EID-SEARCH results and ideally retest samples (such as the Mojiang-seropositive samples) to see what the new panels yield. For our proposed project, some of the new panels will be developed while EID-SEARCH is still running. If this new project is funded, we will work with you to determine what should be done concurrently with EID-SEARCH or after EID-SEARCH is completed and the best way to budget this (mind you, EcoHealth also hopes to get a continuation on our EID-SEARCH project so it is likely both will be concurrent). Assuming the best case, we submit this in February (Due date Feb 3, 2023) and start work in Fall 2023. Depending on how we stage our tasks, re-testing work could start as early as late 2024 or 2025.

One additional note – this project would be subject to new data sharing requirements NIH has enacted for projects starting 2023 or later. In short, all data we generate out of the project would be published at the end of the project period, whether or not publications are complete. We don't anticipate this would be an issue but want to make sure everyone is aware of this from the start of the grant so we can plan data and manuscript publication accordingly.

We'd like to have you as Key Personnel on this proposal. If you are interested, we'd love to have you aboard and get your thoughts and questions. (b) (6) and I will also follow up next week with a proposal budget. We would also need an NIH Biosketch, Other Support form, and Facilities information from you by January 20. We have recent versions of all of these for EID-SEARCH, so we can send templates for you to modify and approve early next week.

Please let us know what you think, and we'd be happy to have a call to discuss.

Best,

(b) (6)

[redacted]

[redacted]

(b) (6)

<2022 EIDCC facilities and equipment TRC EIDCC.docx><Biosketch_((b) (6))_EIDRC_RFA-AI_19-028_(PI-(b) (6))_v02.docx>

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From: (b) (6)
To: (b) (6)
Cc: (b) (6) ; Eric Laing
Subject: Pilot study 2023-Thailand
Date: Sunday, January 15, 2023 10:16:02 PM
Attachments: Submission Checklist_Application.docx
1 Application Cover Sheet.docx
Study Personnel_V.2.docx
Research Performance Sites_V.2.docx
CREID Research Center Collaboration.docx
Mentoring Plan_V.2.docx
Facilities and Resources_V.2.docx
Research aim, methods_V.2.docx
2 Research and Related Other Project Information(b) (6).docx

Dear (b) (6),

I and (b) (6) are writing the Pilot application.
We have some parts not finished which are labeled on the Submission Checklist.

I have attached files to this email for your consideration following;
1. Submission Checklist_Application
2. Proposal Cover Sheet
3. Study Personnel
4. Research Performance Sites
5. CREID Research Center Collaboration
6. Mentoring Plan
7. Facilities, Existing Equipment, and Other Resources
8. Research aim, methods
9. Research and Related Other Project Information_from (b) (6)

I don't know what to do with the IRB part.
Can you give us some advice?
Thank you very much
Best regards,
(b) (6)

(b) (5)

(b) (5)

(b) (5)

(b) (5)

(b) (5)

From: (b) (6)
To: (b) (6)
Cc: (b) (6) ; eric.laing(b) (6)
Subject: Fwd: New NIH proposal on serology methods
Date: Wednesday, January 11, 2023 9:46:05 AM
Attachments: 2022 EIDCC facilities and equipment_TRC EIDCC.docx
Biosketch_(b) (6) _EIDRC_RFA-AI_19-028_(b) (6) _v02.docx

Hi (b) (6),

I have sent the following info and attachments to (b) (6) before, but just resending for your reference.

Attached please find the latest versions of relevant materials I have:

- (b) (6) 's NIH-biosketch - this needs to be updated with the new format (no section D) https://grants.nih.gov/grants/forms/biosketch.htm
- Facilities and equipment
- Other support - this need to be updated with the new format and other grants https://grants.nih.gov/grants/forms/othersupport.htm - *but you will only need this for the Just-In-Time procedure,* ***not*** *now for proposal submission*

Other things you need from (b) (6) are:

- Budget
- Budget justification

And if you need to fill in IRB approval information to use human samples under EID-SEARCH (on the cover page):

- US: Health Medical Lab Institutional Review Board (No. 894ECOH21b) on May 12, 2021
- Thailand: Institute Review Board of the Faculty of Medicine, Chulalongkorn University (No. 211/64) on June 8, 2021

Let me know if anything else I can help with.

Best,
(b) (6)

[redacted]

[redacted]

[redacted]

[redacted].

---------- Forwarded message ---------
From: **Laing, Eric** (b) (6)
Date: Tue, Jan 10, 2023 at 4:54 PM
Subject: Re: New NIH proposal on serology methods
To: (b) (6)
Cc: (b) (6)
Laing, Eric (b) (6)

Hi (b) (6),

My lab is working on expanding our filo/henipa panel to a 28-plex, most of which should be available for further testing under EID-SEARCH. If this proposal is funded (b) (6) and I would like to make sure that there are additional funds in this R01 proposal to support retesting in your lab.

Which groups of the samples (host species and project name) do you plan to test? Now I have moved to work at the new lab, and it isn't easy to access the old samples (before 2020). But it is possible.
For humans and wildlife, how many samples/species have you collected now and what is your projected target for the remainder of the EID-SEARCH POP? What would be really helpful would be a cost estimate for retesting all those samples under this R01 with the optimized in vitro and in silico serologic approaches.

We'd like to list you as a Co-Investigator, which means we'd need a biosketch, LOS, equipment/facilities pages, and that additional budget that we would include in YRS4-5 of this proposal to supplement additional testing. (b) (6) can be involved, just let me know whether salary support is coming from my end as HJF employee or whether he'd be listed as key personnel in your lab directly.

Best regards,
(b) (6)

(b) (6)

On Thu, Jan 5, 2023 at 5:51 AM (b) (6) wrote:

> Dear (b) (6),
> Thank you for your email and invitation to collaborate on the new proposal. I would love to join and have (b) (6) be responsible for lab work if it is funded.
> However, I would like to clarify objective 3 on retesting the archived samples. **Which groups of the samples (host species and project name) do you plan to test?** Now I have moved to work at the new lab, and it isn't easy to access the old samples (before 2020). But it is possible.
>
> Best,
> (b) (6)
>
> [redacted]
>
> ---
>
> **From:** (b) (6)
> **Sent:** Thursday, January 5, 2023 4:48 AM
> **To:** (b) (6)
> **Cc:** (b) (6) Laing, Eric (b) (6)
> **Subject:** New NIH proposal on serology methods
>
> Dear (b) (6),
>
> Happy New Yea! I hope this finds you well. I'm writing because I am putting together a new NIH grant proposal at EcoHealth together with (b) (6) at USU that we wanted to include you in. The aim of the grant is to improve both lab and statistical methods for luminex serology. The main elements of the project would be to:
>
> 1) Develop statistical methods to simultaneously analyze multiplex serology to quantify the probability that signals from multiple beads represent antibodies from exposures to different viruses, cross-reactions of viruses in the multiplex panel, or signals of new, previously unknown viruses.
> 2) Develop discovery-optimized panels for filoviruses and henipaviruses that mix high-specificity and high-sensitivity beads so as to maximize the ability to identify signals of novel viruses.
> 3) Re-test and re-anlayze results from samples from previous serology surveillance studies that have ambiguous results to identify signals of exposure to previously unknown viruses,

and conduct antigenic mapping to estimate where they fall phenotypically in relation to known viruses.

We anticipate sharing all our work across partners over the course of the project (if/when funded). However, we specifically hope to collaborate with you on #3 above. We'd like to use this to look deeper at EID-SEARCH results and ideally retest samples (such as the Mojiang-seropositive samples) to see what the new panels yield. For our proposed project, some of the new panels will be developed while EID-SEARCH is still running. If this new project is funded, we will work with you to determine what should be done concurrently with EID-SEARCH or after EID-SEARCH is completed and the best way to budget this (mind you, EcoHealth also hopes to get a continuation on our EID-SEARCH project so it is likely both will be concurrent). Assuming the best case, we submit this in February (Due date Feb 3, 2023) and start work in Fall 2023. Depending on how we stage our tasks, re-testing work could start as early as late 2024 or 2025.

One additional note – this project would be subject to new data sharing requirements NIH has enacted for projects starting 2023 or later. In short, all data we generate out of the project would be published at the end of the project period, whether or not publications are complete. We don't anticipate this would be an issue but want to make sure everyone is aware of this from the start of the grant so we can plan data and manuscript publication accordingly.

We'd like to have you as Key Personnel on this proposal. If you are interested, we'd love to have you aboard and get your thoughts and questions. (b) (6) and I will also follow up next week with a proposal budget. We would also need an NIH Biosketch, Other Support form, and Facilities information from you by January 20. We have recent versions of all of these for EID-SEARCH, so we can send templates for you to modify and approve early next week.

Please let us know what you think, and we'd be happy to have a call to discuss.

Best,

(b) (6)

[redacted]

(b) (6)

(b) (5)

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From: (b) (6)
To: (b) (6)
Cc: Eric Laing; (b) (6)
Subject: Re: Letter of Intent submission for Pilot Research Program 2023
Date: Sunday, December 11, 2022 8:41:53 PM
Attachments: LOI - EID-SEARCH (Thailand).pdf
Biosketch-(b) (6) .pdf
Biosketch-(b) (6) .pdf

Dear Prof (b) (6) ,

My apologies as I did not send the attachment so here it is attached.

Best regards,
(b) (6)

On Sun, Dec 11, 2022 at 8:16 AM (b) (6) wrote:

> Dear Prof (b) (6)
>
> I applied the NIH pilot study in title "Immune memory bait & capture to identify emerging henipavirus origins" last week, please find the attached file.
> This includes the PBMC study in Mojiang antibody positive from human in Ratchaburi. Dr. (b) (6) is the mentor in this study.
> Please advice how three labs can collaborate on this study.
>
> Thank you very much
>
> Best regards,
> (b) (6)
>
> On Tue 65 BE at 00:32 CREID Info <info@creid-network.org> wrote:
>
> > Dear (b) (6),
> >
> > Thank you for submitting a Letter of Intent for the CREID Network Pilot Research Program. We will follow up with you this week if we have questions. As a reminder, full applications are due by **January 30, 2023 at 5pm ET**.
> >
> > Best wishes,
> > CREID Coordinating Center
> > info@creid-network.org
> > https://creid-network.org/pilot-program

From: (b) (6) >
Sent: Monday, December 5, 2022 3:05 AM
To: CREID Info <info@creid-network.org>
Cc: (b) (6)
Eric Laing (b) (6)
Subject: Letter of Intent submission for Pilot Research Program 2023

To whom it may concern,

We are part of EID-SEARCH research group and we would like to submit for the Pilot Research Program 2023.

Please find attached the three documents including;

1. Letter of Intent (LOI)

2. Biosketch of the applicant PI

3. Biosketch of the applicant Co-PI

If you require any further information, please do not hesitate to contact us.

Best regards,

(b) (6)

(b) (5)

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(b) (5)

From: (b) (6)
To: (b) (6)
Cc: (b) (6) ; eric.laing (b) (6)
Subject: Re: CREID Network pilot research proposal 2023
Date: Friday, December 2, 2022 6:26:22 AM
Attachments: Biosketch (b) (6) .pdf
Biosketch (b) (6) .pdf
1 LOI Thailand.docx

Dear (b) (6) ,

Please find attached the draft of the LOI from Thailand. The abstract was modified by (b) (6).
The attached includes my and (b) (6)s biosketch.

Thank you very much

Best regards,
(b) (6)

On Tue, Nov 29, 2022 at 8:47 AM (b) (6) wrote:

> Dear All,
>
> Thank you for confirming your availability, let's have a quick meeting on:
>
> Wednesday, Nov 30 at 8:00 AM (BKK time)
> Tuesday, Nov 29 at 8:00 PM (Eastern time)
>
> We will discuss the general idea to confirm the information for the LOI (template attached)
>
> I have sent you a calendar invite, also including the Zoom link below, look forward to speaking with you!
>
> (b) (6) - it will be helpful if you're free to join the discussion.
>
> Best regards,
> (b) (6)
>
> Join Zoom Meeting
> https://ecohealthalliance-org.zoom.us/j/94509492583?pwd=eUwyL05vUWZLUDV3RFljbDFaZVJhdz09
>
> Meeting ID: 945 0949 2583
> Passcode: thailand
>
> On Wed, Nov 23, 2022 at 11:09 PM (b) (6) wrote:
>
> > Dear (b) (6) ,
> >
> > Thank you for the great news!
> > I am available on Monday. What time do you prefer?
> > Please let us know.
> >
> > Thank you very much
> > Best regards,
> > (b) (6)
> >
> > On Thu, Nov 24, 2022 at 7:43 AM (b) (6) wrote:
> >
> > > Hi (b) (6) ,
> > >
> > > Great news! I will be free either day.
> > >
> > > V/R,
> > > (b) (6)

Sent from my iPhone

On Nov 23, 2022, at 11:44 PM, (b) (6) wrote:

Dear (b) (6),

Thanks for sharing the proposal abstract!

(b) (6) reviewed all proposed ideas and think this one perfectly aligns with EID-SEARCH's research objectives and will help answer some important questions.

However, can we meet to further discuss the title and writing regarding the significance of this work? The current text sounds a bit like adding additional lab procedures that have already been covered by current EID-SEARCH work, so we need to make it sound more significant...And please read through the instruction here for the proposal preparation https://creid-network.org/documents/pilot-program/2023/CREIDPilotProgram_CallforApplications_2023.pdf to see the research and objective priorities (e.g., well-defined hypothesis, and human subject research in LOI, etc.)

Please let me know when you will be available for a quick *call anytime next Monday or Tuesday.*

I'm re-attaching two successful applications, as well as some LOIs examples for your reference.

Best,
(b) (6)

On Sat, Nov 19, 2022 at 8:42 AM (b) (6) wrote:

Dear (b) (6),

Please find attached the abstract for applying to the CREID Pilot Research Program 2023 from Emerging Infectious Diseases Clinical Center (EIDCC), Thailand.
If you require any further information, please feel free to contact us.

Thank you very much

Best Regards,
(b) (6)

On Wed, Nov 16, 2022 at 5:08 PM (b) (6) > wrote:

Thank you, (b) (6)!

(b) (6),

Attached are the two successful applications for 2021 and 2022 for your reference.

And can you please quickly write up one paragraph of your proposed idea (like the abstract) **before Monday next week**? We have received eight requests to collaborate this year, so (b) (6) and I need to review all of them together on Monday to decide which three to support, and it would be great if you can send something written-down.

Best,
(b) (6)

(b) (6)

On Wed, Nov 16, 2022 at 4:01 PM (b) (6) wrote:

> Dear All,
>
> Attached please find the proposal that we submitted for last year's pilot research program for CREID network for your reference.
> Please note that the letter of intent needs to be submitted by December 5, 2022.
>
> Please feel free to ask me or (b) (6) any questions you may have.
>
> Best regards,
> (b) (6)

<Final - CREIDApplication(b) (6) .Proposal.pdf>
<CREID_Pilot_Program_Application(b) (6) _FINAL_PDF_FOR_SUBMISSION.pdf>
<LOI(b) (6).EID-SEARCH.pdf>
<LOI final signed(b) (6) .pdf>

(b) (5)

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From: (b) (6)
To: (b) (6) ; Laing, Eric (b) (6)
Subject: RE: [EXTERNAL] NIH Biosketch for Liberia R01 proposal
Date: Tuesday, November 29, 2022 12:16:26 PM
Attachments: biosketch(b) (6) .docx

(b) (6)

From: (b) (6)
Sent: Tuesday, November 29, 2022 10:01 AM
To: Laing, Eric (b) (6)
Subject: [EXTERNAL] NIH Biosketch for Liberia R01 proposal

Hello, all!

Can you please send me your current NIH-formatted biosketches for inclusion in the Liberia R01 proposal? I would like to collect them before the holidays, so if you could send them by December 21st, that would be excellent.

I've attached the current format (and here is the NIH biosketch webpage). Please let me know if I can answer any questions.

Thank you,
(b) (6)

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From: (b) (6)
To: (b) (6)
Cc: (b) (6); eric.laing (b) (6)
Subject: CREID Network pilot research proposal 2023
Date: Wednesday, November 23, 2022 11:45:42 AM
Attachments: Final - CREIDApplication (b) (6) .Proposal.pdf
CREID_Pilot_Program_Application (b) (6) G_FINAL_PDF_FOR_SUBMISSION.pdf
LOI (b) (6) .EID-SEARCH.pdf
LOI final signed (b) (6) .pdf

Dear (b) (6),

Thanks for sharing the proposal abstract!

(b) (6) and (b) (6) reviewed all proposed ideas and think this one perfectly aligns with EID-SEARCH's research objectives and will help answer some important questions.

However, can we meet to further discuss the title and writing regarding the significance of this work? The current text sounds a bit like adding additional lab procedures that have already been covered by current EID-SEARCH work, so we need to make it sound more significant...And please read through the instruction here for the proposal preparation https://creid-network.org/documents/pilot-program/2023/CREIDPilotProgram_CallforApplications_2023.pdf to see the research and objective priorities (e.g., well-defined hypothesis, and human subject research in LOI, etc.)

Please let me know when you will be available for a quick *call anytime next Monday or Tuesday*.

I'm re-attaching two successful applications, as well as some LOIs examples for your reference.

Best,
(b) (6)

On Sat, Nov 19, 2022 at 8:42 AM (b) (6) > wrote:

> Dear (b) (6),
>
> Please find attached the abstract for applying to the CREID Pilot Research Program 2023 from Emerging Infectious Diseases Clinical Center (EIDCC), Thailand.
> If you require any further information, please feel free to contact us.
>
> Thank you very much
>
> Best Regards,
> (b) (6)
>
> On Wed, Nov 16, 2022 at 5:08 PM (b) (6) wrote:
>
> > Thank you, (b) (6)!
> >
> > (b) (6),
> >
> > Attached are the two successful applications for 2021 and 2022 for your reference.

And can you please quickly write up one paragraph of your proposed idea (like the abstract) **before Monday next week**? We have received eight requests to collaborate this year, so (b) (6) , and I need to review all of them together on Monday to decide which three to support, and it would be great if you can send something written-down.

Best,
(b) (6)

[redacted]

On Wed, Nov 16, 2022 at 4:01 PM (b) (6) [redacted] wrote:

> Dear All,
>
> Attached please find the proposal that we submitted for last year's pilot research program for CREID network for your reference.
> Please note that the letter of intent needs to be submitted by December 5, 2022.
>
> Please feel free to ask me or (b) (6) any questions you may have.
>
> Best regards,
> (b) (6)
>
> [redacted]

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From: Laing, Eric (b) (6)
To: (b) (6)
Subject: Re: NIH R01 on serology bioinformatics
Date: Wednesday, November 23, 2022 9:42:40 AM
Attachments: (b) (6) -et-al_bangladesh-bats-cocirculation-serology_2022-08-11(b) (6) .docx

---

Hey (b) (6),

I made some edits on a recent flight but completely lost track of the document. If you are still working on this my edits are attached.

(b) (6)

[redacted]

On Wed, Nov 2, 2022 at 12:46 PM Laing, Eric (b) (6) wrote:

> Excellent!
>
> (b) (6) presented PREMISE to the CREID Lab Group - so cool to see a whole program around serology. Not sure whether I like "dark matter" more than "virus shadows"
>
> (b) (6)
>
> [redacted]
>
> On Wed, Nov 2, 2022 at 12:35 PM (b) (6) wrote:
>
> > Already cited! I have some writing time reserved next week, expect an updated outline.
> >
> > Best,

(b) (6)

[redacted]

On Wed, Nov 2, 2022 at 10:12 AM Laing, Eric (b) (6) > wrote:

Here's an interesting article

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7292646/

(b) (6)

[redacted]

On Tue, Sep 6, 2022 at 8:41 AM (b) (6) > wrote:

Whoops! Fixed.

On Tue, Sep 6, 2022, 8:28 AM Laing, Eric (b) (6) wrote:

Hey (b) (6)

The invite is showing up for Monday (yesterday) Sept 5.

On Mon, Sep 5, 2022 at 8:55 AM (b) (6) wrote:

Great, I've sent a Zoom/Calendar invite for Sep 6 at 1:30PM EST. Talk to you soon!

--

[redacted]

On Fri, Sep 2, 2022 at 3:23 PM Laing, Eric [redacted] wrote:

> Hi Noam,
>
> Apologies for my absence, you have my focus now.
>
> Here are my follow-up items:
>
> ***- I'd like to have a conversation with you about nuts and bolts and budgeting.*** I'm headed out for vacation Friday afternoon through Labor day. ***If you want to chat tomorrow or Friday pick a time*** at this link, if not I will write when I return: https://calendly.com/[redacted]/60-minute-chat-extended-hours
> Can we touch base next Tuesday Sept 6th? I'm free after 1pm EST. I'd like to go over some of the assays mentioned in the pdf you attached.
>
> - ***I'll get you a draft*** with a bulleted section on this stuff that you and [redacted] can make sound like a virologist who knows what they are talking about. I expect this ***around September 12.***
> Sounds good.
>
> - I think it would be good for me to ***come down to USU for a day or two in early-mid September*** for us to work through some of this stuff if you agree and have the time. If so, let's schedule that.
> Yes, I'll be out of town from Sept 18 - 22, but in town until that date.
>
> - [redacted]
>
> [redacted]

(b) (6)

On Wed, Aug 24, 2022 at 4:58 PM (b) (6) > wrote:

> Hi (b) (6),
>
> Hi! I hope you are well. I had a call with (b) (6) yesterday about this grant as I know you've been away for family reasons. (CC'ing him to chat with you about this when he has the chance). We discussed things that we could write into the grant on your side, notably validation/calibration testing using the Filovirus standard coming out of Oxford, and also running tests on the sera from experimental infections that are at BSL-4 labs at RMN, UTMB, and maybe South Africa. These could be used to model how we expect real responses to look in related virus and host species.
>
> Here are my follow-up items:
>
> ***- I'd like to have a conversation with you about nuts and bolts and budgeting.*** I'm headed out for vacation Friday afternoon through Labor day. ***If you want to chat tomorrow or Friday pick a time*** at this link, if not I will write when I return: https://calendly.com(b) (6) /60-minute-chat-extended-hours
>
> - ***I'll get you a draft*** with a bulleted section on this stuff that you and (b) (6) can make sound like a virologist who knows what they are talking about. I expect this ***around September 12.***
>
> - I think it would be good for me to ***come down to USU for a day or two in early-mid September*** for us to work through some of this stuff if you agree and have the time. If so, let's schedule that.
>
> Best,
> (b) (6)

(b) (6)

[redacted]

On Mon, Aug 8, 2022 at 8:51 AM (b) (6) [redacted] wrote:

> Hi (b) (6),
>
> After many false starts, I really am planning to submit an R01 on bioinformatics and statistical methods for Luminex serology in the next cycle (due Oct 6). Everyone came back from the BatID conference really jazzed about this. I still think it works best with you and I as co-PIs. If you are still game, want to have a call about it? I'd still like to come down to USU for a day in the next month, too, if you'd like to spend a day working on it and other related issues.
>
> Schedule a call on my calendar here: https://calendly.com(b) (6) /60-min-chat
>
> Last draft of specific aims attached.
>
> Best,
>
> (b) (6)

[redacted]

– DETAILED SCIENTIFIC AGENDA –

FINAL AGENDA

DAY 1 | WEDNESDAY, SEPTEMBER 21

Scientific Meeting Zoom Link

Time (UMT -4)	Session	Location
13:00-14:00	**Opening, Priorities, and Vision** ▪ Welcome from DMID | (b) (6) ▪ CREID Network Priorities | (b) (6) ▪ Welcome from the CREID Network | (b) (6) ▪ CREID Network Shared Vision | (b) (6)	Fitzgerald B/C & Zoom
14:00-14:15	Break	
14:15-15:15	**Collaboration for Outbreak Research Response & Tabletop Introduction** ▪ (b) (6) ▪ (b) (6) ▪ (b) (6)	Fitzgerald B/C & Zoom
15:15-16:15	**CREID Network Stakeholders: Existing Collaborations** Session Chairs: (b) (6) ▪ PREMISE | (b) (6) ▪ WRCEVA | (b) (6) ▪ TGHN/CSPH Virtual Biorepository | (b) (6) ▪ NIBSC | (b) (6) ▪ Abbott Pandemic Defense Coalition | (b) (6) ▪ Benefit sharing and preparing for downstream translation | (b) (6)	Fitzgerald B/C & Zoom
16:15-16:30	Break	
16:30-16:45	**CREID Network Stakeholders: Potential Collaborations** Session Chairs: (b) (6) ▪ (b) (6)	Fitzgerald B/C & Zoom
16:45-17:00	**Day 1 Takeaways** (b) (6)	Fitzgerald B/C & Zoom

DAY 2 | THURSDAY, SEPTEMBER 22

Scientific Meeting Zoom Link

Time (UMT -4)	Session (Virtual Presentation)	Location
11:00-12:00	**CREID Research Center Y2 Review Panels** Session Chair: (b) (6) ▪ EID-SEARCH | (b) (6) ▪ UWARN | (b) (6) ▪ CREID-ESP | (b) (6) ▪ WARN-ID | (b) (6) ▪ EEIDI | (b) (6)	Fitzgerald B/C & Zoom
12:00-13:00	Lunch *Participants obtain on their own.*	
13:00-14:00	**CREID Research Center Y2 Review Panels (continued)** Session Chair: (b) (6) ▪ PICREID | (b) (6) ▪ CREATE-NEO | (b) (6) ▪ CREID-ECA | (b) (6) ▪ WAC-EID | (b) (6) ▪ A2CARES | (b) (6)	Fitzgerald B/C & Zoom
14:00-15:00	**Oral Presentations 1: Identifying and Characterizing Emerging Pathogens** Session Chair: (b) (6) Session Co-Chair: (b) (6) ▪ Rift Valley Fever and Crimean-Congo Hemorrhagic Fever in Senegal: animal seroprevalence as indicator of virus circulation in nature | (b) (6) ▪ Evidence of co-circulation of multiple endemic arboviruses based on syndromic sentinel surveillance in Senegal | (b) (6) ▪ Multi-RC Arbovirus Active Surveillance in Mosquito Enzootic Vectors and Potential Host in Panama | (b) (6) ▪ From malaria to fevers of unknown origins: genomic surveillance in Senegal | (b) (6) ▪ Retrospective investigation of horses with encephalitis reveals unnoticed circulation of West Nile Virus in Northeastern Brazilian states | (b) (6) ▪ Dengue-2 Cosmopolitan genotype detection and emergence in South America | (b) (6)	Fitzgerald B/C & Zoom
15:10-15:30	Break	
15:30-16:40	**Oral Presentations 2: Ecology, Environs and Entomology** Session Chair: (b) (6) Session Co-Chair: (b) (6) ▪ High burden of Arbovirus in Remote Rural Villages under environmental change in Ecuador | (b) (6) ▪ Social constructs of place and their relevance in locating dengue fever outbreaks | (b) (6) ▪ Ecology of Aedes-transmitted arboviruses and their vectors in sylvatic and urban settings of Senegal: entomological findings | (b) (6) ▪ Multi-RC Implications of land used and land coverage in the emergence of Madariaga Encephalitis in an endemic region of Venezuelan Equine Encephalitis Virus in Eastern Panama | (b) (6) ▪ Multi-RC Ecological features of potential Madariaga and Venezuelan equine encephalitis virus enzootic hosts in Panama | (b) (6) ▪ Larval microbiome by Aedes aegypti genotype interactions drive susceptibility to Zika virus | (b) (6)	Fitzgerald B/C & Zoom
16:40-17:00	**Day 2 Takeaways** (b) (6)	Fitzgerald B/C & Zoom

Acronyms (p7)

DAY 3 | FRIDAY, SEPTEMBER 23

Scientific Meeting Zoom Link

Time (UMT -4)	Session (Virtual Presentation)	Location
11:00-12:00	**2021 Pilot Awardee Presentations** Session Chairs: (b) (6) ▪ Investigation of the spatiotemporal dynamics and ecological drivers of enzootic arbovirus circulation in non-human primates in Minas Gerais State/Southeast Brazil | (b) (6) ▪ Hantavirus detection and characterization in humans and rodents from Cambodia | (b) (6) ▪ Defining antiviral humoral immunity against SARS-CoV-2 in Kenya | (b) (6) ▪ Multi-species farming, viral diversity, and risks of cross-species transmission in the Central Highlands of Viet Nam | (b) (6) ▪ Revealing vector species with potential to mediate pathogen spillover from wildlife to livestock in the Pantanal | (b) (6) ▪ Development of a real-time pathogen surveillance system in Jordan | (b) (6)	Fitzgerald B/C & Zoom
12:00-13:00	Lunch *Participants obtain on their own.*	
13:00-14:30	**Lightning Talk Concurrent Sessions** – *see pp4-6 for details* A: Ecology, Entomology and Field Methods B: One Health and Zoonotic Surveillance C: Clinical and Laboratory Science D: Epidemiology and Surveillance Across the Americas E: Epidemiology and Surveillance Across Africa and Asia	 Fitzgerald B/C & Zoom Fitzgerald A North & Zoom Fitzgerald A South & Zoom Warfields & Zoom Grason & Zoom
14:30-14:50	Break	
14:50-16:00	**Oral Presentations 3: Advanced Tools, Techniques and Late Breakers** Session Chair: (b) (6) Session Co-Chair: (b) (6) ▪ An enrichment method for capturing SARS-CoV-2-related whole genome sequences directly from bat samples | (b) (6) ▪ Multi-RC Real-time RT-PCR for Venezuelan equine encephalitis complex, Madariaga and Eastern equine encephalitis viruses: application in clinical diagnostic and mosquito surveillance | (b) (6) ▪ Simple and economical extraction of viral RNA and storage at ambient temperature | (b) (6) ▪ Late breaker: Serological evidence of significant Middle East Respiratory Syndrome coronavirus transmission to humans among camel-owning households in Northern Kenya | (b) (6) ▪ Late breaker: Concurrent non-human primate, bat, mosquito, and human One Health surveillance in the Peruvian Amazon 2021-2022 | (b) (6)	Fitzgerald B/C & Zoom
16:00-16:15	**Closing Remarks** (b) (6)	Fitzgerald B/C & Zoom

2022 Pilot Awardee ePosters

(*see Between Session slides*)

- Investigation of ecological drivers of sarbecoviruses spillover in Myanmar and Nepal | (b) (6)
- Vector surveillance in context of urban transmission and spread of Crimean-Congo hemorrhagic fever virus (CCHFV), Karachi Pakistan | (b) (6)
- In the Air Tonight: Metagenomic Pathogen Discovery as Tools in Pathogen Surveillance | (b) (6)
- (b) (6)
- Pathogenic Mammarenaviruses and Orthohantaviruses in Argentina | (b) (6)
- Surveillance for known and novel viruses with zoonotic potential at the interface between humans and livestock in Kenya | (b) (6)
- Characterization of the mosquito microbiome and its role in arbovirus emergence and maintenance in Senegal | (b) (6)

Lightning Talk Concurrent Session Details

Day 3 | Friday, September 23 | 13:00-14:30

Concurrent Session A: Ecology, Entomology and Field Methods Location: Fitzgerald B/C & Zoom

Session Chair: (b) (6)

Session Co-Chair: (b) (6)

- Building capacity for ecological surveillance and molecular diagnostics of mosquito-borne viruses in Sierra Leone | (b) (6)
- Active surveillance to detect low-level Rift Valley Fever Virus transmission in humans in East and Central Africa | (b) (6)
- The use of satellite imagery to create robust geospatial sampling designs and examine landscape dynamics across an urban to rural gradient in Managua, Nicaragua | (b) (6)
- Higher infestation indices of the vector Aedes aegypti in rural areas than in urban areas in Managua, Nicaragua | (b) (6)
- Rainfall and the prevalence of Aedes aegypti in northern coastal Ecuador | (b) (6)
- Setting the standard for field biosafety at the human-animal interface | (b) (6)
- Effective Recruitment Strategies during an Infectious Disease Surveillance Study at Rural Health Facilities in Liberia; WARN-ID Liberia Team Experience | (b) (6)
- Environmental surveillance of informal sewage systems reveals community SARS-CoV-2 transmission dynamics | (b) (6)
- Mosquito ecology and risk of arboviral infectious disease spillover in southwestern Uganda | Jalika Joyner (EEIDI)
- Report of arbovirosis surveillance prospective cohort as model of early detection of viral circulation | (b) (6)
- Towards the laboratory maintenance of Haemagogus janthinomys, the major neotropical vector of sylvatic yellow fever | (b) (6)

Lightning Talk Concurrent Session Details, continued

Concurrent Session B: One Health and Zoonotic Surveillance Location: Fitzgerald A North & Zoom

Session Chair: (b) (6)

Session Co-Chair (b) (6)

- One Health Pathogen Surveillance in the Bwindi Impenetrable Forest Region of Uganda | (b) (6)
- Use of molecular epidemiological, serological and experimental approaches to study COVID-19 transmission in Hong Kong | (b) (6)
- Evidence of Bourbon virus in ticks and humans in St. Louis Missouri, USA | (b) (6)
- Potential reservoirs hosts zoonotic pathogens in Senegal including emerging viruses such arboviruses, Lassa, Ebola, coronaviruses and henipaviruses | (b) (6)
- A One Health investigation framework for zoonotic and vector-borne disease outbreaks | (b) (6)
- Coronavirus Circulation in Peridomestic Rodent Populations in Sierra Leone | (b) (6)
- One Health surveillance of Lassa fever in rodents and human close contacts in North Central Nigeria | (b) (6)
- Plasmodium vivax infection in two non-human primates in the Amazon | (b) (6)
- YF-neutralizing antibodies in pied tamarins (Saguinus bicolor) captured in Amazon Rainforest fragments in the urban area of Manaus, Brazil | (b) (6)
- CREATE-NEO arboviral surveillance in mosquitoes, febrile humans and non-human primates in transition zones in Panama and Darien | (b) (6)
- Late Breaker: Predicting the zoonotic capacity of mammals to transmit SARS-CoV-2 | (b) (6)
- Late Breaker: Coronavirus surveillance among farmed collared peccaries (Dicotyles tajacu) and caretakers in the Peruvian Amazon | (b) (6)
- Late Breaker: SARS-CoV-2 Genomic Variant Surveillance in Human and Non-Human Primates in Peru | (b) (6)

Concurrent Session C: Clinical and Laboratory Science Location: Fitzgerald A South & Zoom

Session Chair: (b) (6)

Session Co-Chair: (b) (6)

- Acute Neurologic Syndromes associated with Chikungunya virus infections in Salvador, Brazil | (b) (6)
- Predictors of severity in dengue-suspected pediatric patients during 2019 dengue epidemic in Brazil | (b) (6)
- Influence of previous Zika virus exposure on Brazilian dengue outbreak in 2019 | (b) (6)
- Immune escape mutations in the Spike protein of an endemic SARS-CoV-2 variant in Panama | (b) (6)
- The highly conserved stem-loop II motif is important for the lifecycle of astroviruses but dispensable for SARS-CoV-2 | (b) (6)
- Antibody fucosylation predicts disease severity in secondary dengue infection | (b) (6)
- Unexpected Acute Viral Fever Mimicking Dengue-Like Illness in Major City of Pakistan | (b) (6)
- Comparison of the Immunogenicity of five COVID-19 vaccines in Sri Lanka | (b) (6)
- SARS-CoV-2 Variant Detection and Surveillance with an Economical and Scalable Molecular Protocol | (b) (6)
- Multiplexed detection of respiratory viruses and SARS-CoV-2 variants with mCARMEN | (b) (6)
- Developing Rapid Antigen Diagnostics for Emerging Viruses using Antibodies Cloned from Sorted Single Memory B-cells | (b) (6)

- A simplified Cas13-based assay for the identification of SARS-CoV-2 and its variants | (b) (6)
- Late Breaker: Dynamics of infectious virus neutralization from convalescent and vaccine cohorts across global SARS-CoV-2 variant lineages reveals boost protection and novel monoclonal antibody efficacy against Omicron strains | (b) (6)

Concurrent Session D: Epidemiology and Surveillance Across the Americas Location: Warfields & Zoom

Session Chair: (b) (6)
Session Co-Chair (b) (6)

- A Dengue outbreak in Panama, 2022 | (b) (6)
- Seroprevalence of Zika, Dengue, and Chikungunya viruses in a rural area in Brazil | (b) (6)
- Zika virus infection surveillance in Manaus, Amazonas state | (b) (6)
- Real-time genomic surveillance of DENV-1 and DENV-2 in Brazil: improving public health outbreaks response | (b) (6)
- Surveillance and evolutionary analysis of Dengue viruses to understand the epidemiological dynamics of dengue outbreaks, São José do Rio Preto, São Paulo, Brazil | (b) (6)
- Monitoring the genetic diversity of reemerging chikungunya virus in Brazil | (b) (6)
- Genomic and epidemiological monitoring of YFV reemergence in Brazil: unveiling the corridor of spread and the geographic hot spots for predicting and preventing other possible spillover events | (b) (6)
- Genomic surveillance of SARS-CoV-2 in symptomatic vaccinated and unvaccinated asymptomatic patients in Brazil | (b) (6)
- SARS-CoV-2 genomic surveillance and the impact of different lineages circulation in the epidemiological landscape of São José do Rio Preto, São Paulo, Brazil | (b) (6)
- Genomic epidemiology reveals the impact of national and international restrictions on the SARS-CoV-2 epidemic in Brazil | (b) (6)
- Occurrence of SARS-CoV-2 reinfections at regular intervals in Ecuador | (b) (6)
- Genomic characterization of SARS-CoV-2 during the COVID-19 pandemic in Nicaragua | (b) (6)

Concurrent Session E: Epidemiology and Surveillance Across Africa and Asia Location: Grason & Zoom

Session Chair: (b) (6)
Session Co-Chair: (b) (6)

- Flaviviruses and Lassa fever in febrile patients in North Central Nigeria: A cross-sectional study | (b) (6)
- Double Stigma and discrimination: A qualitative study of Lassa fever and hearing loss in Northern Nigeria | (b) (6)
- A brief chronicle of SARS-CoV-2 genomic surveillance in Cambodia | (b) (6)
- Identification of Genetic Variations of SARS-CoV-2 Omicron Strain and their Clinical Significance in Karachi, Pakistan | (b) (6)
- Genetic characterization of Norovirus strains from an unusual diarrheal outbreak in the community in Thailand | (b) (6)
- Factors associated with changing Dengue case numbers during the COVID-19 pandemic in Sri Lanka | (b) (6)
- SARS-CoV-2 genomic epidemiology in Sierra Leone | (b) (6)
- Yellow Fever outbreak in eastern Senegal, 2020–2021 | (b) (6)
- The evolving SARS-CoV-2 epidemic in Africa: Insights from rapidly expanding genomic surveillance | (b) (6)

- Detection and Characterization of Variants of Concern: Insights from the South African Epidemic | (b) (6) [redacted]
- Emergence of novel combinations of SARS-CoV-2 spike receptor binding domain variants in Senegal | (b) (6) [redacted]
- COVID-19 laboratory surveillance at IRESSEF in Senegal | (b) (6) [redacted]
- Dynamics of Variants of Concern (VOC) during the different waves of COVID-19 in Senegal | (b) (6) [redacted]

Acronyms and Abbreviations

A2CARES	American and Asian Centers for Arboviral Research and Enhanced Surveillance
BV-BRC	Bacterial and Viral Bioinformatics Resource Center
CEPI	Coalition for Epidemic Preparedness Innovations
CREATE-NEO	Coordinating Research on Emerging Arboviral Threats Encompassing the Neotropics
CREID	Centers for Research in Emerging Infectious Diseases
CREID CC	CREID Coordinating Center
CREID-ECA	Center for Research in Emerging Infectious Diseases – East and Central Africa
CREID-ESP	Center for Research in Emerging Infectious Diseases – Epidemiology, Surveillance and Pathogenesis
CSPH	Colorado School of Public Health
DMID	Division of Microbiology and Infectious Diseases
EEIDI	EpiCenter for Emerging Infectious Disease Intelligence
EID-SEARCH	Emerging Infectious Diseases: South East Asia Research Collaboration Hub
NIAID	National Institute of Allergy and Infectious Diseases
NIBSC	National Institute for Biological Standards and Controls
NIH	National Institutes of Health
PICREID	Pasteur International Center for Research on Emerging Infectious Diseases
PREMISE	Pandemic Response Repository through Microbial and Immunological Surveillance and Epidemiology
RC	Research Center
TGHN	The Global Health Network
UWARN	United World Antiviral Research Network
WAC-EID	West African Center for Emerging Infectious Diseases
WARN-ID	West African Research Network for Infectious Diseases
WRCEVA	World Reference Center for Emerging Viruses and Arboviruses

From: (b) (6)
To: (b) (6)

[redacted]

Eric Laing; (b) (6)

[redacted]

Cc: (b) (6)

Subject: RE: CREID Scientific Meeting Day 1
Date: Wednesday, September 21, 2022 1:26:52 PM
Attachments: CREIDAgenda_Detailed_Scientific_v2.pdf

Hi all,

Please find an updated agenda with scientific meeting zoom link attached.

Thank you,

(b) (6)

-----Original Appointment-----
From: (b) (6)
Sent: Friday, September 16, 2022 3:58 PM
To: (b) (6)

(b) (6)

[redacted]

(b) (6) [redacted] eric.laing@(b) (6) [redacted]

(b) (6) [redacted]

Cc: (b) (6) [redacted]

Subject: CREID Scientific Meeting Day 1
When: Wednesday, September 21, 2022 1:00 PM-5:00 PM (UTC-05:00) Eastern Time (US & Canada).
Where:

Hello CREID Scientific Meeting Attendees,

Please plan to join the **Scientific Meeting on Day 1** of the Annual Meeting. Final agenda and materials will be shared on Monday, September 19.

In-person attendees, please go to (b) (6) [redacted]
Virtual attendees, please use the following zoom link for this meeting:
https://explorepsa.zoomgov.com/j/1619281154?pwd=T0JlL1FHK3p2cU9nQ3h2TzFLaHdhQT09

Thank you,

(b) (6) [redacted]

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From: (b) (6)
To: (b) (6)
Cc: (b) (6); eric.laing(b) (6)
Subject: Re: Proposal submitted last year for CREID Network
Date: Wednesday, November 16, 2022 5:08:42 AM
Attachments: Final - CREIDApplication(b) (6).Proposal.pdf
CREID_Pilot_Program_Application_S(b) (6)_FINAL_PDF_FOR_SUBMISSION.pdf

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Thank you, (b) (6)!

(b) (6),

Attached are the two successful applications for 2021 and 2022 for your reference.

And can you please quickly write up one paragraph of your proposed idea (like the abstract) **before Monday next week**? We have received eight requests to collaborate this year, so (b) (6), and I need to review all of them together on Monday to decide which three to support, and it would be great if you can send something written-down.

Best,
(b) (6)

[redacted]

On Wed, Nov 16, 2022 at 4:01 PM (b) (6) > wrote:

> Dear All,
>
> Attached please find the proposal that we submitted for last year's pilot research program for CREID network for your reference.
> Please note that the letter of intent needs to be submitted by December 5, 2022.
>
> Please feel free to ask me or (b) (6) any questions you may have.
>
> Best regards,
> (b) (6)
>
> [redacted]

(b) (6)

CREID Annual Meeting

Welcome

Dear Meeting Attendees,

We are pleased to welcome you to the 2022 CREID Network Annual Meeting. With a focus on the science, the 2022 Annual Meeting will showcase the diverse ongoing research by members of the CREID Network.

The CREID Network was established in 2020 with overarching goals of improved rapid and coordinated outbreak research response and capacity building. CREID is a coordinated network operating in regions around the globe where emerging and re-emerging infectious disease outbreaks are likely to occur. Multidisciplinary teams of investigators are conducting pathogen/host surveillance, studying pathogen transmission, pathogenesis, and immunologic responses in the host, and developing reagents and diagnostic assays for improved detection of important emerging pathogens and their vectors.

This is the first Annual Meeting to include in-person attendees and we eagerly anticipate the collaboration and relationship building that will bring. Those of you joining virtually have an equally valuable contribution to make. We look forward to your engaged participation around this meeting and moving forward as CREID continues to develop its global network.

We look forward to a successful meeting,

Sincerely,
DMID and CREID Coordinating Center Teams

CREID
Centers for Research in Emerging Infectious Diseases

Contact

CREID Coordinating Center
info@creid-network.org

Meeting Venue

Sheraton Baltimore North

Address 903 Dulaney Valley Rd, Towson, MD 21204
Phone (410) 321-7400

On-Site Food

Coffee Corner Marketplace

Daily 6:30AM – 2:00PM

Rain 903 Restaurant

Monday - Friday 6:30AM – 10:00AM and 5:00PM – 10:00PM
Saturday—Sunday 7:00AM – 11:00AM and 5:00PM – 10:00PM

CREID
Centers for Research in Emerging Infectious Diseases

Transportation

To the Venue

BWI Airport to Sheraton Baltimore North

Driving Directions

~35 minutes via I-695

https://goo.gl/maps/ELBk7kbMfyGmHw2NA

Metro Directions

~1 hour, 30 minutes via

https://goo.gl/maps/ELBk7kbMfyGmHw2NA

Closest Metro Stop (6 minute walk)

Fairmont & Delaney Valley Rd: CityLink RED Line

Closest Bus Stop (6 minute walk)

Towson Town Center Bay 1: Bus 51

Beyond Towson and Baltimore, MD

Take a CityLink light rail from Towson Town Center Bay 2 and an Amtrak from Baltimore Penn Station into **Washington D.C.** to tour the famous National Monuments and Memorials (use Google Maps for detailed directions).

https://washington.org/visit-dc/monuments-memorials

No-Host Social Hours*

Please join CREID Network members for no-host social hours Tuesday, Wednesday, and Thursday so we can get to know each other outside of the formal meeting.

Tuesday, September 20, 6:00pm

World of Beer

125 E. Joppa Road, Towson, MD

Featuring 500+ global beers, tavern food

~15 minute walk

Wednesday, September 21, 6:00pm

The Point in Towson

523 York Road, Towson, MD

American eats, beers, & cocktails

~15 minute walk

Thursday, September 22, 6:00pm

7 West Bistro Grille

7 W. Chesapeake Avenue, Towson, MD

Mediterranean tapas & creative American dishes

~17 minute walk

* This is an optional activity and there is no central coordination of these events.

CREID
Centers for Research in Emerging Infectious Diseases

Food and Drink Nearby

Towson Town Center

0.3 miles; 7 minute walk

Shopping mall with many dining options:

- P.F. Chang's
- The Cheesecake Factory
- Stoney River Steakhouse and Grill
- Many more

Towson Tavern

0.6 miles; 14 minute walk

516 York Rd, Towson, MD 21204

Pollo Amigo

0.6 miles; 13 minute walk

714 York Road, Towson, MD

Whole Foods Market

0.8 miles; 17 minute walk

300 Towson Row, Towson, MD

FOD Poke Bar

0.7 miles; 15 minute walk

402 York Rd, Towson, MD 21204

The Fresh Market

0.3 miles; 8 minute walk

838 Dulaney Valley Road, Towson, MD

Other Nearby Restaurants

https://goo.gl/maps/f9XhD1Ypr75vNNPt8

CREID
Centers for Research in Emerging Infectious Diseases

Things to Do

Inner Harbor Baltimore

Water Taxi Ride for $20 with 13 destination points

National Aquarium

Maritime Museums Historic Ships in Baltimore, USCG Lightship Chesapeake, Seven Foot Knoll Lighthouse

Local Seafood Eat the Maryland delicacy, crab, and many other seafood delights at one of the many waterfront restaurants (may be pricier on the harbor)

baltimorewatertaxi.com

Baltimore Museums and Monuments

Fort McHenry National Monument used in the War of 1912 to defend the Baltimore Harbor, now a historic site

Baltimore Museum of Art with 19th-century, modern and contemporary art

American Visionary Art Museum with unusual and unique modern art

Reginald F. Lewis Museum Maryland African-American History and Culture

Edgar Allan Poe House and Museum

State Parks and Nature Trails

Patapsco Valley State Park – Hollofield Area; Maryland's largest state park along 32 miles of the Patapsco River south and west of the city

Patterson Park an urban park featuring 173 acres of open fields of grass, large trees, paved walkways, historic battle sites, a lake, playgrounds, athletic fields, a swimming pool, an ice skating rink and other signature attractions and buildings

Jones Falls a 17.9-mile-long stream running through the city

Lake Montebello a reservoir with a 1.4 mile biking and walking path

Sports and Other

Baltimore Orioles watch the Baltimore baseball team play a home game at Oriole Park at Camden Yards Sep. 19-25.

Maryland Zoo located in the historic Druid Hill Park in northwestern Baltimore

mlb.com/orioles/schedule/2022-09

– DETAILED SCIENTIFIC AGENDA –

FINAL AGENDA

DAY 1 | WEDNESDAY, SEPTEMBER 21

Scientific Meeting Zoom Link

Time (UMT -4)	Session	Location
13:00-14:00	**Opening, Priorities, and Vision** ▪ Welcome from DMID | (b) (6) ▪ CREID Network Priorities | (b) (6) ▪ Welcome from the CREID Network | (b) (6) ▪ CREID Network Shared Vision | (b) (6)	Fitzgerald B/C & Zoom
14:00-14:15	Break	
14:15-15:15	**Collaboration for Outbreak Research Response & Tabletop Introduction** ▪ (b) (6) ▪ (b) (6) ▪ (b) (6)	Fitzgerald B/C & Zoom
15:15-16:15	**CREID Network Stakeholders: Existing Collaborations** Session Chairs: (b) (6) ▪ PREMISE | (b) (6) ▪ WRCEVA | (b) (6) ▪ TGHN/CSPH Virtual Biorepository | (b) (6) ▪ NIBSC | (b) (6) ▪ Abbott Pandemic Defense Coalition | (b) (6) ▪ Benefit sharing and preparing for downstream translation | (b) (6)	Fitzgerald B/C & Zoom
16:15-16:30	Break	
16:30-16:45	**CREID Network Stakeholders: Potential Collaborations** Session Chairs: (b) (6) ▪ (b) (6)	Fitzgerald B/C & Zoom
16:45-17:00	**Day 1 Takeaways** (b) (6)	Fitzgerald B/C & Zoom

DAY 2 | THURSDAY, SEPTEMBER 22

Scientific Meeting Zoom Link

Time (UMT -4)	Session (Virtual Presentation)	Location
11:00-12:00	**CREID Research Center Y2 Review Panels** Session Chair: (b) (6) ▪ EID-SEARCH | (b) (6) ▪ UWARN | (b) (6) ▪ CREID-ESP | (b) (6) ▪ WARN-ID | (b) (6) ▪ EEIDI | (b) (6)	Fitzgerald B/C & Zoom
12:00-13:00	Lunch *Participants obtain on their own.*	
13:00-14:00	**CREID Research Center Y2 Review Panels (continued)** Session Chair: (b) (6) ▪ PICREID | (b) (6) ▪ CREATE-NEO | (b) (6) ▪ CREID-ECA | (b) (6) ▪ WAC-EID | (b) (6) ▪ A2CARES | (b) (6)	Fitzgerald B/C & Zoom
14:00-15:00	**Oral Presentations 1: Identifying and Characterizing Emerging Pathogens** Session Chair: (b) (6) Session Co-Chair: (b) (6) ▪ Rift Valley Fever and Crimean-Congo Hemorrhagic Fever in Senegal: animal seroprevalence as indicator of virus circulation in nature | (b) (6) ▪ Evidence of co-circulation of multiple endemic arboviruses based on syndromic sentinel surveillance in Senegal | (b) (6) ▪ Multi-RC Arbovirus Active Surveillance in Mosquito Enzootic Vectors and Potential Host in Panama | (b) (6) ▪ From malaria to fevers of unknown origins: genomic surveillance in Senegal | (b) (6) ▪ Retrospective investigation of horses with encephalitis reveals unnoticed circulation of West Nile Virus in Northeastern Brazilian states | (b) (6) ▪ Dengue-2 Cosmopolitan genotype detection and emergence in South America | (b) (6)	Fitzgerald B/C & Zoom
15:10-15:30	Break	
15:30-16:40	**Oral Presentations 2: Ecology, Environs and Entomology** Session Chair: (b) (6) Session Co-Chair: (b) (6) ▪ High burden of Arbovirus in Remote Rural Villages under environmental change in Ecuador | (b) (6) ▪ Social constructs of place and their relevance in locating dengue fever outbreaks | (b) (6) ▪ Ecology of Aedes-transmitted arboviruses and their vectors in sylvatic and urban settings of Senegal: entomological findings | (b) (6) ▪ Multi-RC Implications of land used and land coverage in the emergence of Madariaga Encephalitis in an endemic region of Venezuelan Equine Encephalitis Virus in Eastern Panama | (b) (6) ▪ Multi-RC Ecological features of potential Madariaga and Venezuelan equine encephalitis virus enzootic hosts in Panama | (b) (6) ▪ Larval microbiome by Aedes aegypti genotype interactions drive susceptibility to Zika virus | (b) (6)	Fitzgerald B/C & Zoom
16:40-17:00	**Day 2 Takeaways** (b) (6)	Fitzgerald B/C & Zoom

DAY 3 | FRIDAY, SEPTEMBER 23

Scientific Meeting Zoom Link

Time (UMT -4)	Session (Virtual Presentation)	Location
11:00-12:00	**2021 Pilot Awardee Presentations** Session Chairs: (b) (6) ▪ Investigation of the spatiotemporal dynamics and ecological drivers of enzootic arbovirus circulation in non-human primates in Minas Gerais State/Southeast Brazil | (b) (6) ▪ Hantavirus detection and characterization in humans and rodents from Cambodia | (b) (6) ▪ Defining antiviral humoral immunity against SARS-CoV-2 in Kenya | (b) (6) ▪ Multi-species farming, viral diversity, and risks of cross-species transmission in the Central Highlands of Viet Nam | (b) (6) ▪ Revealing vector species with potential to mediate pathogen spillover from wildlife to livestock in the Pantanal | (b) (6) ▪ Development of a real-time pathogen surveillance system in Jordan | (b) (6)	Fitzgerald B/C & Zoom
12:00-13:00	Lunch *Participants obtain on their own.*	
13:00-14:30	**Lightning Talk Concurrent Sessions** – *see pp4-6 for details* A: Ecology, Entomology and Field Methods B: One Health and Zoonotic Surveillance C: Clinical and Laboratory Science D: Epidemiology and Surveillance Across the Americas E: Epidemiology and Surveillance Across Africa and Asia	 Fitzgerald B/C & Zoom Fitzgerald A North & Zoom Fitzgerald A South & Zoom Warfields & Zoom Grason & Zoom
14:30-14:50	Break	
14:50-16:00	**Oral Presentations 3: Advanced Tools, Techniques and Late Breakers** Session Chair: (b) (6) Session Co-Chair: (b) (6) ▪ An enrichment method for capturing SARS-CoV-2-related whole genome sequences directly from bat samples | (b) (6) ▪ Multi-RC Real-time RT-PCR for Venezuelan equine encephalitis complex, Madariaga and Eastern equine encephalitis viruses: application in clinical diagnostic and mosquito surveillance | (b) (6) ▪ Simple and economical extraction of viral RNA and storage at ambient temperature | (b) (6) ▪ Late breaker: Serological evidence of significant Middle East Respiratory Syndrome coronavirus transmission to humans among camel-owning households in Northern Kenya | (b) (6) ▪ Late breaker: Concurrent non-human primate, bat, mosquito, and human One Health surveillance in the Peruvian Amazon 2021-2022 | (b) (6)	Fitzgerald B/C & Zoom
16:00-16:15	**Closing Remarks** (b) (6)	Fitzgerald B/C & Zoom

2022 Pilot Awardee ePosters

(*see Between Session slides*)

- Investigation of ecological drivers of sarbecoviruses spillover in Myanmar and Nepal | (b) (6)
- Vector surveillance in context of urban transmission and spread of Crimean-Congo hemorrhagic fever virus (CCHFV), (b) (6)
- In the Air Tonight: Metagenomic Pathogen Discovery as Tools in Pathogen Surveillance | (b) (6)
- (b) (6)
- Pathogenic Mammarenaviruses and Orthohantaviruses in Argentina | (b) (6)
- Surveillance for known and novel viruses with zoonotic potential at the interface between humans and livestock in Kenya | (b) (6)
- Characterization of the mosquito microbiome and its role in arbovirus emergence and maintenance in Senegal | (b) (6)

Lightning Talk Concurrent Session Details

Day 3 | Friday, September 23 | 13:00-14:30

Concurrent Session A: Ecology, Entomology and Field Methods Location: Fitzgerald B/C & Zoom

Session Chair: (b) (6)

Session Co-Chair (b) (6)

- Building capacity for ecological surveillance and molecular diagnostics of mosquito-borne viruses in Sierra Leone | (b) (6)
- Active surveillance to detect low-level Rift Valley Fever Virus transmission in humans in East and Central Africa | (b) (6)
- The use of satellite imagery to create robust geospatial sampling designs and examine landscape dynamics across an urban to rural gradient in Managua, Nicaragua | (b) (6)
- Higher infestation indices of the vector Aedes aegypti in rural areas than in urban areas in Managua, Nicaragua | (b) (6)
- Rainfall and the prevalence of Aedes aegypti in northern coastal Ecuador | (b) (6)
- Setting the standard for field biosafety at the human-animal interface | (b) (6)
- Effective Recruitment Strategies during an Infectious Disease Surveillance Study at Rural Health Facilities in Liberia; WARN-ID Liberia Team Experience | (b) (6)
- Environmental surveillance of informal sewage systems reveals community SARS-CoV-2 transmission dynamics | (b) (6)
- Mosquito ecology and risk of arboviral infectious disease spillover in southwestern Uganda | (b) (6)
- Report of arbovirosis surveillance prospective cohort as model of early detection of viral circulation | (b) (6)
- Towards the laboratory maintenance of Haemagogus janthinomys, the major neotropical vector of sylvatic yellow fever | (b) (6)

Lightning Talk Concurrent Session Details, continued

Concurrent Session B: One Health and Zoonotic Surveillance Location: Fitzgerald A North & Zoom

Session Chair: (b) (6)

Session Co-Chair: (b) (6)

- One Health Pathogen Surveillance in the Bwindi Impenetrable Forest Region of Uganda | (b) (6)
- Use of molecular epidemiological, serological and experimental approaches to study COVID-19 transmission in Hong Kong | (b) (6)
- Evidence of Bourbon virus in ticks and humans in St. Louis Missouri, USA | (b) (6)
- Potential reservoirs hosts zoonotic pathogens in Senegal including emerging viruses such arboviruses, Lassa, Ebola, coronaviruses and henipaviruses | (b) (6)
- A One Health investigation framework for zoonotic and vector-borne disease outbreaks | (b) (6)
- Coronavirus Circulation in Peridomestic Rodent Populations in Sierra Leone | (b) (6)
- One Health surveillance of Lassa fever in rodents and human close contacts in North Central Nigeria | (b) (6)
- Plasmodium vivax infection in two non-human primates in the Amazon | (b) (6)
- YF-neutralizing antibodies in pied tamarins (Saguinus bicolor) captured in Amazon Rainforest fragments in the urban area of Manaus, Brazil | (b) (6)
- CREATE-NEO arboviral surveillance in mosquitoes, febrile humans and non-human primates in transition zones in Panama and Darien | (b) (6)
- Late Breaker: Predicting the zoonotic capacity of mammals to transmit SARS-CoV-2 | (b) (6)
- Late Breaker: Coronavirus surveillance among farmed collared peccaries (Dicotyles tajacu) and caretakers in the Peruvian Amazon | (b) (6)
- Late Breaker: SARS-CoV-2 Genomic Variant Surveillance in Human and Non-Human Primates in Peru | (b) (6)

Concurrent Session C: Clinical and Laboratory Science Location: Fitzgerald A South & Zoom

Session Chair: (b) (6)

Session Co-Chair: (b) (6)

- Acute Neurologic Syndromes associated with Chikungunya virus infections in Salvador, Brazil | (b) (6)
- Predictors of severity in dengue-suspected pediatric patients during 2019 dengue epidemic in Brazil | (b) (6)
- Influence of previous Zika virus exposure on Brazilian dengue outbreak in 2019 | (b) (6)
- Immune escape mutations in the Spike protein of an endemic SARS-CoV-2 variant in Panama | (b) (6)
- The highly conserved stem-loop II motif is important for the lifecycle of astroviruses but dispensable for SARS-CoV-2 | (b) (6)
- Antibody fucosylation predicts disease severity in secondary dengue infection | (b) (6)
- Unexpected Acute Viral Fever Mimicking Dengue-Like Illness in Major City of Pakistan | (b) (6)
- Comparison of the Immunogenicity of five COVID-19 vaccines in Sri Lanka | (b) (6))
- SARS-CoV-2 Variant Detection and Surveillance with an Economical and Scalable Molecular Protocol | (b) (6)
- Multiplexed detection of respiratory viruses and SARS-CoV-2 variants with mCARMEN | (b) (6)
- Developing Rapid Antigen Diagnostics for Emerging Viruses using Antibodies Cloned from Sorted Single Memory B-cells | (b) (6)

- A simplified Cas13-based assay for the identification of SARS-CoV-2 and its variants | (b) (6))
- Late Breaker: Dynamics of infectious virus neutralization from convalescent and vaccine cohorts across global SARS-CoV-2 variant lineages reveals boost protection and novel monoclonal antibody efficacy against Omicron strains | (b) (6)

Concurrent Session D: Epidemiology and Surveillance Across the Americas Location: Warfields & Zoom

Session Chair: (b) (6)
Session Co-Chair: (b) (6)

- A Dengue outbreak in Panama, 2022 | (b) (6))
- Seroprevalence of Zika, Dengue, and Chikungunya viruses in a rural area in Brazil | (b) (6)
- Zika virus infection surveillance in Manaus, Amazonas state | (b) (6)
- Real-time genomic surveillance of DENV-1 and DENV-2 in Brazil: improving public health outbreaks response | (b) (6)
- Surveillance and evolutionary analysis of Dengue viruses to understand the epidemiological dynamics of dengue outbreaks, São José do Rio Preto, São Paulo, Brazil | (b) (6)
- Monitoring the genetic diversity of reemerging chikungunya virus in Brazil | (b) (6)
- Genomic and epidemiological monitoring of YFV reemergence in Brazil: unveiling the corridor of spread and the geographic hot spots for predicting and preventing other possible spillover events | (b) (6)
- Genomic surveillance of SARS-CoV-2 in symptomatic vaccinated and unvaccinated asymptomatic patients in Brazil | (b) (6)
- SARS-CoV-2 genomic surveillance and the impact of different lineages circulation in the epidemiological landscape of São José do Rio Preto, São Paulo, Brazil | (b) (6)
- Genomic epidemiology reveals the impact of national and international restrictions on the SARS-CoV-2 epidemic in Brazil | (b) (6)
- Occurrence of SARS-CoV-2 reinfections at regular intervals in Ecuador | (b) (6)
- Genomic characterization of SARS-CoV-2 during the COVID-19 pandemic in Nicaragua | (b) (6)

Concurrent Session E: Epidemiology and Surveillance Across Africa and Asia Location: Grason & Zoom

Session Chair: (b) (6)
Session Co-Chair: (b) (6)

- Flaviviruses and Lassa fever in febrile patients in North Central Nigeria: A cross-sectional study | (b) (6)
- Double Stigma and discrimination: A qualitative study of Lassa fever and hearing loss in Northern Nigeria | (b) (6)
- A brief chronicle of SARS-CoV-2 genomic surveillance in Cambodia | (b) (6)
- Identification of Genetic Variations of SARS-CoV-2 Omicron Strain and their Clinical Significance in Karachi, Pakistan | (b) (6)
- Genetic characterization of Norovirus strains from an unusual diarrheal outbreak in the community in Thailand | (b) (6)
- Factors associated with changing Dengue case numbers during the COVID-19 pandemic in Sri Lanka | (b) (6)
- SARS-CoV-2 genomic epidemiology in Sierra Leone | (b) (6)
- Yellow Fever outbreak in eastern Senegal, 2020–2021 | (b) (6)
- The evolving SARS-CoV-2 epidemic in Africa: Insights from rapidly expanding genomic surveillance | (b) (6)

- Detection and Characterization of Variants of Concern: Insights from the South African Epidemic | (b) (6) [redacted]
- Emergence of novel combinations of SARS-CoV-2 spike receptor binding domain variants in Senegal | (b) (6) [redacted]
- COVID-19 laboratory surveillance at IRESSEF in Senegal | (b) (6) [redacted]
- Dynamics of Variants of Concern (VOC) during the different waves of COVID-19 in Senegal | (b) (6) [redacted]

Acronyms and Abbreviations

A2CARES	American and Asian Centers for Arboviral Research and Enhanced Surveillance
BV-BRC	Bacterial and Viral Bioinformatics Resource Center
CEPI	Coalition for Epidemic Preparedness Innovations
CREATE-NEO	Coordinating Research on Emerging Arboviral Threats Encompassing the Neotropics
CREID	Centers for Research in Emerging Infectious Diseases
CREID CC	CREID Coordinating Center
CREID-ECA	Center for Research in Emerging Infectious Diseases – East and Central Africa
CREID-ESP	Center for Research in Emerging Infectious Diseases – Epidemiology, Surveillance and Pathogenesis
CSPH	Colorado School of Public Health
DMID	Division of Microbiology and Infectious Diseases
EEIDI	EpiCenter for Emerging Infectious Disease Intelligence
EID-SEARCH	Emerging Infectious Diseases: South East Asia Research Collaboration Hub
NIAID	National Institute of Allergy and Infectious Diseases
NIBSC	National Institute for Biological Standards and Controls
NIH	National Institutes of Health
PICREID	Pasteur International Center for Research on Emerging Infectious Diseases
PREMISE	Pandemic Response Repository through Microbial and Immunological Surveillance and Epidemiology
RC	Research Center
TGHN	The Global Health Network
UWARN	United World Antiviral Research Network
WAC-EID	West African Center for Emerging Infectious Diseases
WARN-ID	West African Research Network for Infectious Diseases
WRCEVA	World Reference Center for Emerging Viruses and Arboviruses

– AGENDA: TABLETOP EXERCISE, APPLIED SESSIONS A and B –

DAY 1 | WEDNESDAY, SEPTEMBER 21

The ***Tabletop Exercise Situation Report*** will be introduced during the ***Collaboration for Outbreak Research Response & Tabletop Introduction*** session during the Scientific Meeting (Time: UMT -4, 14:15-15:15)

TTX Zoom Link

DAY 2 | THURSDAY, SEPTEMBER 22

Time (UMT -4)	Session	Location	Participants
08:30-10:40	**Tabletop Exercise: Applied Session A**		Network Members, DMID, EAC, NIAID
	Breakout Rooms		
	Combined Biorepository and Data Capture & Harmonization (hybrid session) **Facilitators:** (b) (6) [redacted] **Reporters:** (b) (6) [redacted]	Fitzgerald A South & Zoom	
	Laboratory Assays (hybrid session) **Facilitators:** (b) (6) [redacted] **Reporters:** (b) (6) [redacted]	Fitzgerald A North & Zoom	
	Outbreak Research Response A (in-person session only) **Facilitators:** (b) (6) [redacted] **Reporter:** (b) (6) [redacted]	Warfields	
	Outbreak Research Response B (in-person session only) **Facilitators:** (b) (6) [redacted] **Reporter:** (b) (6) [redacted]	Fitzgerald B/C	
	Outbreak Research Response C (virtual session) **Facilitators:** (b) (6) [redacted] **Reporters:** (b) (6) [redacted]	Grason & Zoom	
10:40-11:00	BREAK		
	Scientific Meeting (see detailed agenda)		
11:00-17:00			Network Members, DMID, EAC, NIAID, External and USG Stakeholders

DAY 3 | FRIDAY, SEPTEMBER 23

TTX Zoom Link

Time (UMT -4)	Session	Location	Participants
08:30-10:40	**Tabletop Exercise: Applied Session B**		Network Members, DMID, EAC, NIAID
08:30-09:40	**Breakout Rooms**		
	Combined Biorepository and Data Capture & Harmonization (hybrid session) **Facilitators:** (b) (6) [redacted] **Reporters:** (b) (6) [redacted]	Fitzgerald A South & Zoom	
	Laboratory Assays (hybrid session) **Facilitators:** (b) (6) [redacted] **Reporters:** (b) (6) [redacted]	Fitzgerald A North & Zoom	
	Outbreak Research Response A (in-person session only) **Facilitators:** (b) (6) [redacted] **Reporter:** (b) (6) [redacted]	Warfields	
	Outbreak Research Response B (in-person session only) **Facilitators:** (b) (6) [redacted] **Reporter:** (b) (6) [redacted]	Fitzgerald B/C	
	Outbreak Research Response C (virtual session) **Facilitators:** (b) (6) [redacted] **Reporters:** (b) (6) [redacted]	Grason & Zoom	
09:40-09:50	TRANSITION TIME		
09:50-10:40	**Tabletop General Session: Working Group Report Outs**	Fitzgerald B/C & Zoom	
10:40-11:00	BREAK		
	Scientific Meeting		
11:00-16:15	**Scientific Meeting** (see detailed agenda)		Network Members, DMID, EAC, NIAID, External and USG Stakeholders

Outbreak Research Response Tabletop Exercise Applied Sessions

Annual Meeting, Days 2 and 3

All Network Members

Research Response

Outbreak *simulation* to stress test Network capacity to launch rapid & effective research for future outbreaks.

Network-wide interactive outbreak simulation activity in support of the Network Vision to build capacity and outbreak research response readiness.

An outbreak *Situation Report* will be presented on Day 1 in the General Scientific Session.

Exercise Goals:

- Work as multidisciplinary teams to respond to a *Pathogen X* outbreak
- Build collaborative connections with Network colleagues
- Pressure test existing Network Tools/Inventories to launch coordinated research response
- Identify critical gaps/pinch points for effective research response
- Develop an After-Action Report to guide Network capacity building and outbreak research readiness

Mentimeter Tool

Using your smartphone, laptop, or other mobile device, please go to **mentimeter.com** to participate in interactive polls, quizzes, and post questions.

Applied Session Schedule: Day 2, 8:30-10:40am EDT **|** Day 3, 8:30-10:40am EDT

Additional Information:

See detailed agendas

Email: info@creid-network.org

CREID Centers for Research in Emerging Infectious Diseases

NIH National Institute of Allergy and Infectious Diseases

TTX Activities at Annual Meeting (Day 1 and 2)

15 min

Facilitator presents scripted, cross-cutting scenario (**Sit Rep 1**) and briefs participants on objectives, procedures, & expectations. Research Centers with communication/ coordination with in-country partners in preparation for applied sessions.

Presentation of Sit Rep 1 and plan for applied session

10 min

Facilitator reminds participants of outbreak scenario, reviews expectations and WG-specific objectives for session, and orients participants to the Mentimeter facilitation tool.

APPLIED SESSION A: OUTBREAK IDENTIFIED

1. Presentation of WG-specific module

100 min

Facilitator uses scripted questions in the Facilitator Guide and Mentimeter facilitation tool to generate discussion about how participants would respond to the outbreak scenario presented in Sit Rep 1. Participants discuss, make decisions, and take action in real-time (e.g., test Network tools).

2. Guided Discussion with interspersed polling through menti.com

20 min

Facilitator (or reporter) summarizes key take-aways from Session 1 and presents Sit Rep 2, which will be used in Applied Session 2 ("Outbreak evolves")

3. Wrap-up and Late Breaking news

2022 Annual Meeting Scientific Session (Day 1)

The Coordinating Center presents Situation Report (Sit Rep) #1, which describes the scenario to be discussed on Day 2 in the applied session.

2022 Annual Meeting Concurrent Working Group Sessions (Day 2 from 8:30-10:40 am ET)

Participants meet in WG-specific break-out groups to discuss response to Sit Rep 1.

1. Biorepository Collaboration & Quality/ Data Capture & Harmonization (combined group)
2. Laboratory Assay Oversight and Quality
3. Outbreak Research Response (split into 3 concurrent sessions)

Note: As a cross-cutting activity, capacity building considerations will be integrated into other WG discussions rather than convening a stand-alone session. Capacity Building WG members will have the opportunity to join the other WG session of their choice.

TTX Activities at Annual Meeting (Day 3)

10 min

Facilitator (or reporter) provides a summary of key take-aways from Session 1 and a reminder of Sit Rep 2.

50 min

Facilitator uses Facilitator's Guide and Mentimeter facilitation tool to guide participants through scripted questions and to generate discussion about how participants would respond to the new information presented in Sit Rep 2. Participants discuss, make decisions, and take action in real-time.

10 min

Facilitator (or reporter) summarizes key take-aways from Session 2.

60 min

Reporter from each WG session briefs Network on key discussion and take-aways from Applied Sessions 1 and 2.

APPLIED SESSION B: OUTBREAK EVOLVES

1. Summary of Session 1 and reminder of Sit Rep 2

2. Guided Discussion with interspersed polling using menti.com

3. Wrap-up

Report on Key Take-Aways

2022 Annual Meeting
Concurrent Working Group Sessions
(Day 3 from 8:30-9:40 am ET)

Participants re-convene in their WG-specific breakout groups from Day 2 to discuss Sit Rep #2.

1. Biorepository Collaboration & Quality/ Data Capture & Harmonization (combined group)
2. Laboratory Assay Oversight and Quality
3. Outbreak Research Response (split into 3 concurrent sessions)

2022 Annual Meeting
General Session
(Day 3 from 9:40-10:40 am ET)

Pathogen X-Like Outbreak of Suspected Animal Origin

Situation Report 01

01 September 2022 (Epi Week 36)

1. Situation at a Glance	8 Affected Countries	Cases	Deaths	CFR
		56	4	7.1%

- Since 05 August 2022, a total of 56 suspected cases have been identified in 8 countries, with geographic clusters in West Africa (Sierra Leone, Guinea) and Central Africa (DRC, Uganda). Isolated cases have also been identified in Brazil, China, France, and Senegal.
- Four deaths have been reported to date. One death has been linked to an immunocompromised patient. No further information is available about the other deaths.
- Many cases (n=31) report a history of animal exposure, including rodents, bats, monkeys (species unknown), wild pig, domesticated dogs, and livestock (poultry, pigs).
- All cases report fever and malaise. Other common symptoms include rash, swollen lymph nodes, cough, anorexia, myalgia, nausea, and skin lesions.
- Separate and distinct epidemiological linkages have been established among cases in West African cases (Sierra Leone and Guinea) and Central Africa (DRC and Uganda).
- Epidemiological investigation and laboratory analyses are ongoing.

2. Epidemiological Summary

Since 05 August 2022, WHO has received reports of a pathogen X-like illness in Brazil, China, Democratic Republic of Congo (DRC), France, Guinea, Senegal, Sierra Leone, and Uganda (Table 1). Clustering of cases has occurred in West Africa, along the Sierra Leone-Guinea border, as well as Central Africa, along the DRC-Uganda border. Among cases reported in West and Central Africa, 29 have a recent history of animal exposure, most commonly rodents. Cases also report exposure through livestock (poultry, pigs), domesticated dogs, or preparation or consumption of bushmeat (bats, monkeys, "bush rats", wild pigs).

Table 1. Suspected Cases, Deaths, and Samples for Pathogen X-like Illness (05 to 31 August 2022)

Country	Cases	Deaths	Samples Collected
Sierra Leone	21	1	18
DRC	16	2	16
Guinea	6	0	6
Uganda	7	1	7
Senegal	2	0	2
China	2	0	2
Brazil	1	0	1
France	1	0	1
Total	56	4	53

Epidemiological investigation suggests two separate and distinct transmission chains, with the index cases located in Sierra Leone and DRC. The index cases and transmission dynamics are further described in **Section 3 (Description of Cases)**. There is no known evidence of epidemiological linkages between the cases in West Africa and Central Africa.

Isolated cases have also been identified in Brazil, China, France, and Senegal; Among these cases, five have reported recent travel to West or Central Africa and two have reported animal exposure.

Clinical presentation. All cases presented with fever and malaise. Other common symptoms include rash, swollen lymph nodes, cough, anorexia, myalgia, nausea, and skin lesions. A total of four deaths have been reported (2 in DRC; 1 in Uganda; 1 in Sierra Leone). One death has been linked to an immunocompromised patient. No further information is available about the other deaths.

Diagnostic Activities. Blood samples have been collected from a total of 53 cases (see Table 1) and sent to the National Reference Laboratory in each affected country for diagnosis and viral identification by real-time PCR. Diagnostic testing results are not yet available. All samples collected in Sierra Leone, Guinea, and Senegal were sent to the Institut Pasteur de Dakar (Dakar, Senegal) for additional diagnostic and confirmatory testing.

3. Description of Cases

Suspected Clusters in Sierra Leone and Guinea. On 05 August 2022, the National IHR Focal Point in Sierra Leone notified WHO of a cluster of cases of a Pathogen X-like illness in the Kailahun District. All cases presented with fever and malaise; Other symptoms included rash, swollen lymph nodes, skin lesions, and respiratory symptoms. Epidemiological investigation indicates that the index case was a 21-year-old male with a history of animal exposure, including household exposure to rodents and domestic animals or livestock (dogs, poultry, pig) and consumption of bushmeat (bat, "bush rat"). The majority of cases (8) were identified in Yenga, a village located in a remote forest area that sits at the main international border crossing with Guinea. On 15 August 2022, health authorities in Guinea reported a household cluster of three cases presented with symptoms similar to the Sierra Leone cases. The household cluster is located in Nongoa, within the Guéckédou prefecture, and approximately 9 miles from the Sierra Leone border. Epidemiological investigations are ongoing; preliminary information indicates that one of the Guinea cases has an established travel link to affected areas in Sierra Leone and all three Guinea cases have a history of animal exposure, including rodents, bats, monkeys (unknown species), and domesticated animals or livestock. The proximity of the affected areas to international borders, cross-border movement between the Sierra Leone and Guinea, and the potential transmission of the unknown pathogen between animal vectors and humans poses an increased risk for cross-border spread. These factors also suggest a high risk at the national and regional level, given that the Kailahun District is well connected to the Guéckédou prefecture in Guinea and Lofa County in Liberia.

Suspected Clusters in DRC and Uganda. On 25 August 2022, the Minister of Health in DRC reported 16 cases of Pathogen X-like illness in the North Kivu Province. Epidemiological investigation indicates that the suspected index case and majority of suspected cases reside in the Beni Health zone, located on the border with Uganda. This report of the cluster in Beni directly follows the 22 August 2022 announcement by the DRC Minister of Health of a newly identified Ebola virus disease (EVD) outbreak in the same health zone. The suspected index case is a 5-year-old male who presented to a public health facility with a 10-day history of fever, malaise, swollen lymph nodes, and appearance of rashes on his skin. Three household contacts presented with similar symptoms, although the time of symptom onset was less than seven days. Before presenting to the health facility, the child and his family sought care from a traditional health practitioner who provided local herbs for the fever and rash. The suspected index case and his household contacts report a history of animal exposure, including rodents, monkeys of unknown species, and livestock (poultry, goats) within two weeks of symptom onset. The mother of the suspected index case also reports frequent trips to neighboring villages, including several villages in the Kasese District of Uganda to sell bush meat.

Suspected Cases in Senegal, China, Brazil, and France. Isolated cases of Pathogen X-like disease have also been reported in Senegal, China, Brazil, and France, five cases reported recent travel to West or Central Africa (2 from China and 1 each from Senegal, Brazil, and France). Among these five patients, only those from Senegal and France reported travel to an area with an identified cluster. Two suspected cases (1 from China and 1 from Senegal) also report a recent animal exposure.

CREID Preparedness Session 1b: Now open for YOUR input via XLEAP!

Activity Goals

- *Collectively identify evidence gaps and critical research questions that can be addressed by the Network during inter-outbreak periods*
- *Promote knowledge-sharing and relationship-building across CREID Research Centers and Sites*
- *Define mechanisms for rapid and effective research coordination, collaboration, and execution when an outbreak occurs*

Overview and Activity Methodology

Outbreak Research Preparedness Session 1b is a follow-up to the Zoom-based Session 1 on Lassa Fever (LASV) and Chikungunya Fever (CHIKV), which was held in July 2022.

For this asynchronous crowd-sourcing activity (Session 1b), the CC is employing a virtual facilitation tool called **XLeap**. Please visit the CREID Private Portal to access:

- Brief instructions on how to use and navigate within XLeap
- An informational demo of this activity in XLeap
- https://creid-network.org/login

XLeap is a secure, password protected site. Please use the login details below to access the XLeap platform and provide your responses to the four questions posed regarding CHKV and LASV within the XLeap platform **by September 26th, 2022.** Your responses will be reviewed and prioritized by a CREID Steering Committee-assigned panel of Subject Matter Experts in early October.

XLeap Login

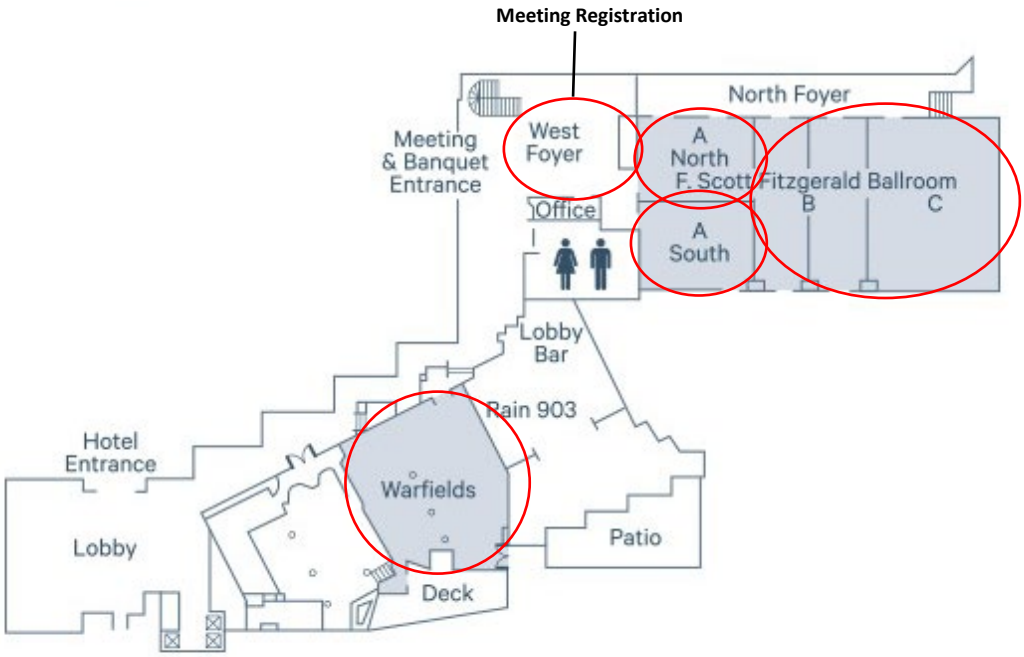
Website Link: https://rti.xleap.net/fr9o9uz47ck7/login
Your name: *Please use your full name and CREID Network affiliation* (i.e., Research Center)
Your email: *Please use your email address as your login ID*
Access Code: (b) (6)

Additional Information

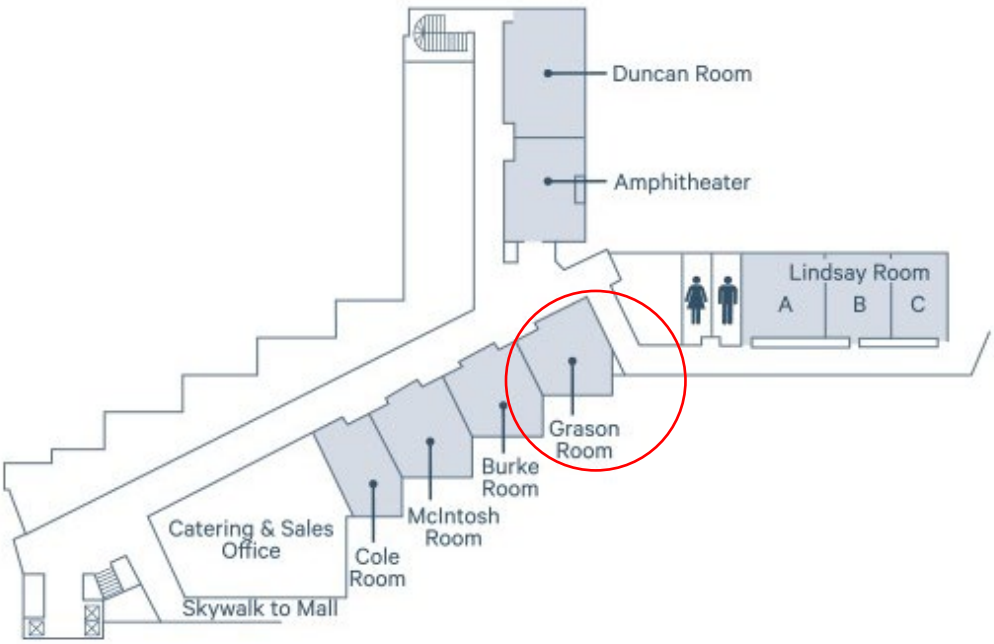
Email: info@creid-network.org

Sheraton Baltimore North Hotel: Venue Map

PLAZA LEVEL



SECOND LEVEL



2022 CREID Network Annual Meeting
COVID-19 Guidance
Updated: 09/19/2022

To In-Person CREID Annual Meeting Participants:
Included here is information about the COVID-19 Community Level in Baltimore County, Maryland, the site of the Annual Meeting, and the mitigation efforts the meeting organizers are putting in place. All information and mitigation efforts are based on guidance from the U.S. Centers for Disease Control and Prevention (CDC).

Current (as of September 19, 2022) transmission in Baltimore County: **Low**

We will update the COVID-19 information shared with meeting attendees and if the COVID-19 Community Level changes, our guidance to meeting participants will change accordingly. If you have any questions, please contact info@creid-network.org.

Annual Meeting COVID-19 logistics (for low transmission of COVID-19)

Based on updated NIH policy (as of August 24, 2022) meeting attendees are no longer required to provide vaccination attestations or a negative COVID test.

Onsite COVID-19 Mitigation

- Meeting organizers will provide masks and hand sanitizer onsite at the hotel.
- We ask all attendees to conduct a self-assessment utilizing the *CDC Coronavirus Self Checker* before attending the meeting and we ask that anyone exhibiting COVID-19 symptoms to participate in the meeting virtually: Symptoms of COVID-19: https://www.cdc.gov/coronavirus/2019-ncov/symptoms-testing/symptoms.html

Baltimore County COVID-19 Information
According to the CDC COVID-19 County Check for **Baltimore County, Maryland** on **September 19, 2022**, where the Sheraton Baltimore North Hotel is located, the COVID-19 Community Level is **Low**, which is determined by "hospital beds being used, hospital admissions, and the total number of new COVID-19 cases in an area." *Source*: https://www.cdc.gov/coronavirus/2019-ncov/your-health/covid-by-county.html

CDC Low Community Level guidance is:

- Stay up to date with COVID-19 vaccines (https://www.cdc.gov/coronavirus/2019-ncov/vaccines/stay-up-to-date.html)
- Get tested if you have symptoms (https://www.cdc.gov/coronavirus/2019-ncov/symptoms-testing/testing.html)
- People may choose to mask at any time. People with symptoms, a positive test, or exposure to someone with COVID-19 should wear a mask.

Additional information from the CDC COVID Data Tracker: County View https://covid.cdc.gov/covid-data-tracker/#county-view?list_select_state=Maryland&data-type=CommunityLevels&list_select_county=24005)

CREID Annual Meeting COVID Guidance
Updated: 09/19/2022

Health Facilities Near Meeting Location (Sheraton Baltimore North Hotel)

Health Facility	Location and Phone	Website	Hours of Operations	Distance from Meeting
Patient First Primary and Urgent Care, Towson	950 York Road, Towson, MD 21204; (410) 372-6373	https://www.patientfirst.com/locations/baltimore/towson?utm_source=local&utm_medium=organic&utm_campaign=gmb	8:00am – 10:00pm	.8 miles
Minute Clinic	1001 York Road, Towson, MD 21204; (866) 389-2727	https://www.cvs.com/minuteclinic/virtual-care/e-clinic?WT.mc_id=LS_MC_GOOGLE_2216_get_online_care_button	8:30am-7:30pm	.8 miles
University of Maryland St. Joseph Medical Center Emergency Room	7601 Osler Drive, Towson, MD 21204; (410) 337-1000	https://www.umms.org/sjmc/locations/emergency-department?utm_source=local-listing&utm_medium=organic&utm_campaign=website-link	Open 24 hours	2.5 miles
Greater Baltimore Medical Center	6701 N. Charles Street, Towson, MD 21204; (443) 849-2000	https://www.gbmc.org/	Open 24 hours	3.8 miles
University of Maryland Urgent Care, Belvedere	600 E. Belvedere Ave, Suite A, Baltimore, MD 21212; (410) 296-0018	https://www.umms.org/health-services/urgent-care/locations/belvedere-square?utm_source=local-listing&utm_medium=organic&utm_campaign=website-link	8:00am-8:00pm	5 miles
MedStar Good Samaritan Hospital	5601 Loch Raven Blvd., Baltimore, MD 21239; (443) 444-8000	https://www.medstarhealth.org/locations/medstar-good-samaritan-hospital?utm_campaign=mhs_citations&utm_medium=ad_listings&utm_source=rio_seo&utm_term=hospital	Open 24 hours	7.8 miles

From: (b) (6)
To: (b) (6)

Eric Laing; (b) (6)

Cc: (b) (6)
Subject: CREID Network Annual Meeting: Final Agenda and Materials
Date: Monday, September 19, 2022 2:22:33 PM
Attachments: CREID_welcomepacket.pdf
CREIDAgenda_Detailed_Scientific.pdf
TTX Materials_v3.pdf
Preparedness_Session1b.pdf
Venue Map_v3.pdf
COVIDguidance_09192022.pdf

Dear CREID Network Members,

We are looking forward to seeing you – in person or virtually – on Wednesday as our CREID Network Annual Meeting gets underway.

Please find final materials attached for your reference:

1. Welcome Packet
2. Final Scientific Agenda (Zoom links included)
3. Tabletop Agenda and Materials (Zoom links included)
4. Preparedness Session 1b flyer
5. Venue Map
6. COVID guidance

For those attending in person:

- A registration table will be set up (see venue map) for you to pick up your badges and a hard copy of meeting materials starting Tuesday afternoon (for about an hour at 4pm EDT) or in the mornings before the sessions start.
- Good news! COVID transmission in Baltimore County is now low; updated COVID information is attached.
- We hope you take advantage of the no-host social hours – Tuesday, Wednesday, and Thursday evenings – to get to know one another better. See the Welcome Packet for location details.
- Look for the CREID CC team members with the yellow ribbons on their name badges if you have questions or concerns during your stay.

If you have any questions, please email info@creid-network.org.

For those who are traveling, safe travels!

(b) (6)

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From: (b) (6)
To: Laing, Eric
Cc: (b) (6)
Subject: Fwd: Draft manuscript: Co-circulation dynamics of viruses in a bat population
Date: Wednesday, September 7, 2022 2:57:50 PM
Attachments: (b) (6) -et-al_bangladesh-bats-cocirculation-serology_2022-08-11.docx

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(b) (6) let us know that you should be on the attached paper as you developed some of the proteins used in the panel run in our previous Bangladesh study. Glad to have you on, as this way we'll have another joint reference for the grant.

We're doing final revisions on it now before sending to preprint before the end of the month. If you have any feedback, maybe we touch on it when we meet next week?

Best,
(b) (6)

---------- Forwarded message ---------
From: (b) (6)
Date: Fri, Aug 19, 2022 at 11:22 AM
Subject: Draft manuscript: Co-circulation dynamics of viruses in a bat population
To: (b) (6)
Christopher Broder (b) (6)
Cc: (b) (6)

Colleagues,
I hope you're all doing well. I'm excited to share a draft manuscript, led by (b) (6), that details the serodynamics of henipaviruses, filoviruses and rubulaviruses in a population of *Pteropus medius*. We plan to submit this to *Nature Communications* in September. Please send Noam and I your comments by **September 10th**.

Cheers,
(b) (6)

(b) (6)

[redacted]

[redacted]

[redacted]

(b) (5)

From: (b) (6)
To: Laing, Eric
Cc: (b) (6)
Subject: Fwd: NIH R01 on serology bioinformatics
Date: Wednesday, August 24, 2022 4:58:43 PM
Attachments: EcoHealth_SerologicalStats_conceptnote-Aug2022.pdf

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Hi (b) (6)

Hi! I hope you are well. I had a call with (b) (6) yesterday about this grant as I know you've been away for family reasons. (CC'ing him to chat with you about this when he has the chance). We discussed things that we could write into the grant on your side, notably validation/calibration testing using the Filovirus standard coming out of Oxford, and also running tests on the sera from experimental infections that are at BSL-4 labs at RMN, UTMB, and maybe South Africa. These could be used to model how we expect real responses to look in related virus and host species.

Here are my follow-up items:

- ***I'd like to have a conversation with you about nuts and bolts and budgeting.*** I'm headed out for vacation Friday afternoon through Labor day. ***If you want to chat tomorrow or Friday pick a time*** at this link, if not I will write when I return: https://calendly.com(b) (6) /60-minute-chat-extended-hours

- ***I'll get you a draft*** with a bulleted section on this stuff that you and (b) (6) can make sound like a virologist who knows what they are talking about. I expect this ***around September 12.***

- I think it would be good for me to ***come down to USU for a day or two in early-mid September*** for us to work through some of this stuff if you agree and have the time. If so, let's schedule that.

Best,
(b) (6)

[redacted]

On Mon, Aug 8, 2022 at 8:51 AM (b) (6) wrote:

> Hi (b) (6),
>
> After many false starts, I really am planning to submit an R01 on bioinformatics and statistical methods for Luminex serology in the next cycle (due Oct 6). Everyone came back from the BatID conference really jazzed about this. I still think it works best with you and I as co-PIs. If you are still game, want to have a call about it? I'd still like to come down to USU for a day in the next month, too, if you'd like to spend a day working on it and other related issues.
>
> Schedule a call on my calendar here: https://calendly.com(b) (6) /60-min-chat
>
> Last draft of specific aims attached.
>
> Best,
>
> (b) (6)

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From: (b) (6)
To: Laing, Eric
Subject: Re: For you to edit and review by August 15 Monday_CREID EAC report
Date: Wednesday, August 17, 2022 2:11:42 PM
Attachments: 2022 EAC Report_EID-SEARCH_draft v03.docx
2022 EAC Report EID-SEARCH FINAL_formatted.docx

Hi (b) (6),

Just sharing an updated version of draft v03 where (b) (6) from UNC addressed some of your comments on "Host range, transmissibility, and antigenicity of a pangolin coronavirus", in case you want to know (the paper is under revision, and no preprint). Also attached the final version from (b) (6) for your information.

Cheers,
(b) (6)

On Mon, Aug 8, 2022 at 2:37 PM Laing, Eric (b) (6) wrote:

> Hey (b) (6),
>
> One edit. The cross RC collaboration is with (b) (6). I incorrectly wrote (b) (6)
>
> (b) (6)
>
> On Mon, Aug 8, 2022 at 2:21 PM (b) (6) wrote:
>
> > Thank you so much, (b) (6), for your quick response!
> >
> > Sending a reminder that please review and edit the document **by August 15 next Monday.** Or if you don't have anything to add, please simply reply to approve the content for submission. Thank you very much!!
> >
> > Best regards,
> > (b) (6)

(b) (6) [redacted]

[redacted]

[redacted]

On Tue, Aug 2, 2022 at 3:36 PM Laing, Eric (b) (6) [redacted] wrote:

Hi (b) (6) [redacted],

Some edits and suggestions are attached.

(b) (6) [redacted]

[redacted]

[redacted]

On Tue, Aug 2, 2022 at 1:23 AM (b) (6) [redacted] wrote:

Dear All,

As mentioned at the EID-SEARCH meeting in July, we'll submit an External Advisory Committee (EAC) report for our Research Center in August, so the EAC can evaluate our work as an individual Research Center and the overall CREID Network to give feedback at the CREID annual meeting with DMID, USG, and other stakeholders in September.

Attached please find the very first draft report for your edit and review. We'll greatly appreciate everyone's input on incorporating the EAC recommendations and aligning with NIAID CREID Network Program Priorities as described in the Appendix.

For unpublished data, I tried to keep the information general, will also make sure the CREID keeps all information for internal use only (to the EAC), but please let me know if you have any concerns.

We hope to submit the report on August 19, and your feedback **by August 15 Monday** will be appreciated! Thank you all very much in advance!

All the best,

(b) (6) [redacted]

(b) (6)

[redacted]

(b) (5)

From:	(b) (6)
To:	Laing, Eric
Subject:	NIH R01 on serology bioinformatics
Date:	Monday, August 8, 2022 8:52:38 AM
Attachments:	EcoHealth_SerologicalStats_conceptnote-Aug2022.pdf

---

Hi (b) (6),

After many false starts, I really am planning to submit an R01 on bioinformatics and statistical methods for Luminex serology in the next cycle (due Oct 6). Everyone came back from the BatID conference really jazzed about this. I still think it works best with you and I as co-PIs. If you are still game, want to have a call about it? I'd still like to come down to USU for a day in the next month, too, if you'd like to spend a day working on it and other related issues.

Schedule a call on my calendar here: https://calendly.com(b) (6) /60-min-chat

Last draft of specific aims attached.

Best,

(b) (6)

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From: (b) (6)
To: (b) (6) Eric Laing
Cc: (b) (6)
Subject: Summary of discussion w NSF PIPP program officers
Date: Sunday, June 19, 2022 7:31:02 PM
Attachments: Discussion_NSFprogramofficers_17June2022.docx
ATT00002.bin
(b) (6) NSF PIPP Panel Summary and reviews_3June2022.docx
ATT00004.bin

Dear all,

We (me, (b) (6)) had a 1 hour call w NSF on Friday afternoon to discuss our unfunded NSF PIPP proposal. Here are my notes summarizing the call.

In short, we ranked very high but will not get funded. They encouraged us to try and fund parts of the proposal via other mechanisms, but these could involve putting whole proposals together (~1 year time frame before funding), but we will pursue other mechanisms that could be quicker like EAGER grants. I will also follow up w (b) (6) (the BIO NSF PIPP program officer) to get her thoughts.

In any case, this is very frustrating as we were "highly competitive" and clearly came so close to getting funded. I still believe (as I summarized in my previous email to the NSF program officers) that the reviewers just flat out got some things wrong that we laid out quite clearly, esp. 2 of the main negative aspects in the panel summary - but seems we have no real recourse here but to try other funding mechanisms, and/or apply for a Center level grant in ~1 year. We also agreed it would be great to not lose momentum before the RFP for Center level grants, and maybe find ways to do some of what we proposed (albeit without funding!). Chime in if you have some specific ideas.

Appreciate everyone's hard work on this. Upward and onward.

Cheers,
(b) (6)

P.S. Sending the panel summary and reviews again too, as I didn't send them to everyone here before.

Notes - PIPP planning call - 2 September 2021

Participants:

- *EHA*: (b) (6)
  - Unable to attend: (b) (6)
- *Univ. of Rochester*: (b) (6)
  - Unable to attend: (b) (6)
- *USU*: (b) (6)
- *Georgetown Univ*ersity:
  - Unable to attend: (b) (6)

Action Items:

- (b) (6) to send around 1-page 'Project Summary' by early next week
- (b) (6) to request biosketches and other required documents from senior personnel by early next week
- (b) (6) to share his method for anomaly detection of serological results, with (b) (6)
- (b) (6) to follow up with Eric to discuss novel serological ideas that could be included on the proposal
- PIPP team (especially (b) (6)) to review meeting notes from the call with Facebook. Explore various links to preprints/datasets/online tools
  - (b) (6) to start a notes document and write down ideas about how to leverage the datasets referenced in the notes for our proposal
- All to continue thinking about 'broader impacts' and draft any text related to training or outreach at your institution

Agenda/Notes:

- *Introduce* (b) (6) *- serology and biosurveillance*
  - (b) (6) works at Uniformed Services University (USU). Extensive experience with serological surveillance
  - (b) (6) I'm a molecular virologist by training, but I work on biosurveillance. Doing a lot of COVID-19 work recently

- *Brainstorm intellectual and engineering innovations on "Detecting zoonotic disease exposure at scale" (Serology)*
  - (b) (6) We have a current NIH project sampling people at high risk communities in SE Asia (e.g. bat guano harvesters, people presenting symptoms at health facilities). Using serological platforms to find evidence of spillover (perhaps of novel bat viruses)
  - (b) (6) First question for (b) (6) Are you working with any engineers/product designers to think about scaling up technologies (cheaper, smaller, easier to use)?
    - (b) (6) No we aren't. We haven't looked at anything that's point of care focused. No handheld devices. No antigens on lateral flow.

        - This is possible to do, but you wouldn't be able to do multiplex
        - You could develop a small test for a specific virus (e.g. Nipah) but nobody wants to develop it. No money in that venture.
    - (b) (6)
    - I spoke with (b) (6) (of Duke-NUS) who works on serology. He's less focused on point of care, more focused on working at a larger scale (e.g. working with blood banks to access lots of samples at a larger scale/larger fractions of the population)
        - The main goal of this Phase 1 proposal is to plan for the Phase 2 proposal. Who do we want to bring together to brainstorm big ideas for Phase 2? Question is about piloting different approaches. Show what could be possible in a 5-year project.
        - (b) (6) Also thinking about our working group structure. (b) (6), given your familiarity with the serological workshop space, **who else would you bring to the table, re next-generation serological multiplexing for zoonoses?** These are the things you (b) (6) could help us think about
            - (b) (6) Serology is challenging. Always limited by what it is you're looking for. CoVs, paramyxos, filos potentially have the greatest risk for pandemic potential. So do you make rapid tests for those viral families? We have everything we need to develop test for Nipah, but no monetary interest there. What would be needed for ebolaviruses? **Also, I don't know how you'd make a 'disease x' assay**
                - (b) (6) NSF is thinking big picture. So one idea is a 'serochip' assay where you test for 1000s of viruses at once. Maybe this doesn't work that well compared to standard methods, but **this planning grant is focused on thinking about big picture ideas.**
        - (b) (6) You can look at this pandemic as a blueprint for moving forward. The research diagnostic field moved very quickly (ELISAs were rapidly approved, etc.)
            - **Re the serochip idea, the big question is more about: Do we have the analytics in place to understand what the data means?**
                - (b) (6) Probably not. Even for multiplex of 15 tests, we barely have the bioinformatics set up to run those and have rapid interpretation. **So the rapid analytics side of things needs to be further explored.**
                - (b) (6), would you and (b) (6) be able to assist with anomaly detection of antibody positives?
                - (b) (6) I have a method that I can share with you (b) (6).
                    - (b) (6) That's maybe more in (b) (6) s field. I'm more about scaling up methods. I'm happy to take a look though.

(b) (6) There are several ideas for scaling serological surveillance. 1. Strategy to test more people, would involve having a more centralized high throughput lab. 2. Another strategy would involve moving more towards point of care. We could talk to (b) (6) Developed lateral flow assay that is optical reader so that lateral flow assay can be calibrated to give quantitative output at point of care scan. It measures light absorption that otherwise would just read positive or negative. 3. **Think about which of these methods would require having a rapid analytical pipeline in place.**

- (b) (6) **How do you detect exposure for a virus you're not looking for (e.g. one that's truly novel, i.e. the 'Disease X' problem?) Problem that I don't know the answer to**
    - With PCR, you can apply a viral family wide primer set, but you don't have that with serology
        - (b) (6) Ideally, you make serological tests as specific as possible
- (b) (6) In 1st aim (scaling up the measurement of human-animal contact), the idea is to identify (with more granularity and more geographic specificity), populations that are likely encountering probable viral hosts, and to identify the types of contact that are likely to lead to exposure. I think of serology in the same way. We can think about the degree of regional specificity. Looking at particular classes of viruses in certain areas, etc. Or some other stratified, scaling approach where one has higher cost multiplex approaches covering many different viruses, then using that to do more intensive/geographically extensive sampling for particular viruses. **This would be a surveillance design question.**
    - (b) (6)) **The problem is that we don't know the geographic extent of pathogen groups. So we can't necessarily target serological testing of particular viruses in particular geographies.**
    - (b) (6) So the knowledge gap is that viruses emerge where testing isn't deployed and that viruses diverge enough to avoid seroglocial testing.
        - So if you implemented more serological platforms at more interfaces (e.g. markets), would this be helpful or should we focus on hospitals/clinics?
        - (b) (6) There's a low level virus exposure that may not be human to human transmitted that we want to detect.
            - But logical framework is that with many viruses emerging, one of them will go pandemic.

    - (b) (6) Given the widespread nature of SARS-CoV-2, identifying people who have been exposed to CoVs won't be very helpful (unless we can discriminate between SARS-CoV-2 and other related viruses)
    - (b) (6) PCR - too much of a "needle in haystack" problem, could be overcome by novel, scaled-up serology approaches.
    - (b) (6) , we should circle back with you on some novel seroglocial ideas.
        - (b) (6) There's a nearby company that does 3D printing. Could 3D print the lateral flow plastic component. Issue would be access to positive controls. If we had outbreak, we could run it and look at acute periods vs. convalescent periods of exposure. Need to think about where we'd get validating serum samples
            - (b) (6) Re: positive control to EHA could potentially help procure samples (perhaps from EHA project w EIDRC on Nipah). We'll follow up on this

- *Debrief from Facebook (FB) <-> Rochester discussion earlier today*
    - (b) (6) FB would primarily support Aim 1 re human-animal contact (image recognition) as well as Aim 3 (post-exposure human mobility data)
        - Next steps will be to have discussions with the FB team about exploring the available data and discuss methodology used to weight the co-location dataset. (b) (6) (form FB) will set up discussions with his colleagues.
        - **Think this would be a nice private-public partnership to highlight for NSF**
    - (b) (6) I like the fact that FB is thinking seriously about privacy concerns, and that they're transparent about data sharing. Also data is available on a global scale.
    - (b) (6) I agree that it's important we highlight the importance of data privacy
    - PIPP team will review the notes from the discussion with Facebook
        - (b) (6) will start notes document as he works through datasets mentioned during Facebook call
    - (b) (6) Co-location dataset is available and updated weekly. Activity space dataset should be made available within a month or so. Might be available to us now, just not made public yet?
    - (b) (6) **For image recognition, do we want to hone in on a specific geography?**
        - (b) (6) **SE Asia is obviously of interest. Probably pretty good coverage from Facebook too. Central Africa not so much.**
        - (b) (6) For surveys, we'll have to refine our geographic scope.

- (b) (6) **We should include a schematic in the proposal for how we hone down our strategy** (e.g. conduct global image recognition of human bat contacts, and where we identify high rates of contact we push out surveys, etc)
- (b) (6) **It'd be helpful if we could map out all of the wildlife markets in a particular geography (e.g. Thailand), then use the activity map space to look at who's coming in and out of the areas where those markets exist**. Could be an interesting way to look at human flow in and out of markets. Perhaps we could implement a pilot study of this where markets have already been mapped out (Sulawesi bat markets that (b) (6) helped to identify). We could take the GPS coordinates of those markets and look at how human movement in and out of there changes over time.

- *Brainstorm "Broader Impacts" of our proposed 18 months*
  - (b) (6) **Curious what we want our broader impacts to be?**
  - (b) (6) Perhaps mention how methodologies could be used for societal good? (e.g. widespread implementation of FB surveys).
  - (b) (6) In the past, NSF's broader impacts have been very specific. Typically, they're heavily aimed at bringing underrepresented groups into the research field, with a big focus on the training and education sides of things. **On that front, the best thing to do was to already have a program at your institution that you could partner with. e.g. Are there REU programs that target recruitment of underrepresented groups?** Seems NSF has relaxed a bit about how narrow broader impacts are
  - (b) (6) **I think it's important to be creative about your broader impacts.** Rochester has a lot of programs focused on increasing diversity that track success of participants over time.
    - https://www.rochester.edu/college/kearnscenter/
    - At the same time, I don't think this piece of the proposal is quite as important as intellectual merit side of the proposal
  - (b) (6) We could highlight the EcoHealthNet (EHN) program. Mention that although the project is wrapping up in 2022, that we can extend the EHN model through to this PIPP project. We need to make sure the proposal includes funding for this though (and that we draw from financial numbers from EHN to accurately budget for this)
  - (b) (6) I had a friend that was part of an IB program in Maryland. They introduced me to a program that connected high school students to various research opportunities. I'll try to remember what this was.
  - (b) (6) We should think about this more. Need to be cognizant of diversity on our proposal as well.

- (b) (6) **Other things that I've seen that have been well received include: making lectures public (through Zoom/Zoom recordings) and making datasets open access**

- *AOB*

From: (b) (6)
To: (b) (6) ; Eric Laing
Cc: (b) (6)
Subject: Re: [Rescheduled] PIPP call this week, Thursday (9/2) from 3-4 PM EDT
Date: Tuesday, September 7, 2021 11:38:03 AM
Attachments: Notes - PIPP planning call - 2 September 2021.docx
ATT00002.bin

Dear all,

Attached are detailed notes from our NSF PIPP proposal planning call last week Thursday. Hope you all had a good Labor Day weekend. Will be following up throughout this week to start getting our proposal text together, NSF biosketches and Conflict of Interest lists from you, etc.

Cheers,
(b) (6)

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From: (b) (6)
To: (b) (6) ; Eric Laing (b) (6)
Cc: (b) (6)
Subject: Re: DEEP VZN proposal submitted!
Date: Thursday, June 17, 2021 5:21:09 PM
Attachments: DEEP VZN Decision Tree_v12.pdf
DEEP VZN Consortium Technical Application[37].docx

Hi everyone!

It has indeed been a great experience for almost 6 months working on this since we first started brainstorming on DEEP Vision. I want to thank everyone for the great insights and world-leading inputs along the way!

I've attached here the main technical section of the proposal we submitted last night, plus the optional decision tree we submitted as an annex that shows the flow of the major testing platforms/concepts and then the feedback loops to refine our various strategies along the way. For best effect, open the decision tree in Acrobat and look at it in the two-page (side-by-side) view. A huge shout-out to Eunah Cho at OHI for taking our initial caveman like etch-a-sketch drawings and turning them into a really neat and unified graphics package!

We of course ask that you keep these documents close hold/confidential within our group since we don't want this floating around somewhere and then somehow interfering with the review process.

I hope everyone has as great weekend, and we'll keep you in the loop as we hear back from USAID… most likely not until August if we're lucky!

Thanks again and all the best,

(b) (6)

From: (b) (6)
Date: Thursday, June 17, 2021 at 10:59 AM
To: (b) (6)

(b) (6)

Eric Laing

(b) (6)

Cc: (b) (6)

Subject: DEEP VZN proposal submitted!

Colleagues,
Our proposal was submitted late last evening and we received confirmation of receipt by USAID this morning.
(b) (6) will follow up shortly with the final version of the technical application so you can see how this all came together. We hope you will be pleased with this collaborative vision for the next steps in combatting emerging pandemic threats. We greatly appreciate your dedication, talents, and generosity in giving your time to pull this together over the last many months (including thousands of pages for the cost application) - we could not have done this without each of you.
Your collective expertise and experience is humbling. We think we have developed an exceptional proposal that reflects our strengths and a strong vision for the future of this work.
We are very grateful to have you all as partners in this endeavor, wherever this leads. We will be in touch as soon as we hear any news.
In the meantime, enjoy the summer!
(b) (6)

On 6/17/21, 4:40 AM, "Discovery & Exploration of Emerging Pathogens – Viral Zoonoses" <deepvzn@usaid.gov> wrote:

Confirming receipt of 8 emails.

Thank you.

On Thursday, June 17, 2021 at 1:20:07 AM UTC-4 (b) (6) wrote:

Dear Ms. (b) (6),

It is with great pleasure that I submit, on behalf of UC Davis and our consortium partners, the attached application to implement the Discovery & Exploration of Emerging Pathogens - Viral Zoonoses (DEEP VZN) project. For your reference, a list of the documents comprising our full application is included below; these documents will be submitted in multiple emails due to the file size limitations noted in the NOFO. Thank you for your consideration.

1. Technical Application Part 1 of 3
2. Technical Application Part 2 of 3
3. Technical Application Part 3 of 3
4. Business (Cost) Application (Excel)
5. Business (Cost) Application (Narrative) Part 1 of 5
6. Business (Cost) Application (Narrative) Part 2 of 5
7. Business (Cost) Application (Narrative) Part 3 of 5
8. Business (Cost) Application (Narrative) Part 4 of 5
9. **Business (Cost) Application (Narrative) Part 5 of 5**

Sincerely,

(b) (6)
[redacted]

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From: (b) (6)
To: Laing, Eric
Cc: (b) (6)
Subject: Re: SEARCH seq approaches
Date: Wednesday, May 12, 2021 11:12:57 PM
Attachments: PREDICTII Consensus PCR Lab Protocols_Mar_2017 - Priority Viral Families.pdf

---

Hi (b) (6),

No problem! The strategy is super simple - there are standardized consensus PCR protocols targeting <700nt of the RdRp gene that were used by all of the PREDICT labs (attached) for each virus family. Recently there was a new paper that came out that suggested that the CoV consensus PCR assay we are all using misses Sars-CoV-2 and some other newly described viruses, and updated the primers. These are the ones were were talking about testing and potentially switching over to: https://www.mdpi.com/1999-4915/13/4/599

Most of the PREDICT work used these cPCR assays and kind of stopped there so we have quite a large number of viruses that were detected and ID'd as being something novel based on short RdRp sequences, but no further genomic characterization was done.

Let me know if you have any other questions or want to chat further about any of this :)

Best wishes,

(b) (6)

On Thu, May 13, 2021 at 11:55 AM Laing, Eric (b) (6) wrote:

> Hi (b) (6),
>
> I have not been involved in PREDICT and don't know the nucleic acid strategies in use for SEARCH. Can someone send me some literature, figures or a brief explanation so I can get up to speed?
>
> (b) (6)
>
> [redacted]

--

(b) (6) [redacted]

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From: (b) (6)
To: (b) (6) ; Eric Laing
Subject: Re: R24 - questions about your section
Date: Wednesday, January 20, 2021 10:59:55 AM
Attachments: R24 Grant (b) (6) JAN15.docx

Hi (b) (6)

I think I have incorporated the changes but please check on the attached. This is the current snap-shot of the proposal, but it has a ways to go, yet.

Thanks,

(b) (6)

On Jan 19, 2021, at 6:05 PM, (b) (6) wrote:

Hello!

Attached you will find some edits from (b) (6). See the message below.

Cheers,
(b) (6)

---------- Forwarded message ---------
From: **Laing, Eric** (b) (6)
Date: Tue, Jan 19, 2021 at 8:02 PM
Subject: Re: R24 - questions about your section
To: (b) (6)

Hi (b) (6),

Nice to meet you. Thanks for the ORF5/ORF7 catch. I changed that and added the correct citations. Will you pass this along to (b) (6)? He has probably updated the GFP text to neonGreen.

(b) (6)

[redacted]

[redacted]

On Tue, Jan 19, 2021 at 7:47 PM (b) (6) [redacted] wrote:

> Hi Dr. (b) (6),
>
> My name is (b) (6) and I am working with (b) (6) at EcoHealth.
> I had two quick questions about your section in the R24. I have attached just that section here with them included in the word document.
>
> Let me know if my comment/questions make sense.
>
> Cheers,
> (b) (6)
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<R24[redacted] Questions[redacted].docx>

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From: Laing, Eric (b) (6)
To: (b) (6)
Cc: (b) (6)
Subject: Re: Grant status
Date: Friday, January 15, 2021 9:21:27 PM
Attachments: R24 Grant (b) (6) 010721 SPECIFIC AIMS-(b) (6) .docx

---

Hi (b) (6),

I added two sections to C.3.3.

(b) (6)

[redacted]

On Tue, Jan 12, 2021 at 9:46 AM (b) (6) [redacted] > wrote:

> All, attached is the very rough skeleton of the R24 proposal. I've noted areas for each of you to work on, but of course please feel free to edit any other places you want. It's certainly going to change a bit before the final submission, but (b) (6) and I want to get it to you sooner rather than later. Keep in mind that R24s are not supposed to be hypothesis-driven; they are really about resource development. The NIAID program officers gave us permission to do experimental challenges (Aim 3) so that's the place where we can do some science. We need to keep the Research Strategy to 12 pages (like an R01), so that means that means your sections should be a page to a page and a half. For your references, please just insert the PMID number and I'll get it the references inserted and formatted. It would be great if you could get your edits to us by Friday, but no later than Monday morning so that (b) (6) and I can tidy up the proposal in preparation for submission on January 22.
>
> For R24s, the Resource Sharing plan is critically important since the mechanism is directed to distribution of materials to the greater research community. We haven't started on that but I expect that once the proposal starts to shape up, this section will be mostly self-populating (but of course everone will get to see it before it is submitted).
>
> (b) (6) here at CSU is assembling the budgets and I think it will start routing through CSU's internal review process. If there are any problems, I'll be sure to let you know ASAP.
>
> (b) (6) and I have lined up several people who will provide letters of support for the proposal and I will be working on draft letters for each of them today.

Let me know if you have questions.

Thanks,

(b) (6)

Received: 8 August 2018 | Revised: 15 January 2019 | Accepted: 7 February 2019

DOI: 10.1002/ece3.5046

ORIGINAL RESEARCH

WILEY Ecology and Evolution Open Access

Pteropus lylei primarily forages in residential areas in Kandal, Cambodia

Kinley Choden[1*] | Sébastien Ravon[1*] | Jonathan H. Epstein[2] | Thavry Hoem[1] | Neil Furey[3,4] | Marie Gely[1] | Audrey Jolivot[5,6] | Vibol Hul[1] | Chhoeuth Neung[1] | Annelise Tran[5,6,7,8] | Julien Cappelle[1,7,8,9]

[1]Institut Pasteur du Cambodge, Phnom Penh, Cambodia

[2]EcoHealth Alliance, New York, New York

[3]Fauna & Flora International (Cambodia), Phnom Penh, Cambodia

[4]Harrison Institute, Sevenoaks, UK

[5]CIRAD, UMR TETIS, Montpellier, France

[6]UMR TETIS, CIRAD, CNRS, IRSTEA, AgroParisTech, Montpellier University, Montpellier, France

[7]CIRAD, UMR ASTRE, Montpellier, France

[8]UMR ASTRE, CIRAD, INRA, Montpellier University, Montpellier, France

[9]UMR EpiA, INRA, Marcy l'Etoile, France

Correspondence
Julien Cappelle, Institut Pasteur du Cambodge, Phnom Penh, Cambodia.
Email: Julien.cappelle@cirad.fr

Funding information
European Commission Innovate program (ComAcross project), Grant/Award Number: DCI-ASIE/2013/315-047; Centre National d'Etudes Spatiales (TeleNipah project), Grant/Award Number: DAR 4800000780

Abstract

1. Bats are the second most species-rich Mammalian order and provide a wide range of ecologically important and economically significant ecosystem services. Nipah virus is a zoonotic emerging infectious disease for which pteropodid bats have been identified as a natural reservoir. In Cambodia, Nipah virus circulation has been reported in *Pteropus lylei*, but little is known about the spatial distribution of the species and the associated implications for conservation and public health.
2. We deployed Global Positioning System (GPS) collars on 14 *P. lylei* to study their movements and foraging behavior in Cambodia in 2016. All of the flying foxes were captured from the same roost, and GPS locations were collected for 1 month. The habitats used by each bat were characterized through ground-truthing, and a spatial distribution model was developed of foraging sites.
3. A total of 13,643 valid locations were collected during the study. Our study bats flew approximately 20 km from the roost each night to forage. The maximum distance traveled per night ranged from 6.88–105 km and averaged 28.3 km. Six of the 14 bats visited another roost for at least one night during the study, including one roost located 105 km away.
4. Most foraging locations were in residential areas (53.7%) followed by plantations (26.6%). Our spatial distribution model confirmed that residential areas were the preferred foraging habitat for *P. lylei*, although our results should be interpreted with caution due to the limited number of individuals studied.
5. *Synthesis and applications*: Our findings suggest that the use of residential and agricultural habitats by *P. lylei* may create opportunities for bats to interact with humans and livestock. They also suggest the importance of anthropogenic habitats for conservation of this vulnerable and ecologically important group in Cambodia. Our mapping of the probability of occurrence of foraging sites will help identification of areas where public awareness should be promoted regarding the

*These authors contributed equally to this work.

ecosystem services provided by flying foxes and potential for disease transmission through indirect contact.

KEYWORDS
distribution model, ecology, epidemiology, flying fox, GPS, habitat use, interface, Nipah virus, telemetry

1 | INTRODUCTION

Bats are the second most species-rich Mammalian order with over 1,300 species worldwide (Voigt & Kingston, 2016) and provide a wide range of ecologically important and economically significant ecosystem services (Kunz, Torrez, Bauer, Lobova, & Fleming, 2011). They are also recognized as reservoir hosts for highly pathogenic viruses such as Nipah virus (NiV; Calisher, Childs, Field, Holmes, & Schountz, 2006).

Nipah virus was first identified in pigs and people in Malaysia in 1998 (Chua, 2000) and has reemerged annually in Bangladesh since 2001 (Luby et al., 2009). NiV causes lethal encephalitis in people, and bats in the *Pteropus* genus are the reservoir (Epstein, Field, Luby, Pulliam, & Daszak, 2006). Transmission of the virus in Malaysia is presumed to have occurred as a result of pigs consuming bat-contaminated fruits, followed by contamination of humans working with pigs (Chua, 2003). In Bangladesh, direct bat-to-human transmission of the virus occurs through the consumption of date palm sap (Luby et al., 2006). NiV has been isolated or detected in several *Pteropus* species in Southeast Asia, including *P. medius* in Bangladesh, *P. lylei* in Thailand and Cambodia, and *P. vampyrus* and *P. hypomelanus* in Malaysia. However, despite its detection in *P. hypomelanus*, a serological study on Tioman Island did not find the virus in any of the local people (Chong, Tan, Goh, Lam, & Bing, 2003) that the bats live among and regularly interact with (Aziz, Clements, Giam, Forget, & Campos-Arceiz, 2017). Seasonal NiV shedding patterns have been suggested for *P. lylei* in Thailand, with peak shedding occurring in May (Cappelle, Hul, Duong, Tarantola, & Buchy, 2014; Wacharapluesadee et al., 2010).

Understanding the capacity of a reservoir to spread the virus at local and regional levels to humans and domestic animals is fundamental to surveillance and prevention initiatives. Knowledge about the distribution and movement patterns of these bat species is thus required, and telemetry (measurement and transmission of data from remote sources) is a valuable tool to monitor the drivers and characteristics of fruit bat movements (Smith et al., 2011). This can be used to develop appropriate host management strategies that maximize the conservation of bat populations and minimize the risk of disease outbreaks in domestic animals and humans.

Telemetry studies have been undertaken on several *Pteropus* species in Asia and Australia. In Australia, tracking of fourteen *P. poliocephalus* males revealed that these are highly mobile between roosts and regularly travel long distances (Roberts, Catterall, Eby, & Kanowski, 2012). For instance, one *P. alecto* was tracked between Papua New Guinea and Australia and traveled more than 3,000 km over 11 months (Breed, Field, Smith, Edmonston, & Meers, 2010). In Southeast Asia, the movements of seven *P. vampyrus* males encompassed Malaysia, Indonesia, and Thailand, indicating the need for regional management plans for such species (Epstein et al., 2009). These studies highlight the difference between migratory and nomadic flying foxes and the need to adapt management strategies to relevant geographic scales.

At a local scale, telemetry studies indicate that *Pteropus* bats make foraging flights on a nightly basis, with distances from the roost ranging from a few kilometers to 20–30 km. Depending on species, foraging sites range from apparently intact forest to cultivated areas. In Bangladesh, the roosting ecology of *P. giganteus* is associated with forest fragmentation, likely because fragmented forests offers more foraging options to the bats, including fruit species cultivated by humans (Hahn et al., 2014). Conversely, in the Philippines, most foraging locations of eight *Acerodon jubatus* were situated in closed forest remote from areas of evident human activity (de Jong et al., 2013). Another study on *A. jubatus* and *P. vampyrus* in the Philippines suggested these species prefer undisturbed forest types and select against disturbed and agricultural areas (Mildenstein, Stier, Nuevo-Diego, & Mills, 2005). Foraging also repeatedly occurred 15–30 km from the roost on average. Similarly, movements of *P. alecto* were very similar between nights with most foraging sites located less than 6 km from roost sites. In Thailand, *P. lylei* also undertakes relatively short foraging movements (2.2–23.6 km) on a nightly basis to fields, plantations, backyards, and mangroves (Weber et al., 2015).

In Cambodia, three flying fox species are thought to occur, large flying fox *P. vampyrus* which is listed as "near threatened" by IUCN, Lyle's flying fox *P. lylei* which is listed as "vulnerable," and island flying fox *P. hypomelanus*, which is listed as "least concern" (IUCN, 2008; Kingsada et al., 2011). Most of the known flying fox roost sites in Cambodia are located on the grounds of pagodas, where hunting is limited by the presence of the monks (Ravon, Furey, Hul, & Cappelle, 2014). Consequently, these are often located in the middle of villages close to human and domestic animal populations, and available foraging areas mostly comprise anthropogenic landscapes. Flying foxes in Cambodia are likely to interact frequently with humans and to depend on human activities for their subsistence. As a consequence, understanding of their preferred foraging areas is important to inform public health and conservation actions.

The objective of our study was to use telemetry data to determine and characterize foraging locations visited by flying foxes

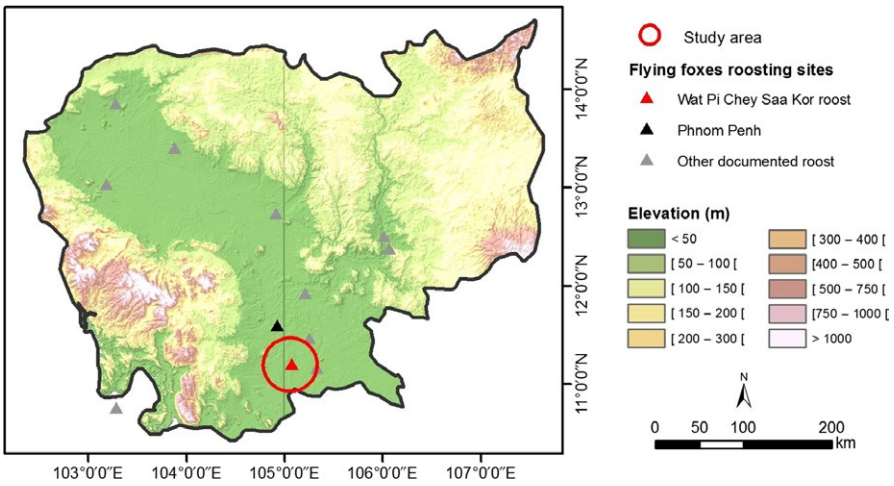

FIGURE 1 Location of the study area and other flying fox roost sites known in Cambodia

inhabiting a *P. lylei* roost in Koh Thom District, Kandal Province, Cambodia.

2 | MATERIALS AND METHODS

2.1 | Study site

The *P. lylei* roost selected for this study was located at Wat Pi Chey Saa Kor (11.200 N, 105.058 E), Kom Poung Kor village, Koh Thom District, Kandal Province (Figure 1). The site comprises a grove of trees on the grounds of a Buddhist pagoda which encompasses 21 roost trees with an estimated population of 4,000 flying foxes (Ravon et al., 2014). The village is bisected by a road with houses on either side and is characterized by a mosaic of agriculture that lacks significant areas of natural vegetation/forest. Land uses in the region include cultivation of rice and other crops, backyards, plantations, and various backyard animal farming activities.

2.2 | Study period

Our study was conducted from April 18, 2016 to May 17, 2016 when shedding of the NiV by *P. lylei* is believed to peak in Cambodia (Cappelle et al., 2014), similar to Thailand (Wacharapluesadee et al., 2010). Nine Global Positioning System (GPS) collars were deployed from April 18, 2016 to April 21, 2016 and five GPS collars from May 3, 2016 to May 6, 2016. Data were collected from these every day for two weeks after each collaring, related to the lifespan of individual collars.

2.3 | Bat collaring

Bats were captured using mist nets between 6 p.m. and 5 a.m. using methods described in (Newman, Field, Epstein, & De Jong, 2011). Weight, forearm length, sex, age, and reproductive status were documented for each bat. Animals were selected for collaring based on weight. Adult males and females without pups weighing at least 400 g were selected so that collars, weighting 20 g, would comprise <5% of body mass (Brigham, 1988). Pregnant and lactating female bats were excluded to avoid adding extra burdens.

Selected bats were anesthetized by injecting medetomidine into the pectoral muscle (Epstein, Zambriski, Rostal, Heard, & Daszak, 2011). GPS devices (FLR V, Telemetry Solutions™, www.telemetrysolutions.com) attached to nylon bands were secured around the neck of each bat using catgut suture (1.0) and three surgical knots (Figure 2), which were presumed to last for at least 30 days. Following collar attachment, atipamezol was injected intramuscularly. Each collared bat was kept in a separate cage during recovery from anesthesia and offered pieces of mango ad libitum prior to release.

We deployed 14 GPS collars on 13 adult males and one adult female (Table 1). Collars 1–5 were programed to transmit one location every 30 min from 5 p.m. to 6 a.m. while collars 6–14 were programed to transmit one location every 30 min for the first night only and one location every 5 min from 5 p.m. to 6 a.m. on following nights. As a consequence, collars 1–5 were expected to last for 1 month and allow observations of foraging behavior across the expected annual excretion peak of NiV. Collars 6–14 were expected to last for 10 days and provide detailed information on *P. lylei* foraging sites, including night roosts. Data were remotely downloaded each morning from active collars with a base station, which automatically connected to the GPS collars when within reading distance (10–20 m).

2.4 | Spatial data and site characterization

Global Positioning System data were transferred each morning to a computer, converted into KML format (QGIS, version 2.14), and mapped to identify foraging locations visited by bats the previous night (Google Earth, version 7.1). Foraging sites were identified based on clusters of two or more locations obtained from individual bats and as many as possible were visited depending on accessibility. Tree species visited by bats and evidence of foraging such as partially

eaten fruits were recorded to facilitate identification of roosting and feeding trees. Nonfruiting trees were also recorded.

2.5 | Habitat use

All locations were classified in three major categories: roost locations (all points less than 30 m from the roost site), foraging locations (a cluster of ≥2 two points separated by <30 m where the bat spent at least 10 min at night (i.e., from 6 p.m. to 6 a.m.)), and commuting locations (isolated points connecting the roost and foraging sites located >30 m from a foraging or roost location). Based on patterns visible in Google Earth, five habitat types were recognized for foraging locations: plantations (including fruit trees within the plantation and trees around the plantation), residential areas (locations within 50 m of human settlements, including pagodas, backyards, roads), agricultural lands (any cultivated land not included in "plantations" and "residential areas"), rivers, and uncultivated areas (all locations not included in the preceding categories).

FIGURE 2 Collared *Pteropus lylei*, southern Cambodia

2.6 | Spatial analysis

The home range of an animal illustrates spatial and temporal use of an area and is defined as the area commonly used for normal activities such as foraging for food, breeding, and caring for young (Burt, 1943). We used the kernelUD() function of the Adehabitat package in R software (Version 3.2.3) to estimate the home range for all bats, using the kernel density method (Calenge, 2006). The function computes the different percentage levels of home range estimation, for example the 50% home range identifies the areas where an individual is likely to occur 50% of the time.

We used QGIS to analyze the trajectories of each bat and to generate heatmaps based on kernel density estimation. The density was calculated based on the number of points in a location, with larger numbers of clustered points resulting in larger values. We also used the sp package in R software to calculate the maximum linear distance traveled from the roost per night.

The spatial distribution of foraging sites in the study area was modeled using the GPS data collected, a set of generated background data and land cover data. We created a map which classified habitats according to their expected influence on foraging site selection by the bats. This map was the product of a classification procedure based on Landsat images (30 m spatial resolution) acquired in 2015 and ground-truthing. Details of the classification are provided as Appendix (Supporting information Appendix S1: Table S1), and the result is illustrated by (Supporting information Appendix S1: Figure S1). The eight different habitats identified in this classification were

TABLE 1 Characteristics of *Pteropus lylei* studied and GPS device performance, southern Cambodia. The proportion of valid data corresponds to the proportion of locations recorded with valid geographic coordinates

Bat ID	Sex	Reproductive Status	Weight (g)	Forearm (mm)	Collar lifespan (nights)	Total recorded data	Proportion of valid data (%)
Bat01	Male	Mature	560	169	26	760	32
Bat02	Male	Mature	565	152.9	3	247	90
Bat03	Male	Mature	540	165.5	11	439	81
Bat04	Male	Mature	435	NA	9	394	40
Bat05	Male	Mature	490	149.4	23	716	88
Bat06	Male	Mature	430	151.9	13	1,904	95
Bat07	Male	Mature	425	149.5	9	1,747	41
Bat08	Male	Mature	420	144.9	12	1,675	95
Bat09	Male	Mature	532	145.9	1	22	41
Bat10	Male	Mature	425	144.5	8	1,200	89
Bat11	Male	Mature	590	153.7	13	1,768	98
Bat12	Male	Mature	414	148.3	12	1,752	99
Bat13	Female	Adult	430	149.4	12	1,592	96
Bat14	Male	Mature	550	152.4	13	1,912	97

speculated to have the following impacts on the distribution of foraging sites. Plantations were expected to be highly attractive to bats because of the high density of fruit available. Tree vegetation was expected to be attractive because of the potential presence of fruit consumed by bats. Water bodies such as rivers were also expected to attract the bats due to the presence of fruit trees on their banks. Residential areas were expected to have mixed effects as a source of disturbance for the bats and a potential source of fruit in backyards. The four remaining habitats in the classification (rice field, bare soil, flooded vegetation, and shrubland) were not expected to attract the bats.

To train and validate the model, we used all GPS locations of foraging sites and generated an equivalent number of background locations in the study area which were used as pseudoabsences by the model. Half of the data were randomly assigned to a training dataset and the other half to a validation dataset. We used a generalized linear model with the training dataset as the response variable with a binomial distribution (1 for presence and 0 for pseudoabsence) and habitat type as an explanatory qualitative variable. To deal with the discrepancy between the spatial resolution of our classification (30 m) and GPS points (1–5 m), we calculated the distances of all data points to the closest habitats with an expected influence on bat habitat selection: plantations, tree vegetation, water bodies, and residential areas. Because of this discrepancy and landscape fragmentation in the study area, GPS locations of bats foraging in attractive habitats could be recorded in an adjacent nonattractive habitat. We therefore generated four explanatory variables (dPlant, dTree, dWater, and dResid) to allow us to capture the spatial structure of the study area. Using the distance to these attractive habitats as explanatory variables in the model would then help take into account the limited spatial resolution of our habitat classification as well as spatial autocorrelation. As a consequence, no further variable was added to the model to deal with the latter. Finally, distance to the roost (dRoost) was added to the explanatory variables as this should be minimized by the bats to optimize their energy efficiency while foraging. We used the results of the model, which was based on data from 14 individuals, to map the probability of presence of the foraging sites of *P. lylei* in the study area. The validation dataset was used to estimate the performance of the model through the calculation of the area under the ROC curve (AUC).

3 | RESULTS

3.1 | Collar performance

A total of 84 bats were caught, 14 of which were selected for collaring (Table 1). Our GPS devices transmitted from 1 to 26 nights, with an average of 11.8 nights (Table 1). A total of 13,646 valid locations were collected over 27 nights from the 14 collared bats. The proportion of valid data (i.e., data with an actual geographic location provided) varied from 32% to 99% of the data provided by each collar. Overall, 84.6% of the data generated were valid locations (n = 13,646/16,128).

3.2 | Habitat use

Tree species identified during visits to foraging sites are listed in Table 2. Partially eaten mango (*Mangifera indica*, n = 15) and sapodilla (*Manilkara zapota*, n = 3) were found at exact GPS foraging locations (Figure 3). It was not possible to detect whether leaves or flowers were also consumed.

TABLE 2 Tree species identified at foraging sites of 14 GPS-collared *Pteropus lylei*, southern Cambodia

Common name	Scientific name	Species at GPS locations (5 m precision)	Species ≤30 m from GPS locations	Known to be consumed by flying foxes[a]
Banana	*Musa paradisiaca*		X	Direct
Banyan	*Ficus benghalensis*		X	Unknown
Custard apple	*Annona reticulate*		X	Direct
Eucalyptus	*Eucalyptus exserta*	X	X	Indirect
Jack fruit	*Artocarpus heterophyllus*		X	Direct
Java apple	*Syzgium malaccense*		X	Unknown
Kapok	*Ceiba pentandra*	X	X	Direct
Longan	*Dimocarpus longan*		X	Indirect
Mango	*Mangifera indica*	X	X	Direct
Neem	*Azadirachta indica*	X	X	Direct
Papaya	*Carica papaya*		X	Direct
Sacred fig	*Ficus religiosa*	X	X	Direct
Sapodilla	*Manikara zapota*	X	X	Direct
Sugar palm tree	*Borassus flabellifer*	X	X	Indirect

[a]Direct means direct evidence from feces or feeding remains, indirect means information based on evidence from location data but with no direct evidence from feces or feeding remains. Based on (Aziz, Clements, Peng et al., 2017; Hahn et al., 2014; Weber et al., 2015; Win & Mya, 2015).

Among the valid data, 29% of the locations (*n* = 3,959/13,646) corresponded to the roost site where the bats were captured, 20.3% (*n* = 2,774) to commuting locations, and 50.7% (*n* = 6,913) to foraging locations and night roosts. Most of the foraging locations were in residential areas: 53.7% (*n* = 3,714/6,913), 26.6% (*n* = 1,836) in plantations, 16.2% (*n* = 1,118) in uncultivated areas, 3.2% (*n* = 219) in agricultural lands, and 0.4% (*n* = 26) on rivers (Table 3). (Supporting information Appendix S1: Figure S2) shows the spatial distribution of the foraging sites in the study area.

3.3 | Movement patterns and flight distances

The maximum distance traveled per bat/night ranged from 6.88 to 105.14 km and averaged 28.3 km (Table 3). All individuals showed fidelity to at least one foraging site, returning on 3–11 nights to the same site (all locations <30 m from the previous one were counted as the same foraging site) during the study period. Thirty-six foraging sites were shared by at least two bats. All bats (excluding bat #9 due to lack of data) shared at least one and as many as eight foraging locations with another bat. Shared foraging locations or night roosts were relatively close to the roost, with an average and maximum distance of 2.85 and 7.75 km, respectively. Eight bats returned to the study roost every night (bats #1–3, #6, #9, #11, #13–14). Of the six remaining bats, four went to a nearby *P. lylei* roost in Prey Veng Province (28 km east, Wat Veal Lbang, Prey Veng, 700 flying foxes), whereas two went to more distant and previously unknown roosting sites: 65 km in one night (site A) and 105 km over two nights (site B) for bat #8 and 50 km during one night (site C) for bat #10 (Figure 4).

3.4 | Spatial analysis

The complete results of the home range estimations for all bats are shown in (Supporting information Table S2). The estimated home ranges were maximal for bats #08 and #10 which went to distant roosts, with 95% home range of respectively 5,984 and 1,158 km^2. For the eight bats that did not join another roost, the 95% home range ranged from 29.5 to 316.8 km^2 with an average 95% home range of 104.5 km^2 (*SD* = 115.5 km^2). The 50% home range of these same eight bats ranged from 4.3 to 41.1 km^2 with an average 95% home range of 14.9 km^2 (*SD* = 13.4 km^2). Our heatmap of GPS locations showed that most foraging sites and night roosts were located <15 km from the roost (Figure 5). The spatial distribution model showed that foraging locations were significantly negatively correlated with the distance to the roost, residential areas, and water bodies. Conversely, foraging locations were significantly and positively correlated with distance to plantations. Residential areas, trees, and plantations were the main foraging habitats used by the bats (Table 4). Our map of the probability of *P. lylei* foraging sites highlights areas close to the roost but also helps to identify further areas where bat–human interfaces could occur (Figure 6). Model performance was very good with a cross-validated AUC of 0.93.

FIGURE 3 Partially consumed mangoes at a GPS foraging location of *Pteropus lylei*, Kandal Province, southern Cambodia

4 | DISCUSSION

Our study yielded two main results. First, our study bats mostly foraged in residential areas (53.7% of foraging locations) rather than in plantations (25.6%) and our spatial model indicated that residential areas were the preferred foraging habitat (Table 4). While other studies have shown that *P. lylei and P. giganteus* can primarily forage in anthropogenic landscapes (Hahn et al., 2014; Luskin, 2010; Weber et al., 2015), our data indicate a particularly strong interface through residential backyards where the potential for contact between bats and humans would be higher due to continuous human presence. This could potentially facilitate NiV transmission to humans and domestic animals and two transmission routes have been documented in previous outbreaks of NiV. The first is directly from bats to humans due to consumption of raw palm sap contaminated by flying foxes, which has led to recurrent outbreaks in Bangladesh (Luby et al., 2009). The second route was suggested for the Malaysian outbreak where pigs were likely infected after consuming fruit contaminated by flying foxes (Chua, 2003) and supported by isolation of the virus from fruit partially eaten by bats in Malaysia (Chua et al., 2002). Consistent with this second route, a direct bat-to-human transmission via ingestion of fruit has been suggested for another paramyxovirus in Malaysia (Yaiw et al., 2007). Thus, by frequently foraging in residential areas, *P. lylei* could contaminate fruit where humans and domestic animals live, increasing the chance of indirect contact. As such, further information on the use by local residents of fruit partially eaten by bats would help to characterize transmission risks and inform preventative actions including promotion of public awareness. Similarly, palm sap collectors in the study area reported seeing flying foxes on palm trees and urine and feces on collection containers. As our data also indicate that *P. lylei* visits these trees (Table 2), research on palm sap collection in the area is needed to assess the risk associated with this potential transmission route.

TABLE 3 Maximum distances traveled per night by *Pteropus lylei* and proportion of foraging areas per category, southern Cambodia

Bat ID	No. of foraging locations and night roosts	Max distance/night (km)	Residential area (%)	Plantation area (%)	Agricultural land area (%)	Uncultivated area (%)	River (%)
Bat01	111	8.95	32	41	17	0	11
Bat02	145	7.91	15	75	10	0	0
Bat03	189	10.28	99	1	0	0	0
Bat04	100	29.60	75	9	16	0	0
Bat05	190	29.35	89	4	0	7	0
Bat06	1,109	23.35	32	31	4	32	1
Bat07	411	27.39	50	2	4	44	0
Bat08	798	105.14	62	17	2	19	0
Bat09	3	6.88	0	100	0	0	0
Bat10	628	52.11	18	60	2	21	0
Bat11	761	10.39	4	76	0	20	0
Bat12	964	50.33	79	8	4	9	0
Bat13	421	25.45	62	29	4	4	2
Bat14	1,083	9.03	93	2	2	2	0
Total	6,913	28.3[a]	54[b]	27[b]	3[b]	16[b]	0[b]

[a]mean of the maximal distance per night for all bats. [b]Proportion of foraging area for all locations of all bats.

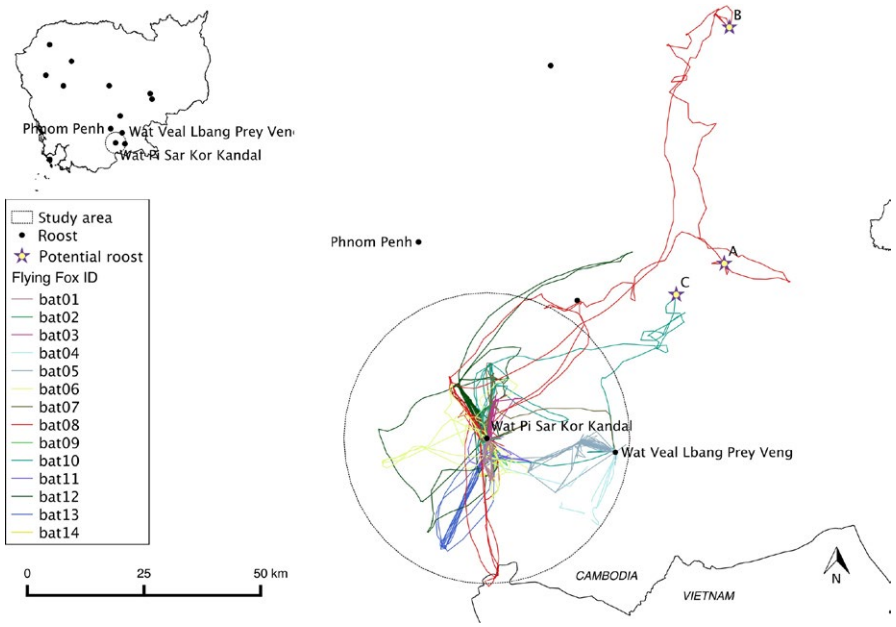

FIGURE 4 Movements of 14 GPS-collared *Pteropus lylei* during the study period in southern Cambodia

Our finding that *P. lylei* mostly forages in residential areas—which mostly correspond to backyards—rather than in plantations was unexpected because human disturbance would likely be higher in the former and food availability greater in the latter. Since our data indicate that *P. lylei* feeds on a variety of fruit in April–May, the greater diversity of fruit typically found in backyards compared to plantations could possibly explain this. More generally, the link between flying fox foraging behavior and the greater diversity of fruits in anthropogenic versus natural environments has been reported elsewhere (Hahn et al., 2014; Luskin, 2010; Weber et al., 2015). All foraging sites in our study were located in anthropogenic landscapes and all individuals showed fidelity to foraging areas, indicating repeated utilization once a food resource was located. This is presumably more energy-efficient than random foraging and is consistent with studies of *A. jubatus* in the Philippines (de Jong et al., 2013) and *P. alecto* in Australia (Palmer & Woinarski, 1999). From an epidemiological standpoint, an infectious flying fox repeatedly shedding virus in the same area could facilitate site contamination and increase the risk of transmission to humans or animals. Indeed, all of our 14 bats shared at least one foraging site during the study. Repeated shedding

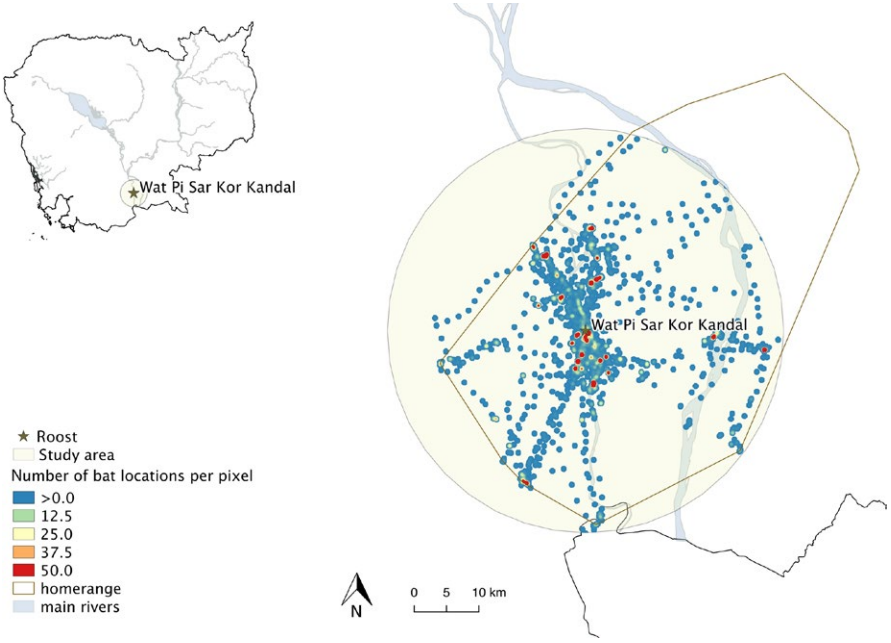

FIGURE 5 Heatmap of *Pteropus lylei* movements and home range (minimum convex polygon) in southern Cambodia

at a shared foraging site or night roost could also increase pathogen transmission in the bat population through fruit contamination. In future analyses, we will use a hidden Markov model to determine different phases of nightly movements and attempt to differentiate foraging sites from night roosts.

From a conservation perspective, the apparent preference for backyards and plantations suggest that our *P. lylei* population is highly dependent on human activities for foraging. As such, understanding of community knowledge, attitudes, and practices regarding bats will be important to develop appropriate conservation and public awareness strategies and is now underway. Nevertheless, that residential backyards were the most strongly selected foraging habitat suggests that conflict with humans may be limited in our study area. This is consistent with the fact that other patches of trees were also attractive to our study bats ("Tree vegetation" in Table 4), albeit less than backyards and plantations. Were major bat–human conflicts to occur in our study area, the few attractive non-human-dominated habitats present could possibly become overselected by the bats. However, our results must of course be interpreted with caution as only 14 bats in the same population were studied.

Second, because six of our 14 study bats visited at least one other roost during our 28-day study, it would appear that movements to other roost sites are relatively frequent. However, these movements were limited in time and the fidelity shown to the day roost by all of our study bats is consistent with the non-nomadic ecology attributed to *P. lylei*. Similar to observations for *P. vampyrus* (Epstein et al., 2009) and *P. medius* (Epstein, unpublished), visits to four other roosts including one 105 km from the study site were observed. These frequent exchanges between roosts are consistent with a regional circulation of different NiV strains in Southeast Asia suggested in previous studies (Epstein, 2017; Wacharapluesadee et al., 2016). From a conservation perspective, they also suggest that *P. lylei* in Cambodia is likely a metapopulation and that conservation strategies should be planned on a regional scale. This is consistent with the results of another telemetry study on the migratory *P. vampyrus*, calling for a comprehensive protection by regional management plans across their international range (Epstein et al., 2009).

The main limitation of our research is the small number of individuals we could study. With only 14 nonrandomly selected individuals tracked out of an estimated 4,000–6,000, our data are unlikely to be representative of the roost population as a whole. Additionally, because foraging behavior is highly dependent on local environments, our results should not be extrapolated to all *P. lylei* colonies in Cambodia. Furthermore, our study group had a strong male bias, with only one female tagged with the GPS device. Though

TABLE 4 Results of generalized linear model. Significant explanatory variables with a *p*-value $<10^{-3}$ are given in bold

Variable	Coefficient (*SE*)	*p*-Value
Intercept	2.844 (0.355)	$1.10\ 10^{-15}$
Habitat type		
Residential area	**2.853 (0.385)**	**$1.34\ 10^{-13}$**
Tree vegetation	**2.178 (0.296)**	**$1.77\ 10^{-13}$**
Plantation	**1.865 (0.519)**	**$3.26\ 10^{-4}$**
Bare soil	0.695 (0.345)	0.044
Water	0.289 (0.670)	0.666
Flooded vegetation	−0.598 (0.499)	0.231
Shrubland	−13.879 (486.4)	0.977
Rice field	Reference	
dResid	**−0.337 (0.111)**	**$2.28\ 10^{-3}$**
dTree	−0.519 (0.411)	0.206
dWater	**−0.599 (0.135)**	**$9.38\ 10^{-6}$**
dPlant	**0.133 (0.040)**	**$8.91\ 10^{-4}$**
dRoost	**−0.220 (0.016)**	**$<2\ 10^{-16}$**

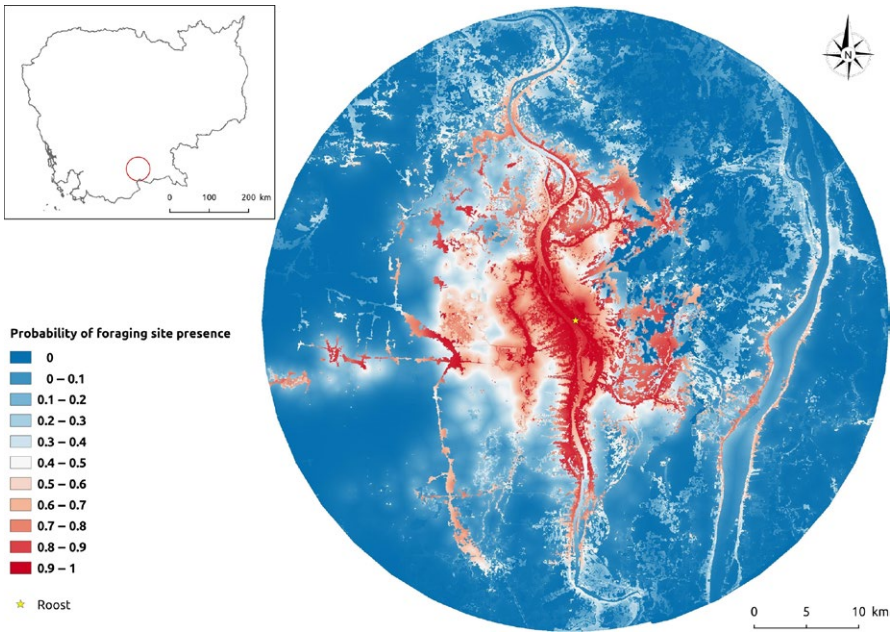

FIGURE 6 Probability of occurrence of *Pteropus lylei* foraging sites based on spatial distribution modeling, southern Cambodia. The model was trained and validated with GPS locations from 14 tracked individuals

other females were caught, these were excluded as they were pregnant or lactating and because limited data are available for female *P. lylei*, it remains unclear if the sexes differ in their foraging behavior. For instance, while female and male *P. poliocephalus* are similar in their movement patterns (Roberts et al., 2012; Tidemann & Nelson, 2004), lactating females of *P. alecto* travel greater distances between roosts and foraging sites than males (Palmer & Woinarski, 1999; Roberts et al., 2012). Nine of the 14 GPS collars we deployed lasted for at least 10 nights (average 11.8 nights), and 80% of the data were valid. Three other collars provided relatively few valid locations, and only one failed to transmit meaningful data. This performance rate was probably influenced by extended battery life due to high temperatures during the study period, while the open agricultural landscape of our study area probably facilitated the acquisition of GPS locations, saving further battery power. We deployed GPS devices on a limited number of individuals, preventing us from any generalization of the observed patterns at the population level. However, the results were consistent between the different individuals and provided useful information on the movement and foraging ecology of *P. lylei* in Cambodia. The GPS devices we used were battery-powered, and the size of the battery was limited by the body weight of the flying foxes. By programing five GPS devices to record locations every 30 min instead of 5 min for the nine other devices, we expected them to last for a month. However, data for only two of these bats were collected for more than 20 days, limiting our capacity to observe any change in foraging patterns over this period. Further studies should then be implemented to assess any variability of foraging patterns over time.

While our data represent a brief snapshot in time, they nonetheless illustrate the potential for foraging behavior to potentially facilitate NiV transmission to humans and domestic animals. To date, no transmission from *P. lylei* to human or animals has been recorded despite the circulation of NiV in this species in Cambodia and Thailand (Cappelle et al., 2014; Reynes et al., 2005; Wacharapluesadee et al., 2010). The presence of a hazard such as the NiV in a reservoir population does not necessarily lead to an emergence (Hosseini et al., 2017). Indeed, despite NiV being detected in *P. hypomelanus* on Tioman Island, no outbreak has occurred there, and no evidence of the virus has been found in people on the island (Chong et al., 2003). As such, close and frequent interfaces between bats and humans, including bats roosting in the middle of villages and feeding on cultivated fruit in residential backyards and orchards (Aziz, Clements, Giam et al., 2017) may not be sufficient to lead to an emergence. Other factors such as cultural and agricultural practices must be taken into account.

Different agricultural practices may lead to different levels of exposure in the countries of Southeast and South Asia. Conditions specific to intensive pig farming in Malaysia or palm sap collection in Bangladesh may explain why the virus emerged in these countries. Nevertheless, understanding the ecology of *P. lylei* may significantly improve our ability to target limited resources for interventions, and educational campaigns that discuss the risks of NiV to people and their domestic animals (Nahar et al., 2014; Parveen et al., 2016). In particular, while based on only 14 individuals, our mapping of the probability of occurrence of foraging sites for the *P. lylei* will help targeting prevention measures to areas where contact between flying foxes and humans can be expected.

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CONFLICT OF INTEREST

None declared.

AUTHOR CONTRIBUTIONS

JC and AT conceived the study and designed methodology; JC and TH coordinated the capture of the bats with the help of KC, SR, NF, VH, and CN; JHE coordinated the deployment of the GPS collars; AT, MG, and AJ collected environmental data and produced the land cover map of the study area; KC and SR collected the GPS data from the collars; KC and SR analyzed the data and led the writing of the manuscript. KC, SR, and JC drafted the first version of the manuscript and all authors contributed critically to the drafts and gave final approval for publication.

DATA ACCESSIBILITY

The data used in this study are available on Movebank (movebank.org, study name "Foraging movements of Lyle's flying foxes in Cambodia (data from Choden et al. 2019)") and are published in the Movebank Data Repository (Choden et al., 2019). https://doi.org/10.5441/001/1.j25661td.

ORCID

Neil Furey https://orcid.org/0000-0003-0439-2446
Annelise Tran https://orcid.org/0000-0001-5463-332X
Julien Cappelle https://orcid.org/0000-0001-7668-1971

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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BOHRN

The Bat / One Health Research Network

The 1st Annual Bat / One Health Research Network Workshop

8-9 November 2018 • Vienna, Austria

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Workshop Overview

Executive Summary

The Bat/One Health Research Network (BOHRN) convened its 1st Annual Research Workshop in Vienna, Austria, 7-8 November 2018, in advance of the International Meeting on Emerging Diseases and Surveillance (IMED). This two-day workshop was organized and hosted by the Defense Threat Reduction Agency, Cooperative Threat Reduction Directorate, Biological Threat Reduction Program (BTRP) in its capacity as a sponsor of life-sciences research-based Threat Reduction Networks (TRNs). This event provided an opportunity to advance BOHRN's core agenda of enabling interdisciplinary collaboration at the interface of biological threat reduction, research, and conservation.

The BOHRN initiative was organized at a side-meeting of the 2nd International Symposium on Infectious Diseases of Bats in Fort Collins, CO on 29 June 2017. During this meeting participants established a Steering Committee and began preliminary actions to build a multi-disciplined, self-sustainable network to better characterize global threats of bat-borne pathogens and formalize community standards and conservation- conscientious practices for One Health disease research. During a series of follow-on meetings, members of the BOHRN Steering Committee identified objectives and developed a research strategy to prioritize and target common needs. The BOHRN 1st Annual Research Workshop in Vienna provided an opportunity to validate its research strategy with a wider audience.

The workshop began with a series of introductory presentations from Dr. (b) (6), DTRA BTRP, who provided background on her organization and the BOHRN effort. There were also a series of presentations from other subject matter experts who provided short lectures on areas were identified as knowledge gaps by members of the Steering Committee at previous BOHRN meetings (note: the full agenda may be found here). Next, workshop attendees participated in two breakout sessions. The first session focused on the research focus areas within the four (4) BOHRN Working Groups and aimed to solicit feed-back in real time on the short and long-term objectives within each network working group.

The second breakout session was initiated by an interactive exercise, facilitated by Dr. (b) (6) (Texas Tech University) and Dr. (b) (6) (EcoHealth Alliance), mapping the intersectional of ecological and epidemiological research questions. Participants were then divided into regional groups with diverse and varying levels of expertise to sketch out hypothesis-driven research projects that mapped to BOHRN working group focus areas. Members of the BOHRN Steering Committee and other experts were on-hand to provide mentorship and guidance. At the end of the workshop, each project was presented orally by a member of the project team in a mock peer review session for feed-back and discussion.

The output and recommendations gathered from the small-group sessions will inform BOHRN next steps, which Dr. (b) (6) described at the conclusion of the workshop as a series of special grant awards for project proposals under BOHRN. She described the process as 'still under construction' but affirmed her leadership's commitment to maintain the network's initial momentum. While the exact mechanism and criteria for award are still being discussed, all interested parties may anticipate a call for proposals via the BOHRN website at some point in the spring of 2019.

There are a number of factors that make bats unique disease reservoirs, including their social behavior, distinct physiology and metabolism, ability to travel long distance, nocturnal activity, species diversity, and long life-span. BTRP anticipates that by taking a lead in funding bat-associated pathogen research, their organization can play a significant role in better characterizing the role of bats in global zoonotic disease ecology, coupled with assessing the impact of human-mediated interactions and environmental changes, to better understand threat reduction value of surveillance and intervention efforts.

Previous BOHRN Events

BOHRN Kick-off Meeting

- Concurrent with 2nd International Symposium on EID
- Sponsored by BTRP
- Took place in Fort Collins, CO – 29 June 2017
- **Outcomes:** (1) established a steering committee; (2) drafted terms of reference; (3) identified research areas of interest

BOHRN Steering Committee Strategy Mapping Meeting – 1

- Concurrent with Prince Mahidol Award Ceremony
- Sponsored by BTRP
- Took place in Bangkok, Thailand – 30 January 2018
- **Outcomes:** (1) prioritized research focus areas; (2) developed targeted action plans; (3) drafted associated workplans and timelines

BOHRN Steering Committee Strategy Mapping Meeting – 2

- Concurrent with International One Health Congress
- Sponsored by BTRP
- Took place in Saskatoon, Canada – 20-21 June 2018
- **Outcomes:** (1) completed workplans and timelines for research focus areas; (2) established BOHRN branding and website; (3) drafted communication and outreach strategy

BOHRN Biological Threat Characterization Discussion

- Concurrent with Western Asia Bat Network (WABNet) Kickoff Meeting
- Sponsored by BTRP, organized by EcoHealth Alliance
- Took place in Tbilisi, Georgia – 20 September 2018
- **Outcomes:** (1) identified and characterized regionally-focused gaps and needs (2) activated communication and outreach strategy;

Workshop Outcomes

Presentation Summaries

The following subject matter experts were invited to present on areas that were identified as knowledge gaps in BOHRN. Event participants received a pdf copy of each presenter's slides.

Dr. (b) (6)

Dr (b) (6), EcoHealth Alliance, presented on *Understanding the Ecology of Emerging Zoonoses*. His presentation focused on the three stages of disease emergence to help understand the complexities of spillover. Starting with the first stage, wildlife and domestic animal interactions, Dr. (b) (6) explained the movement of microbes into domestic animals. Human's increasing interactions with domestic animals leads to the second stage where the microbe has spilled over into the human population causing widespread outbreaks. The third and final stage of disease emergence is the outbreak reaching pandemic levels. Dr. (b) (6) proceeded to present two cases Nipah Virus spillover from Pteropid Bats and Nipah Virus spillover from date palm sap harvesting. Both cases were used to support evidence that the driver of spillover is human activity. However, as Dr. (b) (6) explained, this does not account for why human infections occur a small areas of these bat's known habitats. Therefore, it is important to understand why spillover is only occurring in these small areas, whether it is a rare event or there is a need for more broad spread surveillance.

Dr. (b) (6)

Dr. (b) (6), from the Center for Disease Control and Prevention, presented on *Filovirus Maintenance in Nature: Potential Lessons Learned from Studying Marburg Virus*. Dr (b) (6) presentation focused on the persistence of Marburg Virus (MARV) in nature supported by the recent study findings that Egyptian Rousette bats are identified as a natural reservoir for MARV. The study looked at bats during birthing and breeding seasons in the Python Cave of Uganda and focused on the impact seasonal pluses have on human spillover. From this study, Dr. (b) (6) presented on the need for messaging to miners and the community to emphasize the importance of bats to the ecosystem and the effects of culling the bats in Python Cave. In addition, the presentation focused on discussing virus transmission from bat to bat, long-term immunity in bats, and the potential to recreate the study with Ebola Virus.

Dr. (b) (6)

Dr. (b) (6), from the University of California-Davis, presented on *Synergies Between the Bench and the Field for Virus Discovery and Capacity Building*. Dr (b) (6) focused on the work of the USAID PREDICT program and the Ebola Host Project. Dr. (b) (6) began his presentation by explaining the challenges of targeted, risk-based surveillance the PREDICT program focuses on. He led into a discussion on virus discovery and detection from identifying viruses by consensus polymerase chain reaction (PCR) supplemented by high-throughput screening (HTS) to performing experiments to understand and rank the potential risk of the virus. Dr. (b) (6) then explained the process PREDICT uses to strengthening laboratory efforts and used the Ebola Host Project in Sierra Leone as an example of

these efforts. The Ebola Host project has led to the training of numerous local scientists and the development of community outreach materials. Dr. (b) (6)'s presentation summarized the efforts in Sierra Leone to focus on Filoviruses which has led to identifying new Ebola viruses in insect eating bats before known human or animal sickness.

Dr. (b) (6)

Dr. (b) (6) presented on *Flying Foxes as Bushmeat in Sulawesi Indonesia, Building Community Outreach Initiatives Based on Novel Understanding of Who, Where, and Why*. Dr. (b) (6) began by identifying the common challenges in institutional capacity, identification of stakeholders, interagency coordination, and funding. Her presentation then focused on the flying foxes as bushmeat and the cultural understanding of the drivers for how and why bats are hunted. Dr. (b) (6) used the outreach initiatives in Sulawesi, Indonesia to emphasize that outreach must include regional level coordination to allow for national level communication at both the front and tail end of any project. In addition, outreach should be designed for the community and the importance of assessing effective ways to disseminate information. Dr. (b) (6) explained potential resolutions to the common challenges could include providing training on outreach, incorporating voices from all levels of policy, and demonstrating the value to other sectors for interdisciplinary funding.

Breakout Session 1 Overview

In advance of the first breakout session, Dr. (b) (6) (EcoHealth Alliance) and Dr (b) (6) (Texas Tech University) provided an update from the Steering Committee, summarizing a year's worth of Steering Committee Strategy Sessions. They presented two - three slides per Working Group, summarizing the group's mission, focus areas, objectives, measurements of success, challenges, and timelines. The new participants, who had not been part of previous BOHRN strategy sessions, were able to discuss the slides as a large group, before breaking out into smaller groups to provide constructive feedback and guidance based on their knowledge and experiences. Breakout session discussions led to the development of cross-cutting recommendations on capacity for in region repositories and curation of voucher material and the implementation of a data-sharing culture. The outcome of these suggested recommendations will ultimately build an additional working group.

Steering Committee Presentations

BOHRN planners collated and drafted the following material from the BOHRN strategy sessions, to provide a visual tool to solicit feedback from a group of new stakeholders. This information was presented in slide-form as an introduction to the large-group discussions and break-out group sessions.

Working Group 1: researching host-pathogen biology and interactions

MISSION: EXPLAIN THE DETERMINANTS OF PATHOGEN TOLERANCE, TRANSMISSION, AND SPILLOVER FROM BATS AT INDIVIDUAL AND POPULATION LEVELS

Established Working Group 1 Research Focus Areas

Bat physiology and immunology	Distributions of pathogen amongst species	Bat pathogen community biology	Modeling approaches for host dynamics and epidemiology

Objective		Outcomes
Complete a systematic review of knowledge gaps on model systems	→	Publish a systematic review of modeling systems and knowledge gaps that were defined
Identify modeling systems that are representatives of all geographic and phylogenetic areas	→	Modeling systems are defined, characterized, and validated
Evaluate the transmission risks and spillover pathogens to another animal host	→	Intrinsic and extrinsic risk factors are identified for major diseases and geographic areas

Overall Challenges: (1) Objectives require multidisciplinary team: (2) consortia would be needed for modeling systems review and validation

Established Working Group 1 Research Projects and Activities Priority Timeline

Short-term project / activity pipeline *6 -12 months*	Long-term project / activity pipeline *12 months +*
Map funding landscape ⇒ Identify funders ⇒ Host a funders meeting	Conduct long-term lab and field studies ⇒ Develop cell lines and bat animal models ⇒ IgM immunoassay ⇒ Develop methods for determining the age of bats ⇒ Determine the timing of viral shedding and the effects of environmental stresses ⇒ Determine co-infection in bat species ⇒ Determine temperate versus tropical variables associated with infection (hibernation periods / viral replication) ⇒ Understand climate change with respect to physiology ⇒ Develop heat stable preservatives ⇒ Develop smaller telemetry and physiology sensors

Working Group 2: researching pathogen surveillance, diagnostic capacity and epidemiology

MISSION: FORM REGIONAL NETWORKS TO ESTABLISH A COMMON METHODOLOGY FOR SURVEILLANCE OF HUMAN AND ANIMAL HEALTH; BETTER UNDERSTAND SPILLOVER RISKS AND EPIDEMIOLOGY OF BAT PATHOGENS

Established Working Group 2 Research Focus Areas

Molecular epidemiology	Geographic and phylogenetic distribution of pathogens	Detection, diagnosis, and reporting of bat-borne pathogens	Established guidance and protocols for sampling

Objective		Outcomes
Conduct a gap analysis of existing diagnostic tools	→	Publish review of epidemiology of known bat-borne pathogens
Conduct outreach to various groups of researchers and build awareness amongst public and science community	→	Established and linked regional networks of practice and expertise
Establish a common methodology for surveillance	→	A better understanding of the risks associated with spillover and established standards for surveillance and reporting

Overall Challenges: (1) The logistics and bureaucracy of creating a multidisciplinary team of international experts; (2) funding to support and sustain efforts to standardize surveillance

Established Working Group 2 Research Projects and Activities Priority Timeline

Short-term project / activity pipeline *6 -12 months*	Long-term project / activity pipeline *12 months +*
Conduct a gap analysis of diagnostic tools ⇒ Identify list of labs and contacts ⇒ Create a list for priority interventions / assistance ⇒ Analyze return data; publish resource lists	Conduct surveillance platform assessment ⇒ Conduct a literature review of previous surveillance platform assessments ⇒ Identify most beneficial platform for animal and human health data information sharing ⇒ Identify most logical platform for low resource settings ⇒ Identify the best field-forward platforms

Working Group 3: researching ecology (bat, domesticated animals and wildlife interface)

MISSION: DEFINE HOW AND TO WHAT EXTENT THE ECOLOGICAL CONTEXT OF BATS, AND THE HUMAN INFLUENCE ON THAT CONTEXT, INFLUENCE PATHOGEN DYNAMICS AND SPILLOVER THREATS

Established Working Group 3 Research Focus Areas

Bat behavior, distribution and movement	Effect of anthropogenic disturbance and modification on pathogen dynamics	Domesticated animals and wildlife behavior, distribution, and movement impact on interaction with bats

Objective		Outcomes
Engage the ecological community to define system uniqueness and interdependencies	→	Pathogen research community acknowledges and integrates ecological systems and interdependencies
Advocate for ecological design and analysis frameworks to pathogen research	→	BOHRN research projects are designed using a framework for well-balanced outcomes
Build capacity for disease researchers to gather ecological data to provide context for their studies	→	More funded studies return ecological data
Define emerging ecological principles that could inform spillover threats	→	Emerging ecological principles become widely accepted governing principles for practice
Establish key messages and conduct efforts to promote a culture of conservation amongst One Health researchers, practitioners, and stakeholders	→	BOHRN establishes itself as a consistent and unbiased perspective from the community and its statements are widely accepted and distributed

Overall Challenges: (1) Science communities have polarized and insular view of bats and diseases; (2) lack of collaboration and communication efforts

Established Working Group 3 Research Projects and Activities Priority Timeline

Short-term project / activity pipeline *6 -12 months*	Long-term project / activity pipeline *12 months +*
Conduct conservation / One Health literature review ⇒ Establish parameters ⇒ Conduct literature review ⇒ Quantify interdisciplinary relationships w/ assessment of numbers of publications ⇒ Publish results Establish ecology tool / training aid kits ⇒ Identify and source materials	N/A ⇒ N/A

- ⇒ Collect and build case-control studies for training
- ⇒ Develop training plans
- ⇒ Distribute through BOHRN

Working Group 4: researching human-bat interactions

MISSION: FULLY DEVELOP, UNDERSTAND, AND COMMUNICATE THE BAT AND HUMAN INTERFACE TO KEY STAKEHOLDERS AND COMMUNITIES

Established Working Group 4 Research Focus Areas

Hunting and commodity chain	Human behavioral risk characterization	Interactions in human dwellings	Ecotourism

Objective		Outcomes
Develop and test policy interventions for specific human-bat interfaces	→	Policy interventions for human-bat interfaces are developed and put into place
Communicate key findings to stakeholders	→	Effectively communicate and publish findings of studies
Develop global risk maps to assess existing data and validate risk maps	→	Publish global risk maps highlighting geographic areas of risk
Identify high-risk groups and develop education platforms to measure knowledge, attitude and practices	→	Getting community buy-in and understanding of concepts

Overall Challenges: (1) Truthful responses in behavior research on bat-human interactions; (2) accuracy of risk map and models; (3) cultural barriers and beliefs

Established Working Group 4 Research Projects and Activities Priority Timeline

Short-term project / activity pipeline *6 -12 months*	Long-term project / activity pipeline *12 months +*
⇒ Develop global risk maps ⇒ Survey high-risk groups for their KAP	Conduct research studies / support for ecology ⇒ Develop and validate education platforms ⇒ Research to measure changes in KAP ⇒ Validate ground-truth risk maps

⇒ Adapt education platforms / materials

Recommendations

BOHRN organizers invited many researchers from diverse backgrounds at varying levels of professional experience to the Vienna workshop. This approach facilitated lively discussions and prompted the Steering Committee to consider new objectives, priorities, and perspectives within their previously established Working Group bounds. The following recommendations were captured by note-takers, observers, and other members of the Steering Committee, and will be marked for further discussion and adjudication during BOHRN's next Steering Committee meeting which will be held at International Bat Research Conference (IBRC) in Phuket, Thailand 2019.

Working Group 1: researching host-pathogen biology and interactions

Breakout Session Recommendations: members of the breakout group accepted the overall mission and objectives that Working Group 1 had established and proposed adding two additional research focus areas: (1) the role of bat taxonomy in host-pathogen coevolution and (2) host specificity in bat-borne pathogens. Members of the breakout group also proposed the following additions to the priority timeline:

- ⇒ Establish species identification consensus tools and techniques – such as the role of bar coding and other methods
- ⇒ Host or link to public-facing databases (e.g., Vertnet, National Science Foundation digitized database)
- ⇒ Identify regional resource repositories for voucher materials
- ⇒ Establish sustainable freezer network
- ⇒ Develop funding models for in-country collection curation capacity building / field sample collection transfer (business plans, logistics, maintenance, training)
- ⇒ Establish a database of reagents
- ⇒ Establish a list of international regulatory experts for transport of select agent materials (e.g., Bombali ebolavirus discovery and the issues they had with reporting and transfer)

Working Group 2: researching pathogen surveillance, diagnostic capacity and epidemiology

Breakout Session Recommendations: members of this breakout group generally accepted the mission and objectives that Working Group 2 had established. They proposed amending the research focus area for "Molecular Epidemiology" to include "Molecular and Serological Epidemiology". They also proposed the following additions to the priority timeline:

- ⇒ Establish a set of common research questions and topics related to biosurveillance data-type (syndromic, diagnostic, environmental) associated with bat-borne pathogen threats
- ⇒ Establish a catalog of surveillance models
- ⇒ Develop a sera and antibody collection with a standardized pool of collection
- ⇒ Conduct studies that integrate bat ecology and pathogen research (One Health research team that collects virology and ecological data at the same time)

- ⇒ Establish a list of minimum biosecurity / biosafety protocols for research (lab / field) and recommended sample sets / study
- ⇒ Establish a list of laboratories with bat sample repositories (by region and country
- ⇒ Establish a registry of "Bat Experts" by region and country
- ⇒ Identify diagnostic capabilities (person / institution)
- ⇒ Develop a hypothesis map
- ⇒ Outline funding mechanisms for other BOHRN stakeholders

The breakout group also recommended that any efforts to seek "standardization" (surveillance platforms) should use the phrase "common framework" as methods and implementation will vary in different countries and regions.

Working Group 3: researching ecology (bat, domesticated animals and wildlife interface)

Breakout Sessions Recommendations: during the breakout session, members of this group did not have any substantial modifications to the Working Group's mission, focus areas, or objectives. They did provide several ideas long-term timeline priorities, which included:

- ⇒ Conduct ecological and taxonomic studies that support disease research (and threat reduction), this will create a demand for ecologists to collect samples and will ultimately capacity for ecology through training and networking
- ⇒ Identify ecological and taxonomic gaps at local levels

Since much of Working Group 3's approach was built around the development of training modules, the group discussed training and the importance of tailoring existing projects / programs. They talked about sustainability in bat research programs and mechanisms for incentivization, offering ideas such as scholarships at the end of a short research project or using a training workshop as a research candidate selection opportunity.

Working Group 4: researching human-bat interactions

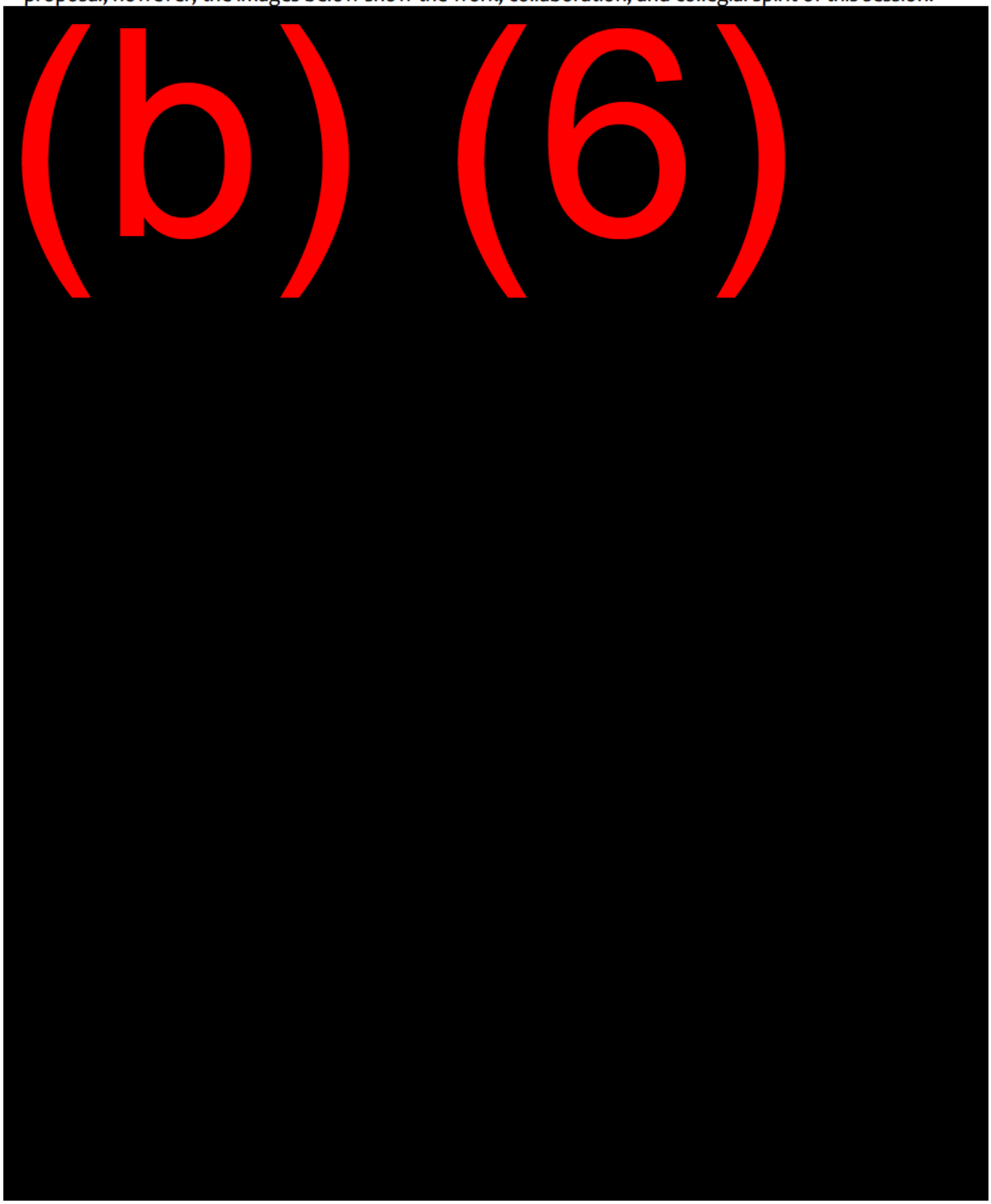
Breakout Sessions Recommendations: members of this breakout group did not have any major changes to the Working Group's mission, focus areas, or objectives. They did, however, want to emphasize the importance determining *where* human behavioral risks are the highest and *what* drives specific human bat interactions and the need to map these interactions accordingly. With regard to the timeline priorities, they made the following recommendations:

- ⇒ Characterize the risk map with priorities
  - DTRA (BTRP) priority pathogens, USG priority pathogen threats, WHO regional threats
  - Chart recent pandemics with drivers (e.g., bush meat markets overlaid with outbreaks)
- ⇒ For database define the approach to obtain data; Bat Conservation International (example), Bat-Plant.com for ecology interactions

Breakout Session 2 Overview

The first breakout session provided a foundation for the second breakout session during which participants formed into regional teams to craft research projects within the bounds of the BOHRN Working Group research focus areas. BTRP intends to fund several high priority threat reduction projects in FY19-FY20 and developed this exercise to test the viability of the network's strategy thus far. The

projects that were developed will not be summarized in this report, as they may be part of future project proposal; however, the images below show the work, collaboration, and collegial spirit of this session.



Participant Feedback

After the BOHRN Workshop participants were sent an anonymous feedback survey via SurveyMonkey. The participants were asked the following six questions:

1. What did you like about this Workshop?
2. Do you think the objectives for the BOHRN Workshop were achieved? Please explain your answer.
3. What do you wish we did differently?
4. What does success of this network look like to you, for your field of study?
5. What do you think was the most important aspect of this Workshop?
6. Any other comments or suggestions?

Overall, the participants responded positively to the efforts accomplished at the first annual BOHRN Workshop. An appreciation for the multidisciplinary networking opportunities, the potential opportunities the network presents, and the alignment of breakout group work with the Workshop presentations was convey by all participants. One participant's comment reflects this in saying "the multidisciplinary networking opportunities for engaging the ecological context of emerging infectious disease and breakout sessions were a nice complement to the big group discussions." Participant feedback indicated that the BOHRN Workshop objectives were achieved but there was a need for further information on next steps and more opportunity for discussion after the final small group session. Suggestions for change were to extend the workshop for two whole days and provide more focus on funding the discussed research.

BOHRN Path Forward

As a result of this workshop, BTRP intends to release an announcement for research project funding in the early part of 2019. The official announcement will be released on the BOHRN website (www.BOHRN.net) and emailed to anyone who has participated in a BOHRN activity.

At the conclusion of the workshop, Dr. (b) (6) presented draft criteria for project award consideration, which included:

- Performed in BTRP engagement countries
- Demonstrated commitment to capacity building in BTRP mission areas (biosafety and biosecurity, and biosurveillance)
- Demonstrated commitment to open science
    - Transparent sharing of knowledge and information
    - Should include a data curation plan and broad statement on information access
    - Sample sharing not require, but strongly encouraged and preferred
- Demonstrated commitment to One Health
    - Inter-disciplinary research teams
    - Local engagement plans or educational outreach

- Include early to mid-career project investigators
- Address cross-cutting themes of BOHRN
    - Projects should be tied to no less than two working groups
    - Projects should be tied to no less than one focus area within each working group
- Include mentorship from member of steering committee or a Steering Committee/Executive Committee-approved designee (correlates to respective working group(s))

These factors are still under consideration and BTRP may change any or all. The only information regarding "Criteria for Eligibility" for a BOHRN grant/project award will be released on BOHRN.net. The timeline for award will also be released on BOHRN.net.

Annex 1: Agenda

Day 1

Time	Session	Objectives
0700 – 0800	**Closed-door Steering Committee Meeting**	
0730 – 0810	**Photo Registration (for non-steering committee members)**	
0810 – 0845	**Welcome and Introductions** (b) (6) *Biological Threat Reduction Program (BTRP)*	Welcome all participants, provide four slides about BTRP and TRNs All participants go around the room and introduce name and organization
0845 – 0900	**BOHRN Overview** (b) (6) *Biological Threat Reduction Program (BTRP)*	Provide an overview about BOHRN, its mission and objectives; make sure to discuss (1) the funding opportunity; (2) the principles of capacity building / mentorship
0900 – 0910	**BOHRN Workshop Agenda, Objectives, and Housekeeping** (b) (6) *Global Systems Engineering*	Provide overview of meeting objectives, scheme of maneuver, and other housekeeping items
Session 1: BOHRN Focus Group Progress and Work		
0910 – 0930	**Understanding the Ecology of Viruses** (b) (6) *EcoHealth Alliance*	Discuss the challenges and understanding of the ecology of viruses such as Nipah and Ebola
0930 – 0950	**Host/Pathogen Interaction** (b) (6) *CDC- Division of High-Consequence Pathogens and Pathology*	Present on work focusing on viruses in the national reservoir hosts and determine the mechanisms by which the viruses are maintained in nature
0950 – 1010	**Laboratory Response** (b) (6) *UC Davis, School of Veterinary Medicine*	Synergies between the Bench and the Field: Rift Valley Fever and Ebola
1010 – 1030	**Building Policy and Community Outreach Initiatives Based on a Novel Understanding of Who, What, and Why** (b) (6) *American Museum of Natural History and National Museum of the Philippines*	Discuss efforts to bridge policy gaps between local, national, regional, and international efforts
1030 – 1110	**Focus Area Research Mentor Progress Reports**	A representative or mentor from each group will present their Focus Area

		objectives, its long / short-term goals schedule, and progress Group 1: 10 min Group 2: 10 min Group 3: 10 min Group 4: 10 min
1110 – 1215	**Breakout Groups**	Participants will be broken into the focus area groups; their placement will be pre-arranged by decision of the steering committee and they will have a sticker on the back of their name card; they will be asked to listen in on the focus area group discussion, see if they could contribute to the group's direction
1215 – 1320	**Working Lunch**	
1320 – 1400	**Breakout Group Open Discussion**	Each group will present any changes to their schedules or objectives Group 1: 10 min Group 2: 10 min Group 3: 10 min Group 4: 10 min
Session 2: BOHRN Project Development Work		
1400 – 1430	**Doing Business with BTRP: Pathways to Contracts, Objectives for BOHRN and Beyond** Dr. (b) (6) *BTRP*	20 Minute presentation of slides that (b) (6) gave in Georgia, plus 1-2 developed with (b) (6), plus 10 minutes for questions from the audience; this presentation will queue funding project development for focus area-specific RFPs
1430 – 1600	**Interactive Illustration Hypothesis Mapping Exercise**	Dr. (b) (6) and Dr. (b) (6) will facilitate an interactive hypothesis mapping session for the group
1600 – 1715	**Breakout Groups**	Breakout into blended project development groups. These groups will be based on seating arrangement (e.g., tables 1 and 2 will work together) to ensure that we have multi-disciplinary efforts.
1715 – 1730	**Close-out Day 1 and Review Day 2**	
1830 – 2000	**Dinner / Social Event** **Quad Chart / Poster Presentations**	

Day 2

Time	Session	Objectives
0900 – 1130	**Small Group Project Development Work**	Groups will come back to the main room to continue work in the smaller group project development
1000 – 1030	**Working Tea Break**	
1130 – 1300	**Working Lunch Break / Small Group Brief-outs**	
1300 – 1315	**Close-out / Group Discussion**	
TBD	**Steering Committee Meeting**	

Annex 2: Participant list

Name	Country	Organization
(b) (6)	Cameroon	National Veterinary Laboratory, Cameroon
	South Africa	University of Pretoria, Dept of Microbiology and Plant Pathology
	Nigeria	Texas Tech University
	Nigeria	Texas Tech University
	Tanzania	Nelson Mandela African Institute of Science and Technology
	Uganda	Makerere University, Kampala
	Uganda	Uganda Virus Research Institute
	Armenia	Yerevan State University
	Georgia	Ilia State University
	Georgia	National Center for Disease Control and Public Health -Georgia
	Georgia	National Center for Disease Control and Public Health - Georgia
	Jordan	Royal Scientific Society
	Jordan	Pasteur Institute in Morocco
	Jordan	Jordan University of Science and Technology
	Bangladesh	University of North Bengal
	Bangladesh	EcoHealth Alliance
	Bangladesh	Jahangirnagar Univeristy
	India	National Centre for Biological Sciences
	Malaysia	University of Kebangsaan Malaysia
	Philippines	University of the Philippines-Los Banos
	Philippines	Research Institute for Tropical Medicine
	Singapore	Duke-NUS, Singapore
	Thailand	Prince of Songkla University
	Thailand	Princess Maha Chakri Sirindhorn Natural History Museum
	Thailand	WHO CC for Research and Training in Viral Zoonoses, King Chulalongkorn Memorial Hospital, Thailand
	Vietnam	Institute of Ecology and Biological Resources
	United States	Metabiota
	United States	University of California- Davis
	United States	Metabiota
	United States	EcoHealth Alliance
	United States	Metabiotia
	United States	University of California- Davis
	United States	University of Georgia

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United States	Colorado State University
United States	Texas Tech University
United States	Uniformed Services Health Service University
United States	EcoHealth Alliance
United States	United States Army Medical Research Institute for Infectious Diseases - Genomic Center
United States	Center for Disease Control and Prevention, Viral Special Pathogens Branch
United States	Royal Scientific Society
United States	DTRA Biological Threat Reduction Program
United States	DTRA A&AS
United States	Global Systems Engineering
United States	Global Systems Engineering
United States	Global Systems Engineering
United States	Global Systems Engineering

From: (b) (6)
To: (b) (6)
; eric.laing(b) (6)
Cc: (b) (6)
Subject: Bat One Health Research Network
Date: Wednesday, February 20, 2019 12:00:39 PM
Attachments: BOHRNVienna_FinalReport[1].pdf
image001.png

Dear all,

On behalf of Dr. (b) (6), please find the final report for the BOHRN Workshop in Vienna.

As discussed in Vienna, there are several action items for the BOHRN network. In order to move forward on several of these items, we ask that you take a few moments to answer the following questionnaire. This survey will help us to identify BOHRN's efforts and progress towards its overarching goals and evaluate the networks threat reduction efforts. Please follow the link and complete the survey no later than 28 February: https://www.surveymonkey.com/r/6FQPQR3

Additionally, please use the following Drop Box link for access to the BOHRN Workshop participant list with pictures and the quad charts submitted by all participants. You may also access the video of the BOHRN Workshop here.

We had hoped to make a more formal announcement regarding solicitation for BOHRN special projects around this time; however, BTRP is internally still reviewing necessary criteria for award and will not be ready to make a more formal announcement until the April / May timeframe. The announcement will be released via the www.bohrn.net website.

Please let us know if you have any questions or concerns.

Kind Regards,

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From: (b) (6)
To: christopher.broder (b) (6)
Cc: (b) (6)
(b) (6)
Subject: Summary report from the Nipah R&D Roadmap Taskforce meeting and revised Nipah R&D Roadmap for your review
Date: Tuesday, August 29, 2023 5:43:08 PM
Attachments: Nipah Roadmap TF Meeting Summary.Final.docx
Nipah RD Roadmap.PostMeetingRevision.August2023.docx

Dear colleagues: First, I want to thank all of you who participated in the Nipah R&D Roadmap Taskforce meeting on July 31 and August 1, 2023. The discussions were highly productive and your input was extremely valuable. Also, it was great to see so many of you in person.

I would also like to again thank those of you who facilitated the meeting: (b) (6) —you all did a great job!

Attached please find a summary report of the meeting and a revised version of the Nipah Virus R&D Roadmap that includes all of the changes that were made based on discussions at the meeting (track changes are included). We also added a couple of additional references.

We would very much appreciate your review of the attached roadmap and any edits or comments that you may have. During the meeting, we mainly focused on the goals and milestones, and that's where the majority of the edits are, but we would very much appreciate your review of the whole document at this point in the process. **Please send any feedback on the document no later than September 15, 2023.**

Also, if you see any glaring omissions or inaccuracies in the summary report, please let me know (although we certainly don't expect you to review and comment on the meeting summary—we are providing it to you for your information only).

Over the coming weeks, I'll be preparing a manuscript about the roadmap and will be sending that to you for your review—tentatively in early October.

Again, thank you all for your support of this important project and for sharing your extensive expertise with us.

Kind regards,

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From: (b) (6)
To: christopher.broder (b) (6)
Cc: (b) (6)
Subject: Revised version of the Nipah R&D roadmap and near-final agenda for the July 31 and August 1 meeting at Wellcome in London
Date: Thursday, July 13, 2023 2:55:19 PM
Attachments: Nipah RD Roadmap.Revision.July2023.ForTFReview.docx
Nipah_Agenda_NearFinal.Draft.docx

Dear Colleagues: Attached please find the revised version of the Nipah R&D Roadmap for your review. This version contains extensive updates from progress made over the past 5 years and includes many additional references from the recent literature.

Please review this version **in advance** of the Nipah taskforce meeting. In the interest of time, during the meeting **we will focus primarily on discussing the goals and milestones**. We would also like to spend some time discussing roadmap implementation.

Following the meeting, we will revise the roadmap and will send it out to taskforce members for your written feedback and comments.

We have also included a near-final agenda of the meeting for your information.

Thank you, again, for your involvement in this important effort. I look forward to seeing many of you either in-person or virtually at the end of the month.

Kind regards,

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From: (b) (6)
To: (b) (6) ; CHRISTOPHER BRODER
Cc: (b) (6)
Subject: Re: RP4
Date: Wednesday, June 7, 2023 9:26:54 AM
Attachments: RP4 Research Strategy-Final-7June23.docx

OK thanks all! I made a few final minor edits and just fired this off to Sponsored Programs.

From: (b) (6)
Sent: Wednesday, June 7, 2023 8:14 AM
To: (b) (6)
CHRISTOPHER BRODER (b) (6)
Cc: (b) (6)
Subject: Re: RP4

External Email Warning: Do not click links or open attachments unless you recognize the sender and expect the content. UTMB Email Phishing Awareness

Ok, I think this is final from me
(b) (6), I added a Fig 4 with sosv data to E3.5, since there was space

---

From: (b) (6)
Date: Wednesday, June 7, 2023 at 8:02 AM
To: (b) (6)
CHRISTOPHER BRODER (b) (6)
Cc: (b) (6)
Subject: Re: RP4

Ok, I am just touching it one last time. Give me 10 min

From: (b) (6)
Date: Wednesday, June 7, 2023 at 7:57 AM
To: (b) (6)
CHRISTOPHER BRODER
(b) (6)
Cc: (b) (6)
Subject: Re: RP4

With a revised final timeline figure….

From: (b) (6)
Date: Wednesday, June 7, 2023 at 8:36 AM

To: (b) (6) CHRISTOPHER BRODER (b) (6)
Cc: (b) (6)
Subject: Re: RP4

References are all set. (b) (6), in the timeline figure (I couldn't edit it), please move Machupo to Prototype with Lassa…

From: (b) (6)
Date: Wednesday, June 7, 2023 at 8:30 AM
To: (b) (6) CHRISTOPHER BRODER (b) (6)
Cc: (b) (6)
Subject: Re: RP4

OK I accepted all of (b) (6)s changes and then some of my own and we were still about half a page over. The only way I could really make this work without ripping the guts out of the approach was to cut back the Background section. This made it work. (b) (6), you will need to fix the references. If anyone is inclined to add some text back to the Background we do have a little space left now. We have about a hour or so before I have to send this to Sponsored Programs. Many thanks!

From: (b) (6)
Sent: Wednesday, June 7, 2023 7:12 AM
To: CHRISTOPHER BRODER (b) (6)
Cc: (b) (6)
Subject: Re: RP4

I'm happy to touch it again now. As needed.
(b) (6)

From: Broder, Christopher (b) (6)
Date: Wednesday, June 7, 2023 at 6:33 AM
To: (b) (6)
Cc: (b) (6)

Subject: Re: RP4

Its nearly there
let me know if you need help this AM
now (b) (6)

On Wed, Jun 7, 2023 at 7:24 AM (b) (6)
wrote:

(b) (6) is active on it now…

From: (b) (6)
Date: Wednesday, June 7, 2023 at 7:17 AM
To: (b) (6)
CHRISTOPHER BRODER (b) (6)
Subject: Re: RP4

I used my bag of tricks and cut it down 1 ½ pages…still ½ page to go…

From: (b) (6)
Date: Wednesday, June 7, 2023 at 6:21 AM
To: (b) (6)
CHRISTOPHER BRODER (b) (6)
Subject: Re: RP4

Thanks (b) (6) (b) (6) is on it now. I will take it when he is done. Get some rest.

From: (b) (6)
Sent: Wednesday, June 7, 2023 5:13 AM
To: (b) (6)
CHRISTOPHER BRODER (b) (6)
Subject: Re: RP4

I'm trimming it now…

From: (b) (6)
Date: Wednesday, June 7, 2023 at 3:02 AM
To: (b) (6)

(b) (6)

[redacted] Chris Broder(b) (6) [redacted]

Subject: RP4

Ok, I got all of the important pieces into this draft and cleaned it up

I need to give it a little rest.

Its 2 pages too long now

(b) (6), if you really reengage at ~5 am (3 hrs from now) I wont likely be up yet. So you could review it, and if you wanted to start cutting the length, that is good. There is methodologic redundancy in SA2 and SA3 that can be cut and converted to “as in SA1 above”

I can do that in the morning if no one gets to it before I get up. But is someone is in process of cutting it, let me know!

Cheers

(b) (6)

[**WARNING** : This email came from an external source. Please treat this message with additional caution.]

[**WARNING** : This email came from an external source. Please treat this message with additional caution.]

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From: (b) (6)
To: (b) (6) CHRISTOPHER BRODER (b) (6)
Cc: (b) (6)
Subject: RE: RP1
Date: Tuesday, June 6, 2023 4:25:15 PM
Attachments: 06JUN23_RP1-BUNYAVIRALES VACCINES--RESEARCH STRATEGY(b) (6)_1033(b) (6)1520.docx
06JUN23_RP1-BUNYAVIRALES VACCINES--RESEARCH STRATEGY(b) (6)_1033(b) (6)1520.pdf

---

Accepted your changes and fixed a small blip I found. Added PDF version of same word document for context incase anything moves.

From: (b) (6)
Sent: Tuesday, June 6, 2023 3:08 PM
To: (b) (6) CHRISTOPHER BRODER
(b) (6)
Cc: (b) (6)
Subject: RE: RP1

Hello Dr (b) (6),

I have a few comments/edits in the attached research strategy. Primarily, there are a few discrepancies between the table legends and text for specific aim 1. I tried to correct as I saw appropriate, but please review and edit as needed.

Thanks,
(b) (6)

From: (b) (6)
Sent: Tuesday, June 6, 2023 10:42 AM
To: CHRISTOPHER BRODER (b) (6)
Cc: (b) (6)
Subject: RE: RP1

Here is the final clean version after (b) (6)' comments. Fixed a few other glitches as well. Added a PDF here for reference in case anything grows legs.

Let me know if anything else needed.

From: Broder, Christopher (b) (6)
Sent: Tuesday, June 6, 2023 8:48 AM
To: (b) (6)
Subject: RP1

hey (b) (6)

here are some ideas to tighten up,
you are ok on space. but i would use 3pt breaks between major sections (less dense)
just have to remove text here and there to delete hanging sentences.

page 11 toi 12 has some weird anchor
might have to cut and move fig or text

there some weird stuff, like forced font capitals on preliminary results header.
i didnt want to keep going but i can, because i figured you have already started another version
reads great
(b) (6)

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From: (b) (6)
To: (b) (6)
Cc: (b) (6)

Subject: Re: OSU/GSU RP5 Final Documents
Date: Tuesday, June 6, 2023 10:55:20 AM
Attachments: RP5_Research Strategy_U19-UTMB_USU_final.pdf
image001.png

Hi (b) (6),

RP5 Research Plan with some minor adjustments.

Thanks,
(b) (6)

From: (b) (6)
Date: Monday, June 5, 2023 at 4:54 PM
To: (b) (6)
Cc: (b) (6)

CHRISTOPHER BRODER (b) (6)
Subject: Re: OSU/GSU RP5 Final Documents

Hi (b) (6),

Here is the research strategy of RP5.

Thanks,
(b) (6)

From: (b) (6)
Date: Monday, June 5, 2023 at 1:39 PM
To: (b) (6)
Cc: (b) (6)

CHRISTOPHER BRODER (b) (6)
Subject: RE: OSU/GSU RP5 Final Documents

Thanks, (b) (6) We do not need a narrative for your research project. This is only included in the overall component.

From: (b) (6)
Sent: Monday, June 5, 2023 12:08 PM
To: (b) (6)
Cc: (b) (6),

(b) (6) CHRISTOPHER BRODER (b) (6)
Subject: Re: OSU/GSU RP5 Final Documents

> **External Email Warning:** Do not click links or open attachments unless you recognize the sender and expect the content. UTMB Email Phishing Awareness

Here is an update for resource sharing plan.

Do we need project narrative?

Thanks,
(b) (6)

From: (b) (6)
Date: Monday, June 5, 2023 at 11:42 AM
To: (b) (6)
Cc: (b) (6)
CHRISTOPHER BRODER (b) (6)
Subject: Re: OSU/GSU RP5 Final Documents

Hi (b) (6),

Please see attached documents from Jianliang and me.
Including:

1. Authentication of key resource (not sure if you received this last time)
2. Abstract (not sure if you received this last time)
3. LOS from me. (not sure if you received this last time)
4. MPI plan
5. Updated vertebrate animal
6. Updated specific aims

Research plan will be ready later today.

Thanks,
(b) (6)

From: (b) (6) >
Date: Thursday, June 1, 2023 at 3:31 AM
To: (b) (6)
Cc: (b) (6)

CHRISTOPHER BRODER (b) (6)
Subject: Re: OSU/GSU RP5 Final Documents

Hi (b) (6),

Here are the requested documents.

Thanks,
(b) (6)

From: (b) (6)
Date: Thursday, June 1, 2023 at 12:20 AM
To: (b) (6)
Cc: (b) (6) CHRISTOPHER BRODER (b) (6)
Subject: Re: OSU/GSU RP5 Final Documents

Hi (b) (6),

Thanks for the reminder. Attached please find my LOS for the application. (b) (6) will be sending Abstract, Authentication of materials and his LOS shortly.

My GSU position starts today, June 1, 2023. I am ccing my GSU emai (b) (6) and I will communicate with the team from my GSU account from now on.

Best regards,
(b) (6)

On Tue, May 30, 2023 at 1:31 PM (b) (6) wrote:

> Hello (b) (6),
> This is a reminder of the documents that we still need to get from you both for RP5 on the UTMB/USUHS U19 application. Our administrative review is this coming Thursday, June 1, so we will need all of the items listed below in final form by **EOB tomorrow, Wednesday, May 31**.
>
> - Abstract- 30 lines, 1 for RP5
> - Authentication of Key Biological Materials- 1 for RP5
> - Letter of Support – If both PI's could provide letters o support for your role on this project, we can include them. This is optional, but strengthens the proposal.
>
> For the final application, please provide the following by **EOB Monday June 5** *(hard deadline!).*

- Research Strategy – Please remember to include references for both items listed below from the RFA for your research project:

Milestone Plan: In a clearly labeled section titled "Project Milestones and Timelines" include a clear delineation of goals with measurable milestones, including detailed quantitative and qualitative criteria for Go/No-Go decision-making, and a timeline for the attainment of each goal and milestone and should be reflected in the Milestone Plan for the overall Program. This plan must include Go/No-Go criteria to be met by the end of Year 3 of the award for continuation to Phase II. Milestones must specify the outcome(s) for each activity. Milestones should be quantifiable and scientifically justified, and include the completion of major research study activities, for example, identification of protective epitopes, animal model development, vaccine or mAb candidate down-selection, identification of correlates of protection, validation of vaccine or mAb strategies for other family members, and analysis, sharing and publication of final data. Milestone criteria should not simply be a restatement of the specific aims. Using a Gantt chart or equivalent tool, describe the associated timelines and identified outcomes for the research Center.

Industry Expertise and Regulatory Considerations: For projects proposing early vaccine development, describe how industry partners will be identified and incorporated into the proposed project including a timeline for inclusion. For projects proposing IND-enabling later stage vaccine development, NIAID requires Centers to include active participation of an industry partner to ensure access to vaccine technology platforms, expertise in manufacturing, clinical development, and regulatory pathways. Applicants should describe the role of this partner in the proposed project and/or team to facilitate discovery, candidate evaluation and/or product development. For the purpose of this FOA, "industry" is defined as a large or small, domestic or foreign, pharmaceutical, biotechnology, bioengineering, or chemical company, or a related non-profit entity.

Please let me know if you have any questions about these or have any concerns about getting them back to us by the deadlines.
Thank you,

(b) (6)
[redacted]

utmb Health
Center for Biodefense &
Emerging Infectious Diseases

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From: (b) (6)
To: (b) (6) CHRISTOPHER BRODER; (b) (6)
Subject: RE: RE:
Date: Monday, June 5, 2023 9:43:47 PM
Attachments: 05JUN23_RP1-BUNYAVIRALES VACCINES--RESEARCH STRATEGY_(b) (6)_2034-clean.docx
05JUN23-UTMB REVAMPP SPECIFIC AIMS-(b) (6) -accepted edits.docx
05JUN23_RP1-BUNYAVIRALES VACCINES--RESEARCH STRATEGY(b) (6)_2034-clean.pdf
05JUN23 UTMB REVAMP ABSTRACT(b) (6).docx

Attached are clean versions of missing three documents. I have been having an issue of things getting moved around and fonts mysteriously changing, so I added a pdf version for reference.

From: (b) (6)
Sent: Monday, June 5, 2023 4:43 PM
To: CHRISTOPHER BRODER (b) (6)
Subject: RE:

I would send a near final version this evening and we can swap out tomorrow morning.

Sent via the Samsung Galaxy Note9, an AT&T 5G Evolution capable smartphone

-------- Original message --------
From: "Broder, Christopher" (b) (6)
Date: 6/5/23 4:41 PM (GMT-06:00)
To: (b) (6)
Subject:

hi
do we have time for (b) (6) RP1?
did a draft get sent for UTMB review?

if so, then (b) (6), can you make an accepted edits version and
we can go from there? we have time to swap in the final version
before clicknig submit

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From: (b) (6)
To: (b) (6)
Cc: (b) (6) ;
CHRISTOPHER BRODER
Subject: submitting edited Core D documents
Date: Monday, June 5, 2023 7:12:13 PM
Attachments: Core_D_ProjectSummary.pdf
Core_D_SpecificAims_FINAL.pdf
Core_D_Research_Strategy_FINAL.pdf
Core_D_Refs_FINAL.pdf

Hi (b) (6) ,

Please find attached the documents we will be submitting within the next hour for Core D. The documents required some editing to fit within the required page limits and to introduce synergies with other parts of the Center.

Please let me know within the next 45 minutes or so if you see anything that needs to be fixed.

We'll report back once UW has submitted the proposal to the sponsor.

Hope everything is going well finishing up your application too!

Best,
(b) (6)

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From: (b) (6)
To: (b) (6)
Cc: (b) (6)

Subject: Re: OSU/GSU RP5 Final Documents
Date: Monday, June 5, 2023 4:55:40 PM
Attachments: RP5_U19-UTMB_USU_Research Strategy.pdf
image001.png

Hi (b) (6),

Here is the research strategy of RP5.

Thanks,
(b) (6)

From: (b) (6)
Date: Monday, June 5, 2023 at 1:39 PM
To: (b) (6)
Cc: (b) (6)
CHRISTOPHER BRODER (b) (6)
Subject: RE: OSU/GSU RP5 Final Documents

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Sent: Monday, June 5, 2023 12:08 PM
To: (b) (6)
Cc: (b) (6) CHRISTOPHER BRODER (b) (6)
Subject: Re: OSU/GSU RP5 Final Documents

Here is an update for resource sharing plan.

Do we need project narrative?

Thanks,
(b) (6)

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To: (b) (6)
Cc: (b) (6)
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To: (b) (6)
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(b) (6) CHRISTOPHER BRODER (b) (6)
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Please let me know if you have any questions about these or have any concerns about getting them back to us by the deadlines.
Thank you,

(b) (6)
[redacted]

utmb Health
Center for Biodefense &
Emerging Infectious Diseases

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From: (b) (6)
To: (b) (6)
Cc: CHRISTOPHER BRODER
Subject: RE: ReVAMPP Bunyavirales Project
Date: Monday, June 5, 2023 4:42:51 PM
Attachments: 05JUN23_RP1-BUNYAVIRALES VACCINES--RESEARCH STRATEGY(b) (6)_1534.docx
05JUN23_RP1-BUNYAVIRALES VACCINES--RESEARCH STRATEGY_(b) (6)_1534.pdf

---

At the point in the grant writing where the document is making unintended changes on its own. Heres the fixed version with pdf in case things jump again

---

From: (b) (6)
Sent: Monday, June 5, 2023 3:30 PM
To: (b) (6)
Subject: Re: ReVAMPP Bunyavirales Project

(b) (5)

From: (b) (6)
Sent: Monday, June 5, 2023 3:02 PM
To: (b) (6)
CHRISTOPHER BRODER (b) (6)
Cc: (b) (6)
Subject: RE: ReVAMPP Bunyavirales Project

Attached is most recent version. I realized late I needed to add the industry section and add go/nogo to gant. Did a lot of editing/cutting to make those two fit.

If you have time, take a look. Working on abstract now.

From: (b) (6)
Sent: Monday, June 5, 2023 1:14 AM
To: (b) (6)
CHRISTOPHER BRODER (b) (6)
Cc: (b) (6)

Subject: RE: ReVAMPP Bunyavirales Project

Attached is the latest version. I still need to deal with the minor highlighted refs and table/figure descriptions but will do it tomorrow. There is a little room to mess around with how the milestones/objectives are presented, I just kept them in paragraph form for now but we can enumerate if it makes more sense to do so. Otherwise space is pretty tight until I can get the refs in and table/figure descriptions fixed.

I'm out of gas for now, welcome any feedback for refinement or if I left something important out.

(b) (6)

From: (b) (6)
Sent: Saturday, June 3, 2023 4:11 PM
To: (b) (6)
CHRISTOPHER BRODER (b) (6)
Cc: (b) (6)
Subject: RE: ReVAMPP Bunyavirales Project

I just talked with (b) (6) to walk through some of RP1 and we have a plan to improve readability and flow. Aims will stay largely the same, but I will add more context and clarify a few items to help with readability and flow. I will send an updated if not complete version to the group by late tomorrow. Adding (b) (6) as a fresh set of eyes to help with review and refinement.

From: (b) (6)
Sent: Saturday, June 3, 2023 2:50 PM
To: (b) (6)
CHRISTOPHER BRODER (b) (6)
Cc: (b) (6)
Subject: Re: ReVAMPP Bunyavirales Project

(b) (5)

(b) (5)

Specific Aims:

(b) (5)

From: (b) (6)
Sent: Saturday, June 3, 2023 10:25 AM
To: (b) (6)
CHRISTOPHER BRODER (b) (6)
Cc: (b) (6)
Subject: Re: ReVAMPP Bunyavirales Project

(b) (5)

(b) (5) [redacted]

From: (b) (6) [redacted]
Sent: Friday, June 2, 2023 3:09 PM
To: (b) (6) [redacted] CHRISTOPHER BRODER
(b) (6) [redacted]
Cc: (b) (6) [redacted]
Subject: Re: ReVAMPP Bunyavirales Project

> **External Email Warning:** Do not click links or open attachments unless you recognize the sender and expect the content. UTMB Email Phishing Awareness

(b) (5) [redacted]

From: (b) (6) [redacted]
Date: Thursday, June 1, 2023 at 11:56 PM
To: CHRISTOPHER BRODER (b) (6) [redacted]
Cc: (b) (6) [redacted]
Subject: RE: ReVAMPP Bunyavirales Project

Getting there, here is the update to now. I hope to finish filling out missing sections by tomorrow evening. Welcome any feedback

(b) (6) [redacted]

From: (b) (6) [redacted]
Sent: Monday, May 29, 2023 1:49 PM
To: Broder, Christopher (b) (6) [redacted]
Cc: (b) (6) [redacted]
Subject: RE: ReVAMPP Bunyavirales Project

Hi (b) (6) [redacted],

Some thoughts…

(b) (5) [redacted]

(b) (5)

(b) (6)

From: Broder, Christopher (b) (6)
Sent: Monday, May 29, 2023 1:09 PM
To: (b) (6)
Cc: (b) (6)
Subject: Re: ReVAMPP Bunyavirales Project

hi all

(b) (5)

(b) (6)

On Sun, May 28, 2023 at 3:35 PM (b) (6) wrote:

> Hey (b) (6)
> (b) (5)
> Best,
> (b) (6)

From: (b) (6)
Date: Saturday, May 27, 2023 at 10:26 PM
To: (b) (6) CHRISTOPHER BRODER (b) (6)
Subject: RE: ReVAMPP Bunyavirales Project

A ways to go on the text, but studies mapped out fairly well at this point, working on filling in text for areas highlighted in blue. Sending now to give the group an idea on direction for aims and subaims

(b) (5)

-
-
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(b) (6)

From: (b) (6)
Sent: Wednesday, May 17, 2023 2:51 PM
To: (b) (6)
;
CHRISTOPHER BRODER (b) (6)
Subject: Re: ReVAMPP Bunyavirales Project

(b) (6),

(b) (5)

Best
(b) (6)

From: (b) (6)
Date: Wednesday, May 17, 2023 at 3:47 PM
To: (b) (6)

(b) (6) CHRISTOPHER BRODER (b) (6)
Subject: RE: ReVAMPP Bunyavirales Project

(b) (5)

From: (b) (6)
Sent: Saturday, May 13, 2023 11:25 AM
To: (b) (6)
CHRISTOPHER BRODER (b) (6)
Subject: Re: ReVAMPP Bunyavirales Project

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(b) (6),

(b) (5)

Best
(b) (6)

From: (b) (6)
Date: Saturday, May 13, 2023 at 12:15 PM
To: (b) (6)
CHRISTOPHER BRODER
(b) (6)
Subject: Re: ReVAMPP Bunyavirales Project

(b) (6)

(b) (5)

Many thanks!

(b) (6)

From: (b) (6)
Sent: Saturday, May 13, 2023 11:10 AM
To: (b) (6)
CHRISTOPHER BRODER (b) (6)
Subject: RE: ReVAMPP Bunyavirales Project

Great, thanks!

(b) (5)

From: (b) (6) >
Sent: Saturday, May 13, 2023 9:38 AM
To: (b) (6)
CHRISTOPHER BRODER
(b) (6)
Subject: Re: ReVAMPP Bunyavirales Project

(b) (5)

From: (b) (6)
Sent: Friday, May 12, 2023 3:52 PM
To: (b) (6) >; CHRISTOPHER BRODER (b) (6)
Subject: RE: ReVAMPP Bunyavirales Project

Here is the first attempt at the specific aims page. Kept it simple to start.

From: (b) (6)
Sent: Thursday, May 4, 2023 1:31 PM
To: (b) (6)
Subject: Re: ReVAMPP Bunyavirales Project

Hi (b) (6)

(b) (5) .

Best,

(b) (6)

From: (b) (6)

Date: Thursday, May 4, 2023 at 11:18 AM
To: (b) (6)
Subject: Re: ReVAMPP Bunyavirales Project

(b) (6),

Please have at it and let us know how we can help.

Best

(b) (6)

From: (b) (6)
Date: Thursday, May 4, 2023 at 2:07 PM
To: (b) (6)
Subject: RE: ReVAMPP Bunyavirales Project

Agree, this SA page from (b) (6) is super helpful. Was going to start putting pen to paper to get a rough draft started for the Bunya RP. Unless you guys have already started?

Is there a format we need to follow for writing the proposal? I think we had 12 pages correct?

Assuming the standard NIH format?

- Significance
- Innovation
- Preliminary data
- Approach/SpecificAim 1-?
- Alternative strategies
- Milestones-Timelines
- Summary statement impact

From: (b) (6)
Sent: Tuesday, April 18, 2023 3:43 PM

To: (b) (6) [redacted]
Subject: Re: ReVAMPP Bunyavirales Project

External Email Warning: Do not click links or open attachments unless you recognize the sender and expect the content. UTMB Email Phishing Awareness

(b) (5) [redacted]

From: (b) (6) [redacted] >
Date: Tuesday, April 18, 2023 at 4:34 PM
To: (b) (6) [redacted]
Subject: Re: ReVAMPP Bunyavirales Project

Just a few thoughts:

1. (b) (5) [redacted]
2. (b) (5) [redacted]
3. (b) (5) [redacted]
4. (b) (5) [redacted]

From: (b) (6) >
Date: Tuesday, April 18, 2023 at 4:15 PM
To: (b) (6)
Subject: Re: ReVAMPP Bunyavirales Project

Hi (b) (6)

(b) (5)

. (b) (6)?

From: (b) (6)
Date: Tuesday, April 18, 2023 at 12:53 PM
To: (b) (6)
Subject: ReVAMPP Bunyavirales Project

All,

(b) (5)

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From: Broder, Christopher (b) (6)
To: (b) (6)
Subject: RP2 Res Strategy
Date: Monday, June 5, 2023 12:02:32 PM
Attachments: RP2-Research strategy_formatted-Final-060523.docx
RP2-Research strategy_formatted-Final-060523.pdf

(b) (6) / all

here is RP2 Res strategy, in case it gets late
(b) (6) are looking at this final version from me

(b) (6) and I have qual exams this PM

Are we supposed to remove the hyperlinks in the References also?

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From: (b) (6)
To: (b) (6) CHRISTOPHER BRODER
Subject: UTMB and USUHS ReVAMPP Overall Research Strategy Final
Date: Monday, June 5, 2023 12:01:01 PM
Attachments: Overall_Research-Strategy_PABVAX-12pages-Final.docx

---

Here is the final Research Strategy for the Overall for the UTMB and USUHS ReVAMPP Center. I think there is still one question from (b) (6) that (b) (6) needs to address for the Overall Aims.

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From: (b) (6)
To: (b) (6)
Cc: (b) (6); CHRISTOPHER BRODER; (b) (6)
Subject: Re: overall 12 page res plan
Date: Monday, June 5, 2023 10:04:08 AM
Attachments: Overall_Research-Strategy_PABVAX-12pages-060523v2.docx

Removed three non-critical references and Endnote updated to NIH style to include PMCID numbers.

On Jun 5, 2023, at 7:56 AM, (b) (6) wrote:

OK here are edits from (b) (6) and then me. This looks really good. (b) (6), I have two questions. Are References limited to 100? If so we will need to lose three? Second, do we have to put PMCID numbers into references?

From: (b) (6)
Sent: Monday, June 5, 2023 6:42 AM
To: (b) (6) CHRISTOPHER BRODER (b) (6)
Cc: (b) (6)
Subject: Re: overall 12 page res plan

Here it is – resolved my two comments and deleted some extraneous text left over from RFA instructions…

From: (b) (6)
Date: Monday, June 5, 2023 at 7:32 AM
To: (b) (6) CHRISTOPHER BRODER (b) (6)
Cc: (b) (6)
Subject: Re: overall 12 page res plan

I think there is something that (b) (6) mentioned on a comment from you. Can you go through it and then I will go through it after you? Many thanks!

From: (b) (6)
Sent: Monday, June 5, 2023 6:31 AM
To: (b) (6) CHRISTOPHER BRODER (b) (6)
Cc: (b) (6)
Subject: Re: overall 12 page res plan

I haven't touched this. Happy to though if you want me to do a review.

From: (b) (6)

Date: Monday, June 5, 2023 at 7:29 AM
To: CHRISTOPHER BRODER (b) (6)
Cc: (b) (6)
Subject: Re: overall 12 page res plan

(b) (6),

I don't want to duplicate effort. Are you working on this now?

Many thanks!

(b) (6)

From: Broder, Christopher (b) (6)
Sent: Sunday, June 4, 2023 9:30 PM
To: (b) (6)
Cc: (b) (6)
Subject: Re: overall 12 page res plan

here is 12 pages
there is ~10% left space on page 12

i did not mess with formatting, so if you want an UNformatted
version , then use this to unformat

i edited throughout to tighten up, did not remove any reffs

Only (b) (6) 's comment (2) remain to finalize

On Sun, Jun 4, 2023 at 8:46 PM (b) (6) wrote:

> Okay, here is where I'm at on the overall research strategy (tracked changes and clean versions). We still need to get rid of a little over half of a page.
>
> On Jun 4, 2023, at 9:56 AM, (b) (6) wrote:
>
> Got it, yes I will add connection to RP5, I initially missed this, but caught it when trying to make a small directional figure to add to RP1 to demonstrate connections to other cores.

From: Broder, Christophe (b) (6)
Sent: Sunday, June 4, 2023 9:35 AM
To: (b) (6)
Cc: (b) (6)
Subject: Re: overall 12 page res plan

problem is (b) (6) aims draft did even mention
alloy mice and LagV or bunya

you can definitely say you wll test nanobodies in SNTs to arenas
and then retest nanobody bi-specifics and then end with testing candidates
in vivo
we could use both big figures one in Admin core the other in the overalll

On Sun, Jun 4, 2023 at 9:45 AM (b) (6) wrote:

> I am crashing on RP1 today in order to get the final to the group for another round of review by tomorrow morning.
>
> Might be good if someone else has some time today to go through and accept the edits and fill in the refs. I can send the associated endnote library from my stuff if needed. Also, I'm good if we need to condense any of my section to be more concise, I figured I'd put more than needed and we could reduce and refine to make it all fit.
>
> I'm in the dark on a lot of the other projects beyond the specific aims as I've not even seen working drafts for really anything other than cores C, D, and E. So I'm trying my best to ensure cohesion with at least these cores, but might be good to ensure I'm on the right track in terms of ensuring formatting is consistent and I'm not missing any sections. I can't say how my RP will interact much with the other RPs as I don't fully know what they are doing and/or if it even makes sense to interact. BTW, did we ever get a specific aims page from Jim? I'm not sure I fully understand the direction he is going in and if I need to demonstrate interaction with his RP.
>
> ---
>
> **From:** Broder, Christophe (b) (6)
> **Sent:** Sunday, June 4, 2023 7:26 AM
> **To:** (b) (6)
> **Cc:** (b) (6)
> **Subject:** Re: overall 12 page res plan
>
>
>
> hi all
>
> i am working on a final version of RP2 now, (b) (6) got
> stuff to me last night. i am putting in the last section on the milestones/timelines/industry
> interfacing ec,,,
> i see (b) (6) your comment about alloy mice and RP4 but it was never clear to me what his plan was
>
> the new fig in the overall is really cool,
> i see (b) (6)'s point about the RP titles. ect,,, but the fig does have a lot more info
> that can be referred to, in order to see how it could be edited and using it larger

someone has to take this version, and go through an accept edits and get the remaining refs in remove comments unless there is a need for one more round to discuss

then we can see where it stands?

(b) (6)

On Sun, Jun 4, 2023 at 7:12 AM (b) (6) wrote:

> Thanks (b) (6). I had a few light edits and a couple of comments.
>
> **From:** (b) (6)
> **Date:** Sunday, June 4, 2023 at 1:03 AM
> **To:** CHRISTOPHER BRODER (b) (6)
> **Subject:** RE: overall 12 page res plan
>
> Now attached
>
> **From:** (b) (6)
> **Sent:** Sunday, June 4, 2023 12:03 AM
> **To:** Broder, Christopher <(b) (6)
> **Subject:** RE: overall 12 page res plan
>
> Added sections on Bunyas for overall, my sections had endnote added. Also sending a separate version also with endnote citations added in case anything gets fudged up. Can also export and send the ENL if needed.
>
> (b) (6) asked me to add the first draft of (b) (6) 's overall figure to this version to see how it fit in place of the first version. Looks good, maybe a few small refinements to streamline. CC'd (b) (6) here so she can get up to speed on the document as well and ensure she gets timely feedback.
>
> Working on RP1 tomorrow and will send full version tomorrow as soon as I can, likely late afternoon or evening.
>
> **From:** (b) (6)
> **Sent:** Saturday, June 3, 2023 4:38 PM
> **To:** Broder, Christopher (b) (6)
> **Subject:** RE: overall 12 page res plan
>
> Filling in the bunya sections today, will have to you all by tonight.
>
> **From:** Broder, Christopher (b) (6)
> **Sent:** Wednesday, May 31, 2023 5:59 PM
> **To:** (b) (6)

(b) (6)

Subject: overall 12 page res plan

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ok,
based on the RFA i made sections to address the requirements
some bits are from the CETR

The biggest thing this RFA wanted was the Milestones and go and no go
and timelines and diagrams.

needed items are green. need your magic guys especially industry and mabs and bunyas

there are never to many refs needed, yellow, so best to
have (b) (6) do this (add them from your library) like the admin ect,,

pending space, the other bits are yellow and optional depending on what you think

(b) (6)

<Overall_Research-Strategy_PABVAX-12pages-060523.docx>

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From: (b) (6)
To: (b) (6)
Cc: (b) (6) .; CHRISTOPHER BRODER; (b) (6)
Subject: RP3 Files for Review
Date: Sunday, June 4, 2023 11:56:03 AM
Attachments: RP3 - Abstract 06032023.docx
(b) (6) - RP3 - Facilities 06032023.pdf
(b) (6) - RP3 EQUIPMENT 06032023.pdf
BIOSKETCH - (b) (6) .pdf
(b) (6) - VERTEBRATE ANIMALS.pdf
(b) (6) - LOS DALGARD TAGC.pdf
(b) (6) - RESOURCE SHARING PLAN.pdf
(b) (6) - AUTHENTICATION KEY.pdf
Budget justification (b) (6) -RP3 5-24-2023 v4 .docx
image001.png

Hello,
We have made a pass through the files that have been sent over from both of you for RP5 on the UTMB/USUHS U19 (PABVAX). Please let me know if you have any last changes or comments for the files attached before we upload them into the application. Also, please send over to us as soon as possible the items listed below by the end of the day **Monday, June 5.**

- Final Specific Aims – 1 pg
- Final Research Strategy – 12 pgs plus bibliography

Thank you,

(b) (6)

utmb Health
Center for Biodefense &
Emerging Infectious Diseases

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From: (b) (6)
To: (b) (6)
Cc: (b) (6) ; CHRISTOPHER BRODER (b) (6)
Subject: RP5 Files for Review
Date: Sunday, June 4, 2023 11:37:54 AM
Attachments: Biosketch (b) (6) 2023-FINAL.pdf
biosketch (b) (6) U19 UTMB-USU.pdf
OSU_Budget Justification_sjs.docx
RP5 - Equipment 06042023.docx
RP5 - Facilities 06042023.pdf
RP5 - Resource Sharing Plan - 06042023.docx
RP5 - Vertebrate Animals OSU-GSU 06042023.pdf
RP5 - GSU (b) (6) Budget Justification.docx
image001.png
Importance: High

Hello,
We have made a pass through the files that have been sent over from both of you for RP5 on the UTMB/USUHS U19 (PABVAX). Please let me know if you have any last changes or comments for the files attached before we upload them into the application. Also, please send over to us as soon as possible the items listed below by the end of the day **Monday, June 5.**

- Abstract/Summary – 30 lines (Word doc)
- Multi-PI Plan – (Word doc) - *if either (b) (6) or (b) (6) prefer to be Project Lead and the other Co-I, we will not need this. If Co-Leading, we will need a plan to describe how the responsibilities will be shared and the justification for the shared leadership.
- Authentication of Key Biologicals and Chemical Resources
- Resource Sharing Plan – (Word doc attached; please be sure to add information about sharing research tools and model organisms)
- Final Specific Aims (Word doc) – 1 pg
- Final Research Strategy (Word doc) – 12 pgs plus bibliography

Thank you,

(b) (6)
[redacted]
utmb Health
Center for Biodefense & Emerging Infectious Diseases

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From: (b) (6)
To: (b) (6)
Cc: Christopher Broder; (b) (6)
Subject: BRODER - SCHAEFER DOCUMENTS TO UTMB-USUHS REVAMPP U19
Date: Friday, June 2, 2023 4:26:51 PM
Attachments:
BIOSKETCH - (b) (6) .pdf
BIOSKETCH - (b) (6).pdf
BIOSKETCH - (b) (6).pdf
BIOSKETCH - (b) (6) .pdf
PROJECT PERFORMANCE SITE - USU-HJF.pdf
KEY PERSONS RP2 and RP3.pdf
(b) (6) - RESEARCH STRATEGY - DRAFT.docx
(b) (6) - ABSTRACT.pdf
(b) (6) - AUTHENTICATION KEY.pdf
(b) (6) - BUDGET JUSTIFICATION.pdf
(b) (6) - BUDGET.pdf
(b) (6) - EQUIPMENT.pdf
(b) (6) - FACILITIES.pdf
(b) (6) - LOS.pdf
(b) (6) - RESOURCE SHARING PLAN.pdf
(b) (6) - SELECT AGENTS.pdf
(b) (6) - SPECIFIC AIMS.pdf
(b) (6) - ABSTRACT.pdf
(b) (6) - AUTHENTICATION KEY.pdf
(b) (6) - BUDGET JUSTIFICATION.pdf
(b) (6) - EQUIPMENT.pdf
(b) (6) - FACILITIES.pdf
(b) (6) - LOS DALGARD TAGC.pdf
(b) (6) - RESEARCH STRATEGY - DRAFT.pdf
(b) (6) - RESOURCE SHARING PLAN.pdf
(b) (6) - SELECT AGENTS.pdf
(b) (6) - SPECIFIC AIMS - DRAFT.pdf
(b) (6) - VERTEBRATE ANIMALS.pdf
(b) (6) - BUDGET.pdf
(b) (6) - DATA MGMT PLAN.xlsx
(b) (6) - DATA MGMT PLAN.xlsx
(b) (6) CONSORTIUM LOI-(b) (6) -v2(b) (6) -HJF-6-2-23.pdf
HJF LETTER OF INTENT-(b) (6) -USU-SUBAWARD-UTMB-6-2-2023.pdf
HJF LETTER OF INTENT - (b) (6) -USU-UTMBsubaward-6-2-23.pdf
(b) (6) CONSORTIUM LOI-v3-signed(b) (6) -HJF-USU-(b) (6) -6-2-2023.pdf
image002.png

Hello Dr. (b) (6),

This email is being sent on behalf of Drs. (b) (6), Site Principal Investigators for the Uniformed Services University of the Health Sciences to collaborate with Dr. (b) (6), Prime PI with The University of Texas Medical Branch at Galveston (UTMB). Below is a listing of the attached documents. If you require additional information, please do not hesitate to contact me. Upon receipt of the attachments, please send a confirmation. Thank you for doing business with HJF.

1. Key Person Biosketches – 5 pages, NIH format– please address your role and expertise for this U19 in personal statement
2. Key Person Information (including person to be contacted on matters for this application for your institution)
3. Site Information
4. Abstract/Project Summary (30 lines)
5. Facilities & Resources (Word doc)
6. Equipment (Word doc)
7. Resource Sharing Plan (Word doc)
8. Vertebrate Animals (Word doc as applicable)
9. Select Agent Research Plan (as applicable)
10. Authentication of Key Biological/Chemical Resources
11. Budget (SF424) & Justification (Word doc) – please include justification for Core A effort as well Project Leads at least 1.2 person months effort
12. Signed Letter of Intent/Consortium Agreement- for projects that include only effort but no salary or other costs, we will provide a collaborative agreement for signature instead.
13. Specific Aims- 1 page

14. Research Strategy: 12 pages for Research Project
15. Letters of Support

Yours in good health,

(b) (6)
[redacted]

[redacted]

Henry M. Jackson Foundation for the
Advancement of Military Medicine
6720A Rockledge Drive, Suite 100
Bethesda, MD 20817

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From: (b) (6)
To: (b) (6) .; CHRISTOPHER BRODER; (b) (6)
Cc: (b) (6)
Subject: Re: ReVAMPP Bunyavirales Project
Date: Friday, June 2, 2023 4:10:24 PM
Attachments: 01JUN23_RP1-BUNYAVIRALES VACCINES--RESEARCH STRATEGY(b) (6) .docx

---

I added some language for the mAb as alternative to a vaccine sections…

From: (b) (6)
Date: Thursday, June 1, 2023 at 11:56 PM
To: CHRISTOPHER BRODER (b) (6)
Cc: (b) (6)
Subject: RE: ReVAMPP Bunyavirales Project

Getting there, here is the update to now. I hope to finish filling out missing sections by tomorrow evening. Welcome any feedback

(b) (6)

From: (b) (6)
Sent: Monday, May 29, 2023 1:49 PM
To: Broder, Christopher (b) (6)
Cc: (b) (6)
Subject: RE: ReVAMPP Bunyavirales Project

Hi (b) (6),

Some thoughts…

On the PREP, this is just the one study being proposed as proof of concept. Will let (b) (6) elaborate, but we will need to wordsmith to suggest that if successful this may be an avenue to look at with other viruses with the support of SAC and program.

On the cellular and humoral part. Space is a big issue here and I'm not sure we will need to describe in detail in every RP or core, but it needs to be outlined somewhere clearly and that will eat up some space. I put it in my RP since there was no room in the animal core and (b) (6)'s core didn't detail it too much either (so far). My version will likely need to be whittled down as well to make everything fit, so but not so much that the gist is lost. I wonder if we could find a small amount of space to describe the immunology work up I described will be carried out in the scientific cores (and in

collaboration with RP1) as outlined in RP1? That said, we can maybe refer to methods used in RP1 for the core as well since that write-up is already having issues with space? Also note that I'm not doing any immunology on rodents since the patch core mentioned they would do rudimentary serology and ELISPOTs, all this advanced immunology work will be done with primates only due to lack of reagents.

(b) (6)

From: Broder, Christopher (b) (6)
Sent: Monday, May 29, 2023 1:09 PM
To: (b) (6)
Cc: (b) (6)
Subject: Re: ReVAMPP Bunyavirales Project

hi all
RP2 is not looking anything like this post-vaccination subject analysis on the CD4/CD8 and cellular responses or the serological studies.

We were allowing the Upitt core to explore the mice with cellular responses as the pilot.
The plan for the MNP vaccines is to test efficacy in ferrets then proceed to AGMs.

Not sure what to do or how to proceed here! Are there analysis text sections from what the animal core can or should propose here or outline here, as (b) (6) laid out? I dont have anything like this in hand,

also, i saw the comment on PREP
We dont have any plans for PREP for NiV/HeV included here either, our best candidates are already in Mapp's pipeline, or in the CETR

(b) (6)

On Sun, May 28, 2023 at 3:35 PM (b) (6) wrote:

> Hey (b) (6)
> Looks great! I added some language to use or lose in the Engineering Glycoproteins for CCHF section and the gel we used in the Core D preliminary results.
> Best,
> (b) (6)
>
> ---
>
> **From:** (b) (6)

Date: Saturday, May 27, 2023 at 10:26 PM
To: (b) (6) CHRISTOPHER BRODER (b) (6)
Subject: RE: ReVAMPP Bunyavirales Project

A ways to go on the text, but studies mapped out fairly well at this point, working on filling in text for areas highlighted in blue. Sending now to give the group an idea on direction for aims and subaims

Will work on fleshing out significance/innovation sections/and approach text tomorrow. Tables will be shrunk to fit, but followed (b) (6)'s color scheme to be consistent for now.

- (b) (6) I adapted the immunology support sections from our pilot grant to start with. Will very likely need to majorly consolidate, but better to have too much and refine to essentials once all the content is there.

- (b) (6), I just included the pilot study for the PREP study assuming you and (b) (6) already budgeted for it. You mentioned go-no-go, but I did not budget for any "go" studies, so maybe you mean PREP for henipaviruses?

- (b) (6), open to thoughts on the CCHF proteins for prelimary data or innovation. I had some serology which we did using your proteins on human survivors to demonstrate reactivity which I think is helpful. Open to other thoughts.

- (b) (6), I added another group for each of the NHP studies to allow for 2 groups to be evaluated either with different doses or different vax formulations. Was thinking this might be ideal for grantsmanship to have at least another option. Thinking was that we would not use all 5 controls for all studies and may be able to make these up?

(b) (6)

From: (b) (6)
Sent: Wednesday, May 17, 2023 2:51 PM
To: (b) (6)
CHRISTOPHER BRODER (b) (6)
Subject: Re: ReVAMPP Bunyavirales Project

(b) (6),

This is what is in Core D so far:

(b) (5)

Feel free to tweak so it fits with what you are thinking about. I can also give you some innovation language if you'd like on this topic.

Best
(b) (6)

From: (b) (6)
Date: Wednesday, May 17, 2023 at 3:47 PM
To: (b) (6)
CHRISTOPHER BRODER (b) (6)
Subject: RE: ReVAMPP Bunyavirales Project

I'm trying to shoe horn the prophy into RP1, will ciruculate to the group as a whole shortly. If we add this PEP study into RP1, we will probably need to renaim RP1 to something like: **Novel Vaccine Approaches for Emerging Arenaviruses and Nairoviruses:** (b) (6)

From: (b) (6)
Sent: Saturday, May 13, 2023 11:25 AM
To: (b) (6)
CHRISTOPHER BRODER (b) (6)
Subject: Re: ReVAMPP Bunyavirales Project

> **External Email Warning:** Do not click links or open attachments unless you recognize the sender and expect the content. UTMB Email Phishing Awareness

(b) (6),

I like the idea of it as a sub-aim. Core D will refer to it (manufacturing the mAb as well as performing PK to select the best Fc mutant). Depending on how much you are mentioning about the protocols in the Animal Core, you could also mention it there (e.g. "For studies looking at long-acting mAbs as an alternative to a vaccine, NHPs will receive a single dose 1 month prior to challenge…).

I can also if preferred, describe the study in Core D rather than RP1. I'm certainly setting up the idea of it with the PK work…

Best
(b) (6)

From: (b) (6)
Date: Saturday, May 13, 2023 at 12:15 PM
To: (b) (6)
CHRISTOPHER BRODER
(b) (6)
Subject: Re: ReVAMPP Bunyavirales Project

(b) (6)

How do you want to handle the Junin NHP prophylaxis study? At the very least I think RP1 has to mention it somewhere. Does it go into RP1 as a subaim? I don't think we can say it is alternate strategy as that route is usually taken when something proposed fails. Does it get pitched as a subaim to derisk the project? Not quite sure here.

Many thanks!

(b) (6)

From: (b) (6)
Sent: Saturday, May 13, 2023 11:10 AM
To: (b) (6)
CHRISTOPHER BRODER (b) (6)
Subject: RE: ReVAMPP Bunyavirales Project

Great, thanks!

Yeah it was challenging to add a lot into the SA page. But we can refine to make it more inclusive of all points addressed. Definitely will stretch out in the Research strategy.

We can maybe add some reverse genetics, especially for missing MACV lineages…maybe CCHF for the same reason using the cassette approach. Need to think about it a little today, but this may work.

Do we need to add the JUNV prophy study into RP1? Or is that in the core?

From: (b) (6)
Sent: Saturday, May 13, 2023 9:38 AM
To: (b) (6)
CHRISTOPHER BRODER
(b) (6)
Subject: Re: ReVAMPP Bunyavirales Project

Thanks (b) (6)! One thing that we need to think about as (b) (6) outlined for the rescue of Langya in the RP2 Aims is are we going to tackle reverse genetics at all for arenaviruses and CCHF? If so will need to tie that into the Animal Core in terms of rescue. Other thing is tie into Mapp Core for the Junin NHP prophylaxis study in Year 1 as an alternative

approach to vaccination. And then need to think about if we need an approach to tie into RP4 and RP5. All of this of course does not need to be on the Aims page and can be addressed in the 12 page Research Strategy. If you tackle reverse genetics it could be its own Aim along the lines of what (b) (6) has in RP2.

From: (b) (6)
Sent: Friday, May 12, 2023 3:52 PM
To: (b) (6)
CHRISTOPHER BRODER
(b) (6)
Subject: RE: ReVAMPP Bunyavirales Project

Here is the first attempt at the specific aims page. Kept it simple to start.

From: (b) (6)
Sent: Thursday, May 4, 2023 1:31 PM
To: (b) (6)
Subject: Re: ReVAMPP Bunyavirales Project

Hi (b) (6)

Here's a draft of an SBIR that (b) (6) wrote in case that's helpful. It only has data from the first Guinea pig study.

Best,

(b) (6)

From: (b) (6)
Date: Thursday, May 4, 2023 at 11:18 AM
To: (b) (6)
Subject: Re: ReVAMPP Bunyavirales Project

(b) (6)

Please have at it and let us know how we can help.

Best

(b) (6)

From: (b) (6)
Date: Thursday, May 4, 2023 at 2:07 PM
To: (b) (6)
Subject: RE: ReVAMPP Bunyavirales Project

Agree, this SA page from (b) (6) is super helpful. Was going to start putting pen to paper to get a rough draft started for the Bunya RP. Unless you guys have already started?

Is there a format we need to follow for writing the proposal? I think we had 12 pages correct?

Assuming the standard NIH format?

- Significance
- Innovation
- Preliminary data
- Approach/SpecificAim 1-?
- Alternative strategies
- Milestones-Timelines
- Summary statement impact

From: (b) (6)
Sent: Tuesday, April 18, 2023 3:43 PM
To: (b) (6)
Subject: Re: ReVAMPP Bunyavirales Project

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Oh, and here is the specific aims page of (b) (6)'s proposal from a few years ago…looks like it would fit pretty well

---

From: (b) (6)
Date: Tuesday, April 18, 2023 at 4:34 PM
To: (b) (6)
Subject: Re: ReVAMPP Bunyavirales Project

Just a few thoughts:

1. It looks like we can have multiple prototypes per family if justified. I think lassa and junin would be great prototypes for old and new world arenas…seems easy to justify as we don't know if a single product could cover both and pursuing both could help to answer the question.
2. Might be good to confirm with program, but as I initially read it, it looks like if we start with CCHF we'd be expected in Y4-Y5 to expand to other nairos. Hantas may be a little easier – Jim has some mAbs and Mapp does as well, but those are encumbered a bit with (b) (6)'s CETR so we'd have to have some conversations to make sure that would be kosher.
3. The RFA has a bit of mixed message on vaccines and mAbs. I do think they go well together and mAbs can be an alternative option to a vaccine.
4. Do the microneedle people have some experience transitioning to the clinic? I'm looking at this language: "Each ReVAMPP Center is expected to have an established, or have plans to establish when appropriate, collaboration with an industry partner which will provide access to vaccine expertise in manufacturing, clinical development, and regulatory pathways." Mapp can fill that role for mAb vaccines, but not so well for traditional vaccines. It looks like we don't have to have them lined up yet, but might be nice to have a letter of support if needed. DVC is an option as is IBT.

From: (b) (6)
Date: Tuesday, April 18, 2023 at 4:15 PM
To: (b) (6)

Subject: Re: ReVAMPP Bunyavirales Project

Hi (b) (6)

We do have the Junin PBMCs you sent and I like the idea of including Junin in the proposal because we already have cell lines in house to express Machupo, Junin and Lassa GPs. I think we could hit the ground running on arenavirus antibody discovery.

I don't have strong feelings on Hanta vs CCHF. (b) (6)?

From: (b) (6)
Date: Tuesday, April 18, 2023 at 12:53 PM
To: (b) (6)
Subject: ReVAMPP Bunyavirales Project

All,

(b) (5)

(b) (6)

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From: (b) (6)
To: (b) (6)
Cc: (b) (6) ; CHRISTOPHER BRODER
Subject: Re: UTMB update on REVAMPP materials.
Date: Friday, June 2, 2023 11:05:00 AM
Attachments: UTMB Core D-Project Summary and Relevance-2June23.docx
Specific Aims Core D-2June23.docx
UWashington-Core D-Research Strategy-2June23.docx

Here you go. We need you to add a little that is highlighted as we were not sure of the final Center structure. (b) (6), I did not think they you were proposing new reporter viruses or animal models for RVFV or SFTSV. If that is not correct we still have some room left in the Research Strategy for additions.

From: (b) (6)
Sent: Thursday, June 1, 2023 8:38 PM
To: (b) (6)
Cc: (b) (6)
CHRISTOPHER BRODER
(b) (6)
Subject: Re: UTMB update on REVAMPP materials.

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Gentlemen,

We really need completed science documents.

Thanks,
(b) (6)

On Thu, Jun 1, 2023 at 9:08 AM (b) (6) wrote:

> It is mostly written. (b) (6) is in the process of integrating some preliminary data.
>
> ---
>
> **From:** (b) (6)
> **Sent:** Thursday, June 1, 2023 10:28 AM
> **To:** (b) (6)
> **Cc:** (b) (6)
> CHRISTOPHER BRODER (b) (6)
> **Subject:** RE: UTMB update on REVAMPP materials.
>
> Looping (b) (6) in here for an update.
>
> ---

From: (b) (6)
Sent: Thursday, June 1, 2023 9:02 AM
To: (b) (6)
Cc: (b) (6)
CHRISTOPHER BRODER (b) (6)
Subject: Re: UTMB update on REVAMPP materials.

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Hi (b) (6) et al,

Hope all is well.

I am checking in to confirm that you will be able to please send us a near final draft of the science write-up for your Core in the next few hours?

Thanks in advance
(b) (6)

On Tue, May 30, 2023 at 12:27 PM (b) (6) wrote:

> Thank you (b) (6)!
>
> On Tue, May 30, 2023, 11:46 AM (b) (6) wrote:
>
> > Hello (b) (6),
> >
> > We have received internal approval on the budget, and I am working to get the info loaded into the SF424 form now. I expect to have the budget, justification and LOI to you by the end of the day.
> >
> > Thanks,
> > (b) (6)
> >
> > ---
> >
> > **From:** (b) (6)
> > **Sent:** Tuesday, May 30, 2023 12:25 PM
> > **To:** (b) (6) >
> > **Cc:** (b) (6)
> > CHRISTOPHER BRODER (b) (6)
> > **Subject:** Re: UTMB update on REVAMPP materials.
> >
> > **External Email Warning:** Do not click links or open attachments unless you recognize the sender and expect the content. UTMB Email Phishing Awareness

Thanks [redacted] -- please find responses in-line below.

Best,
[redacted]

- The admin documents were submitted to our OSP officials on Friday and we have checked for an update. [redacted] is working on this and will respond when more info is available.

Thank you. Do we have an estimated ETA for the arrival of the documents at UW?

- Milestones/Goals for the Overall document. Per the RFA, there are no milestones required for cores, but we can make mention of the core's role in supporting RP milestones if that is what was meant?

Correct, thanks!

- We are still passing the core's science document around and adding needed needed supporting data, hope to have this in a sharable form by this Thursday.

Could we please get a draft Specific Aims page today? This is necessary to allow us to prepare to weave Core D into the rest of the proposal.

--
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From: Broder, Christopher (b) (6)
To: (b) (6)
Cc: (b) (6)
Subject: Re: UWA Core Aim
Date: Friday, June 2, 2023 8:24:03 AM
Attachments: UWashington-Core D-Research Strategy-02Jun23.docx

---

this looks done.

you are going to format this right?

On Thu, Jun 1, 2023 at 5:45 PM (b) (6) wrote:

> This is looking good. I split out the Aims page (first document) as it is separate and we then have 6 pages for the Core text. I inserted the references. I think once you get those in that will save some space. Also, I went back through the documents from (b) (6) and they are not proposing any animal model development so I fixed that.
>
> ---
>
> **From:** Broder, Christopher (b) (6)
> **Sent:** Thursday, June 1, 2023 1:48 PM
> **To:** (b) (6)
> **Cc:** (b) (6)
> **Subject:** Re: UWA Core Aim
>
> **External Email Warning:** Do not click links or open attachments unless you recognize the sender and expect the content. UTMB Email Phishing Awareness
>
> guys
> i think the core is 6 pages plus a Specific Aims page (total 7)
> if im wrong then cut out fig 2
>
> its getting close. last section needed. insert your refs now, and
> it will get space.
> there are a lot of bits that can be wordsmithed out in the animal methods.
>
> if you make a near final formatted version with section added for RVFV and SFTSV
> send back and i will edit it down to fit
>
> On Thu, May 25, 2023 at 2:50 PM Broder, Christopher (b) (6) wrote:
>
> > i know,
> > best to have too much and cut to start off
> >
> > i am working back on Overall 12 pages
> >
> > On Thu, May 25, 2023 at 2:18 PM (b) (6) wrote:

Thanks (b) (6) We are going to have a lot of stuff to cram into 6 pages. I will start working on this but may do this over the weekend.

From: Broder, Christopher (b) (6)
Sent: Thursday, May 25, 2023 1:01 PM
To: (b) (6)
Subject: UWA Core Aim

(b) (6)

here is revised Aim for us, for the 6 page Core D for UWA

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From: (b) (6)
To: (b) (6) CHRISTOPHER BRODER; (b) (6)
Subject: Re: overall 12 page res plan
Date: Thursday, June 1, 2023 10:44:23 AM
Attachments: Overall_Research-Strategy_PABVAX-12pages-V1-05312023(b) (6).docx

---

OK I made some edits. It looks like (b) (6) is asking for some input from (b) (6). Looping (b) (6) in on the Figure and any thoughts or changes there.

From: (b) (6)
Sent: Thursday, June 1, 2023 8:17 AM
To: (b) (6) CHRISTOPHER BRODER (b) (6)
Subject: Re: overall 12 page res plan

Gang,

Some minor edits and text for the green highlighted sections that were in my wheelhouse…

Best
(b) (6)

From: (b) (6)
Date: Thursday, June 1, 2023 at 8:40 AM
To: (b) (6) CHRISTOPHER BRODER (b) (6)
Subject: Re: overall 12 page res plan

Yes, that is correct. Here is a draft of the Core E Research Strategy. I am going to try to tighten it up today.

From: (b) (6)
Sent: Thursday, June 1, 2023 7:38 AM
To: CHRISTOPHER BRODER (b) (6)
Subject: Re: overall 12 page res plan

(b) (6) …am I correct in assuming you are including animals for therapy studies with the mAbs/nanobodies?

From: (b) (6)
Date: Wednesday, May 31, 2023 at 7:21 PM
To: Broder, Christopher (b) (6)
Subject: Re: overall 12 page res plan

Thanks (b) (6) – looks like a great start. If anyone works on this today, please shoot me the latest version when you are done. I'll work on it first thing tomorrow EST and send back to the group asap.

From: Broder, Christopher (b) (6)
Date: Wednesday, May 31, 2023 at 6:59 PM
To: (b) (6)
Subject: overall 12 page res plan

ok,
based on the RFA i made sections to address the requirements
some bits are from the CETR

The biggest thing this RFA wanted was the Milestones and go and no go
and timelines and diagrams.

needed items are green. need your magic guys especially industry and mabs and bunyas

there are never to many refs needed, yellow, so best to
have (b) (6) do this (add them from your library) like the admin ect,,

pending space, the other bits are yellow and optional depending on what you think

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From: (b) (6)
To: (b) (6)
Cc: (b) (6) CHRISTOPHER BRODER
Subject: RE: Data Mgmt Core Final Documents - UTMB/USUHS U19
Date: Tuesday, May 30, 2023 2:55:57 PM
Attachments: REVAMPP_Org_Chart_Core_Enhancement2.pdf
F-U19 - UTMB_CORE C - (UPITT and UMD) - Abstract-Project Summary - 05-19-2023.docx
Abstract-Summary.docx
UTMB Core E-Project Summary and Relevance-25May23.docx
Abstract-RP2(b) (6) -v2pdf.pdf
Abstract-RP3(b) (6) -v2.pdf
RP4(b) (6) project Summary_Abstrast_30 lines.docx
RP5-Specific Aim_U19-UTMB_USU_23-05-18.pdf
Overall-PABVAX-Project Summary-and-Relevance-V1-05232023(b) (6) .docx
image001.png

(b) (6),
An org chart is attached as well as abstracts/specific aims for the cores and research projects and overall for the center goals. (all but RP1).

Core C: Microneedle Patch Vaccines (UMD; (b) (6) /UPitt; (b) (6))
Core D: Early and IND-Enabling Translational Research for Vaccines and mAbs (MappBio; (b) (6) - WSU; (b) (6) -UTMB; (b) (6))
Core E: Animal Model Development and Preclinical Evaluation (UTMB; (b) (6))
RP1: Antigen Design and Testing of Arena virus and Nairovirus Vaccines (UTMB; (b) (6) - WSU; (b) (6))
RP2: Vaccines and Antibodies to Henipaviruses (USUHS; (b) (6))
RP3: Cedar henipavirus Animal Model Development (USUHS; (b) (6))
RP4: Monoclonal Antibodies Against Henipaviruses, Arenaviruses, and Nairoviruses (VUMC; (b) (6)
RP5: Nanovody Therapeutics Against Henipaviruses and Arenaviruses (OSU; (b) (6) /GSU; (b) (6))

Let me know if this helps or you need anything else.

Best,
(b) (6)

[redacted]

[redacted]

utmb Health
Center for Biodefense & Emerging Infectious Diseases

From: (b) (6)
Sent: Tuesday, May 30, 2023 1:13 PM
To: (b) (6)
Cc: (b) (6) CHRISTOPHER BRODER (b) (6)
Subject: Re: Data Mgmt Core Final Documents - UTMB/USUHS U19

Hi (b) (6),

The following documents are not needed for the data management core:

- Resource sharing plan - I'm providing the data management sharing plan instead for the final submission.
- Authentication of key biologicals - don't have any.

For an Abstract, I would need the organizational chart for the entire proposal to integrate what was asked for earlier.
Based on other U19, this is what my abstract will include:

- Aims " Aim 1 will….. In Aim 2 we will….". And include 1-2 sentences following each aim to elaborate a little.
- Overall goal
- **Mention of Cores and RPs to emphasize synergy**
- End with a statement about how the team of experts at UTMB has been assembled

I can skip the synergy part for now, add it based on the organizational chart if we have one, or pull from the methods from core and RPs.

Please advise.

Best,
(b) (6)

From: (b) (6)
Sent: Tuesday, May 30, 2023 12:22 PM
To: (b) (6)
Cc: (b) (6) CHRISTOPHER BRODER (b) (6)
Subject: Data Mgmt Core Final Documents - UTMB/USUHS U19

Hello Dr. (b) (6)
This is a reminder of the documents that we still need to get from you for Core B (Data Mgmt) on the UTMB/USUHS U19 application. Our administrative review is this coming Thursday, June 1, so we will need all of the items listed below in final form by **EOB tomorrow, Wednesday, May 31**.

- Abstract – 30 lines
- Resource sharing plan
- Authentication of key biologicals

For the final application, please provide the following by **EOB Monday June 5** *(hard deadline!).*

- Research Strategy + Bibliography

Please let me know if you have any questions about these or have any concerns about getting them back to us by the deadlines.
Thank you,

(b) (6)
[redacted]

[redacted]

utmb Health
Center for Biodefense &
Emerging Infectious Diseases

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From: (b) (6)
To: (b) (6)
Cc: (b) (6) CHRISTOPHER BRODER; (b) (6)
Subject: Re: ReVAMPP RP2/RP3 Document Reminder
Date: Friday, May 26, 2023 2:38:55 PM
Attachments:
Biosketch-RP3-(b) (6) r-v2.pdf
Biosketch (b) (6) r-TO BE EDITED.pdf
Biosketch (b) (6) _UTMB-USU-ReVAMPP_RP2-v2.pdf
Biosketch (b) (6) r_RP2_v1.pdf
Key Persons_RP2 and RP3.pdf
Project-Performance Site_UTMB.pdf
Abstract-RP3-(b) (6) -v2.pdf
Abstract-RP2-(b) (6) -v2pdf.pdf
Equipment-(b) (6) _RP2-v2.pdf
Facilities_(b) (6) _RP2-v2.pdf
Equipment-RP3-(b) (6) -v2.pdf
Facilities-RP3-(b) (6) -v2.pdf
Resource Sharing plan_(b) (6) _RP2-v2.pdf
Vertebrate Animals-RP3-(b) (6) r-v3.pdf
Select Agents-RP3-(b) (6) -v2.pdf
Select Agents_(b) (6) _RP2-v2.pdf
Authentication-(b) (6) _RP2-v2.pdf
Authentication of Key Resources Plan-RP3-(b) (6) -v2.pdf
RR Budget_UTMB_(b) (6) -v2.pdf
Budget justification-(b) (6) -RP3 5-24-2023 v4.pdf
RR Budget_UTMB-(b) (6) r-v2.pdf
Budget Justification_(b) (6) _RP2-v3.pdf
image001.png

Hello (b) (6),

Most of our documents are pretty good draft's. Maybe minor editorial changes from our grants office.

1. Key Person Biosketches – 5 pages, NIH format– please address your role and expertise for this U19 in personal statement **(Attached) (b) (6) would like to make some revisions to his.**
2. Key Person Information (including person to be contacted on matters for this application for your institution) **Attached.**
3. Site Information **Attached**
4. Abstract/Project Summary (30 lines) **Attached**
5. Facilities & Resources (Word doc) **pdf documents attached**
6. Equipment (Word doc) **pdf documents attached**
7. Resource Sharing Plan (Word doc). **pdf document attached for (b) (6), will send document for (b) (6)**
8. Vertebrate Animals (Word doc *as applicable*) **pdf attached**
9. Select Agent Research Plan (*as applicable*) **pdf attached**
10. Authentication of Key Biological/Chemical Resources **pdf attached**
11. Budget (SF424) & Justification (Word doc) – *please include justification for Core A effort as well*
    - Project Leads at least 1.2 person months effort **attached, 1.2 cal months verified, no Core A effort; pdf attached of justification**
12. Signed Letter of Intent/Consortium Agreement- for projects that include only effort but no salary or other costs, we will provide a collaborative agreement for signature instead. **this will be provided by grants office no later than June 2, 2023**
13. Specific Aims- 1 page *(draft received 5/18/23 for RP2; draft received 5/12/23 for RP3)*
14. Research Strategy: 12 pages for Research Projects **pdf for (b) (6) attached (draft); will send**

document for (b) (6)

Thanks much....(b) (6)

On Fri, May 26, 2023 at 11:51 AM (b) (6) wrote:

> Hello (b) (6),
>
> Could you please send us your most current/near final versions of the documents below for USUHS for RP2 and RP3 as soon as possible? Apologies if you have sent any of these items already, but I only have the draft aims for these components so far in my folders.
>
> 1. Key Person Biosketches – 5 pages, NIH format– please address your role and expertise for this U19 in personal statement
> 2. Key Person Information (including person to be contacted on matters for this application for your institution)
> 3. Site Information
> 4. Abstract/Project Summary (30 lines)
> 5. Facilities & Resources (Word doc)
> 6. Equipment (Word doc)
> 7. Resource Sharing Plan (Word doc)
> 8. Vertebrate Animals (Word doc *as applicable*)
> 9. Select Agent Research Plan (*as applicable*)
> 10. Authentication of Key Biological/Chemical Resources
> 11. Budget (SF424) & Justification (Word doc) – *please include justification for Core A effort as well*
>   - Project Leads at least 1.2 person months effort
> 12. Signed Letter of Intent/Consortium Agreement- for projects that include only effort but no salary or other costs, we will provide a collaborative agreement for signature instead.
> 13. Specific Aims- 1 page *(draft received 5/18/23 for RP2; draft received 5/12/23 for RP3)*
> 14. Research Strategy: 12 pages for Research Projects
>
> Thank you!
>
> (b) (6)
>
> [redacted]

(b) (6)

utmb Health
Center for Biodefense &
Emerging Infectious Diseases

From: (b) (6)
Sent: Friday, May 19, 2023 2:53 PM
To: (b) (6)
Cc: (b) (6)
CHRISTOPHER BRODER <(b) (6)
Subject: ReVAMPP RP2/RP3 Document Reminder

Hello (b) (6),

This is a reminder as you are working on collecting the required documents for RP2 and RP3 on Dr. (b) (6) and Dr. (b) (6) ReVAMPP U19 application. If you need us to resend any templates for the documents below, please let us know as soon as possible so we can get that over to you. Otherwise, we hope to receive final or near final versions of everything listed below (expect strategy and aims, final due May 26) by end of day **Monday, May 22**.

1. Key Person Biosketches – 5 pages, NIH format– please address your role and expertise for this U19 in personal statement
2. Key Person Information (including person to be contacted on matters for this application for your institution)
3. Site Information
4. Abstract/Project Summary (30 lines)
5. Facilities & Resources (Word doc)
6. Equipment (Word doc)
7. Resource Sharing Plan (Word doc)
8. Vertebrate Animals (Word doc *as applicable*)
9. Human Subjects Study Record (*as applicable*)
10. Select Agent Research Plan (*as applicable*)

11. Authentication of Key Biological/Chemical Resources
12. Budget (SF424) & Justification (Word doc) – *please include justification for Core A effort as well*
    1. 5 years
    2. Project Leads at least 1.2 person months effort, Scientific Core Leads at least 0.6 person months effort, Data Mgmt. Core Lead at least 1.2 person months effort
    3. NIH salary cap, M&O, travel, publications, service fees, indirects, etc.
13. Signed Letter of Intent/Consortium Agreement- for projects that include only effort but no salary or other costs, we will provide a collaborative agreement for signature instead.
14. Specific Aims- 1 page *(draft received 5/18/23 for RP2; draft received 5/12/23 for RP3)*
15. Research Strategy: 12 pages for Research Projects; 6 pages for Scientific and Data Mgmt. Cores

    1. Milestone Plan: Annual Go/No Go's to be met by end of Y3 (Phase I); Gantt chart/Timeline, PhI/PhII
    2. Tie in Industry Expertise
    3. Address Regulatory Barriers
    4. Bibliography (no page limit)

Please let us know if you have any questions or concerns about meeting this deadline for any of these documents. If you have anything completed, feel free to send us what you have ready, so we may begin our review process and start uploading documents to the application.

Thank you!

(b) (6)
[redacted]

utmb Health
Center for Biodefense &
Emerging Infectious Diseases

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(b) (6) [redacted]

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From: (b) (6)
To: (b) (6) ; CHRISTOPHER BRODER; (b) (6)
Subject: RE: UTMB/USUHS ReVAMPP Specific Aims draft - DUE Friday 5/12
Date: Thursday, May 18, 2023 10:50:30 AM
Attachments: 17MAY23_RP1-BUNYAVIRALES VACCINES(b) (6).docx
RP2_Specific Aims-UTMB-USU-05102023-v3.docx
ReVAMPP RP3 Specific Aims 5-12-2023.pdf
RP5-Specific Aim_U19-UTMB_USU (b) (6) _2023y05m12d.docx
Specific_Aims_Core_A.docx
Preliminary AIMS DMC core.docx
ReVAMPP U19 - (b) (6) CORE C - 05-13-2023.docx
Mapp Specific Aims draft.pdf
Specific Aims Core E.docx

Hello everyone,

The Specific Aims draft for Research Projects 1-3, 5, and all Cores are attached for your review.

Thank you,
(b) (6)

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 286, NO. 24, pp. 21678–21686, June 17, 2011

Structural Characterization of the Crimean-Congo Hemorrhagic Fever Virus Gn Tail Provides Insight into Virus Assembly*[S]

Received for publication, December 25, 2010, and in revised form, April 6, 2011 Published, JBC Papers in Press, April 20, 2011, DOI 10.1074/jbc.M110.216515

D. Fernando Estrada and Roberto N. De Guzman[1]

From the Department of Molecular Biosciences, University of Kansas, Lawrence, Kansas 66045

The RNA virus that causes the Crimean Congo Hemorrhagic Fever (CCHF) is a tick-borne pathogen of the *Nairovirus* genus, family *Bunyaviridae*. Unlike many zoonotic viruses that are only passed between animals and humans, the CCHF virus can also be transmitted from human to human with an overall mortality rate approaching 30%. Currently, there are no atomic structures for any CCHF virus proteins or for any *Nairovirus* proteins. A critical component of the virus is the envelope Gn glycoprotein, which contains a C-terminal cytoplasmic tail. In other *Bunyaviridae* viruses, the Gn tail has been implicated in host-pathogen interaction and viral assembly. Here we report the NMR structure of the CCHF virus Gn cytoplasmic tail, residues 729–805. The structure contains a pair of tightly arranged dual $\beta\beta\alpha$ zinc fingers similar to those found in the *Hantavirus* genus, with which it shares about 12% sequence identity. Unlike Hantavirus zinc fingers, however, the CCHF virus zinc fingers bind viral RNA and contain contiguous clusters of conserved surface electrostatics. Our results provide insight into a likely role of the CCHF virus Gn zinc fingers in *Nairovirus* assembly.

Recent outbreaks of the Crimean Congo Hemorrhagic Fever (CCHF)[2] virus along with the reported ability of the virus to transfer between humans have raised concerns of a widespread pandemic (1). The virus is transmitted to humans by tick bite or by direct handling of infected animal meat or blood (1, 2). Infection causes a hemorrhagic fever and myalgia resulting in mortality rates approaching 30% (1–3). The virus contains an antisense RNA genome divided into three segments, and named according to lengths as the S, M, and L (for Small, Medium, and Large) segments (4). The viral proteins are the nucleocapsid protein, two membrane glycoproteins Gn and Gc (also referred to as G1 and G2 in other *Bunyaviridae*) (5, 6), a nonstructural protein (NSm) (7), and an RNA polymerase (4). In the mature virion, the Gn glycoprotein contains a 176 residue ectodomain followed by a 24 residue transmembrane region and terminates in a long cytoplasmic tail consisting of ~100 residues (5, 7).

Recent results from other related *Bunyaviridae* viruses suggest the role of the Gn tail in viral assembly. For example, alanine mutagenesis of the cytoplasmic tails of Uukuniemi virus (genus *Phlebovirus*) (8) and Bunyamwera virus (genus *Orthobunyavirus*) (9) affect the ability of virus-like particles (VLPs) to effectively incorporate ribonucleoproteins, thus intimating a role for Gn tails in genome packaging. More recently, the Gn tail of Puumala virus (genus *Hantavirus*) was shown to co-immunoprecipitate with the Puumala nucleocapsid protein (10). These results suggest that the CCHF virus Gn tail plays an equally important role in viral assembly of genus *Nairovirus*.

The sequence of the CCHF virus cytoplasmic tail is somewhat variable in Nairoviruses (~24% identity) and even more so when compared with other Bunyaviruses (12% identity with Hantavirus Gn tails). However, one characteristic feature present in four of the five genera of *Bunyaviridae* is a conserved dual **C**-X-**C**-X-**H**-X-**C** motifs of cysteine and histidine residues with X representing any amino acid (Fig. 1). Others have suggested that the high cysteine content of the CCHF virus Gn tail could be due to extensive disulfide bonding (5). Recently, we reported that the cysteines in the Andes hantavirus Gn tail fold into a novel arrangement of back-to-back classical $\beta\beta\alpha$ zinc fingers (11). Despite low sequence identity between the Gn tail of Nairoviruses and Hantaviruses, the spacing of the dual CCHC motif in the CCHF virus most closely resembles that of Hantaviruses, suggesting the presence of a similar dual zinc finger structure. To test this hypothesis, we determined the NMR structure of the CCHF virus Gn cytoplasmic tail from residues 729–805. We report here the first known atomic structure of any protein component of the CCHF virus and demonstrate that the high cysteine content of the Gn cytoplasmic tail is partly due to the presence of dual, back to back $\beta\beta\alpha$-type zinc fingers similar to those found in Hantaviruses. Unlike Hantaviral zinc fingers, however, the electrostatic surface of the CCHF virus zinc finger reveals a clear distribution of conserved electrostatic charges. Moreover, we demonstrate using electrophoretic mobility shift assays (EMSA) that these conserved electrostatics may play a role in forming a surface for binding viral

* This work was supported in part by AHA 0755724Z, K-INBRE, and NIH P20 RR016475 (to R. N. D.) and the Madison and Lila Self Graduate Fellowship (to D. F. E.).

The atomic coordinates and structure factors (code 2L7X) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

NMR assignments were deposited at the Biological Magnetic Resonance Bank (BMRB ID 17383).

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S3.

[1] To whom correspondence should be addressed: Dept. of Molecular Biosciences, University of Kansas, 1200 Sunnyside Ave., Lawrence, KS 66045. Fax: 785-864-5294; E-mail: rdguzman@ku.edu.

[2] The abbreviations used are: CCHF, Crimean Congo Hemorrhagic Fever; EMSA, electrophoretic mobility shift assay; GB1, the B1 domain of *Streptococcus* protein G; HSQC, heteronuclear single-quantum coherence spectroscopy; NOE, nuclear Overhauser effect; R_1, longitudinal or spin-lattice relaxation rate; R_2, transverse or spin-spin relaxation rate; ZF1, first CCHC zinc binding array, residues 736–756; ZF2, second CCHC zinc binding array, residues 761–780.

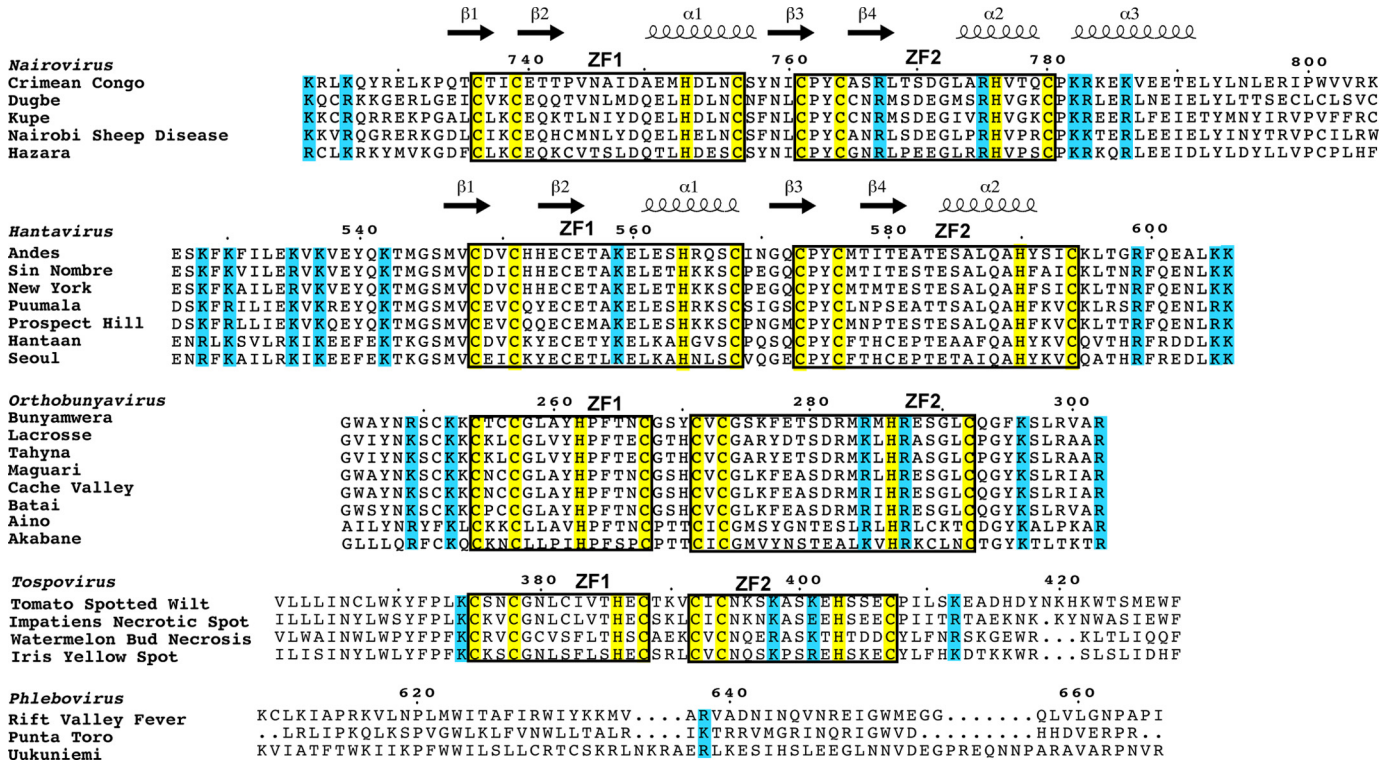

FIGURE 1. **Sequence alignment of the Gn tails of representative members of family *Bunyaviridae*.** *Bunyaviridae* is comprised of five genera: Nairovirus, Hantavirus, Orthobunyavirus, Tospovirus, and Phlebovirus. The conserved CCHC-zinc finger motifs (*boxed*) are present in four of the five genera, with Phlebovirus the lone exception. Another recurring feature is the clustering of conserved basic residues (*in blue*) in the vicinity of the CCHC-motifs. Notably, these basic residues overlap with ZF2 in Nairovirus, Orthobunyavirus, and Tospovirus, but are located outside ZF2 in Hantavirus.

RNA. Together, these data provide insight into the role of the Gn tail in *Nairovirus* assembly.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification—Various constructs of the CCHF virus (strain SPU103/87) Gn cytoplasmic region (spanning residues 719–819) were subcloned from a synthetic gene (GenScript) into the expression vectors pDZ1 and pDZ3 (12), which expressed His_6-tagged GB1 fusion proteins with TEV protease cleavage sites. For NMR structure determination, the soluble Gn construct spanning residues 729–805 ($Gn^{729-805}$) was expressed and purified under native conditions following the method reported previously for the Andes hantavirus zinc finger domain (11). Briefly, ^{15}N- and $^{15}N/^{13}C$-labeled proteins were expressed in *Escherichia coli* BL21(DE3) grown in 1 liter M9 minimal media supplemented with 0.1 mM $ZnSO_4$ before and after induction. Cells were grown at 37 °C, induced with 1 mM isopropyl-β-D-thiogalactopyranoside at A_{600} ~0.8, and cell growth was continued in a 15 °C shaker incubator overnight (to a final A_{600} ~2.0). Cells were harvested by centrifugation, resuspended in buffer A (20 mM Tris-HCl pH 8.0, 20 mM NaCl, 1 mM DTT, 0.1 mM $ZnSO_4$), and lysed by sonication. Cellular debris was removed by centrifugation, and to the supernatant was added one-tenth volume of 1% polyethyleneimine (pH 8) to precipitate the nucleic acids. Following centrifugation, the supernatant was bound to a 40 ml of Q column (GE Healthcare) and eluted with a 280 ml linear gradient of buffer B (20 mM Tris-HCl, pH 8.0, 0.5 M NaCl, 1 mM DTT, 1 mM $ZnSO_4$). For TEV protease digestion, fractions containing the fusion protein were pooled and dialyzed at 25 °C overnight in buffer (50 mM Tris-HCl pH 8.0, 20 mM NaCl, 1 mM DTT, 1 mM $ZnSO_4$) with 0.16 mg recombinant TEV protease (13) per 10 ml of fusion protein. The TEV digestion mixture was dialyzed back into buffer A and passed again through a 40 ml Q column (GE Healthcare). The GB1 tag (theoretical pI of 5.6) was retained on the column while $Gn^{729-805}$ (theoretical pI of 8.6) was present in the flow-through. The 50 ml flow-through fraction was concentrated using Ultra-15 centrifugal filters (Amicon) and dialyzed in NMR buffer (10 mM $NaPO_4$ pH 7.0, 10 mM NaCl, 1 mM DTT, 0.1 mM $ZnSO_4$). The $Gn^{729-805}$ construct retained three residues (Gly-His-Met) cloning artifacts at the N terminus.

NMR Spectroscopy—NMR data were acquired at 25 °C using a Bruker Avance 800 MHz spectrometer equipped with a cryoprobe, processed with NMRPipe (14), and analyzed with NMRView (15). Backbone assignments were obtained from two-dimensional ^1H-^{15}N HSQC (16) and three-dimensional HNCA (17), CBCA(CO)NH (17), and HNCACB (18). Secondary structures were identified from the Cα, Cβ, and Hα chemical shifts (19). Side chain assignments were obtained from two-dimensional ^1H-^{13}C HMQC (20), three-dimensional HBHA(CO)NH (21), and three-dimensional ^{13}C-edited HMQC-NOESY (22) (t_{mix} = 120 ms). The histidine ring nitrogen atoms coordinated to Zn^{2+} ions were identified from two-dimensional ^{15}N HMQC (23) using a nitrogen sweep width of 160–230 ppm. NOE (nuclear Overhauser effect) crosspeaks were identified from three-dimensional ^{15}N-edited NOESY-

HSQC (24) (t_{mix} = 120 ms) and three-dimensional ^{13}C-edited HMQC-NOESY (22) (t_{mix} = 120 ms).

Backbone ^{15}N relaxation parameters were acquired on a 0.5 mM ^{15}N-labeled sample in NMR buffer. The steady-state heteronuclear {^1H}-^{15}N NOE was acquired as a pair of two-dimensional datasets in an interleaved manner (where portions of each two-dimensional spectrum were acquired sequentially until both datasets were completed) (25). The first two-dimensional dataset contained a 3-s proton saturation (achieved with a series of 120° pulses) whereas the second two-dimensional dataset contained a 3-s delay. The heteronuclear {^1H}-^{15}N NOE was calculated as the ratio of the intensities for each peak in the two datasets. Each two-dimensional dataset was acquired with 2048 (^1H) × 128 (^{15}N) complex points, 32 scans per point, and a 5 s recycle delay. Error bars were estimated using the standard deviation of the background signal of each spectrum. The ^{15}N backbone relaxation rates R_1 and R_2 were acquired as described (26). The time delays used to determine R_1 were 10, 60, 120, 240*, 400, 900, and 1100 ms, and the time delays used to determine R_2 were 20, 40*, 50, 60, 70, 90, 100, 120, and 150 ms (asterisk denotes spectra acquired in duplicate to estimate reproducibility). Peak intensities were obtained from NMRView (15) and fitted using GNUPLOT (27). Deviations from fitting were reported as error bars. Because of peak overlap, residues 749, 787, 791, and 796 were not used in the analysis.

Structure Calculation—The protocol used for NMR structure calculation has been described previously (11). Briefly, unique NOE distance restraints were classified into upper bounds of 2.7, 3.5, 4.5, and 5.5 Å and lower bound of 1.8 Å based on peak volumes. Backbone dihedral angles in the α-helical regions identified by the secondary Cα, Cβ, and Hα chemical shifts (19) were restrained to φ (−60 ± 20°) and ψ (−40 ± 20°). Initial structures were generated using CYANA (28), followed by molecular dynamics and simulated annealing in AMBER7 (29); first *in vacuo*, then with the generalized Born (GB) potential. Initial structural calculations were performed in CYANA without the Zn^{2+} restraints to confirm that the zinc finger domain will fold from NOE-derived restraints only. Once the topology of the Zn^{2+}-coordinated residues were confirmed, subsequent CYANA structure calculations used distance restraints that imposed tetrahedral Zn^{2+}-coordination to Cys and His residues (22). Iterative cycles of AMBER calculations followed by refinement of NMR-derived restraints were performed until the structures converged with low restraint violations and good statistics in the Ramachandran plot. A family of twenty lowest energy structures were analyzed using PROCHECK (30) and molecular graphics were generated using PYMOL (31). The surface electrostatic potentials were calculated using APBS (32) and visualized in PYMOL (31).

In Vitro Transcription—A DNA oligonucleotide representing the M genomic segment panhandle was assembled by PCR primer extension and used for *in vitro* transcription. *In vitro* transcription was carried out following manufacturer's protocol (MAXIscript Kit, Ambion). Briefly, a 20-μl reaction was carried out for 1.5 h (37°) and terminated by adding 2 μl 0.25 M EDTA and heating to 90° followed by rapid cooling on ice. Reaction mixtures were then treated with DNase I and subjected to ethanol precipitation. The RNA transcripts were resuspended in RNase-free ddH_2O and analyzed for purity on a native 12% acrylamide gel stained with SYBR Green II dye (Invitrogen).

RNA Binding Assays—To assess protein-RNA binding by gel electrophoresis, RNA transcripts were incubated on ice for 15 min with increasing amounts of either CCHF virus $Gn^{729-805}$ or Andes hantavirus $G1^{543-599}$ in binding buffer (30 mM $NaPO_4$, 30 mM NaCl, pH 7.4). Samples were mixed with one-half volume 50% glycerol and loaded onto a native 12% acrylamide Tris borate gel. The gel was run in a cooling water bath at 90 V for 1 h in Tris borate buffer, pH 8.3 and visualized by staining with SYBR Green II dye. For nucleic acid size determination, each gel included a 100 bp DNA ladder (NEB, NO467S).

CD Spectroscopy—CD spectra were collected in triplicate at 25° on a JASCO J-815 Spectro-polarimeter using a scanning speed of 50 nm/min. Protein concentrations were kept at 1 μM in buffer (10 μM $NaPO_4$, 10 μM NaCl, 0.1 mM $ZnSO_4$). EDTA and $ZnSO_4$ titrations were applied to the same sample.

RESULTS

Protein Expression and Purification—Our previous work with Hantavirus glycoprotein cytoplasmic tails indicates expression of the tail is toxic to *E. coli* (11). Therefore, all constructs of the CCHF virus Gn cytoplasmic tail were expressed as GB1 fusion proteins. The GB1 tag contained His_6 for nickel affinity purification and a TEV protease cleavage site to recover the native Gn zinc finger domain. The fusion protein was expressed in soluble form in *E. coli*, purified under native conditions, and digested with TEV protease to obtain the Gn zinc finger domain. Longer constructs comprising the entire predicted cytoplasmic tail ($Gn^{719-819}$) expressed as insoluble inclusion bodies. $Gn^{729-819}$, which was missing the first ten residues following the transmembrane region, expressed as soluble protein but with low yield. $Gn^{729-805}$ represented the longest construct containing the conserved **C**-X_2-**C**-X_{11-12}-**H**-X_3-**C** (where X is any amino acid) that also expressed in high enough yield to give high resolution NMR data.

Zn^{2+} Is Required for Proper Folding—To examine the reliance of Zn^{2+}-coordination on the proper folding of the CCHF virus Gn tail, we recorded the two-dimensional ^{15}N HSQC of the $Gn^{729-805}$ in the presence of 4 mM EDTA (Fig. 2*A*). The spectrum in the presence of EDTA is collapsed between ppm values of 6.5 and 8.6, whereas the folded spectrum in the absence of EDTA is well dispersed between 6.5 and 9.3 ppm. Narrowing of the spectrum suggests a loss of tertiary structure upon removal of Zn^{2+}, indicating the requirement for Zn^{2+} binding in folding of the domain. A similar titration using circular dichroism (CD) spectroscopy demonstrates that the presence of EDTA causes a downward spectral shift, indicating a transition toward an unfolded protein (Fig. 2*B*). Here we also demonstrate that the addition of Zn^{2+} ion back into the sample recovers the trace of the original native spectrum. Therefore, Zn^{2+} is required for proper folding of the CCHF virus Gn tail.

NMR Structure Determination—CCHF virus $Gn^{729-805}$ showed a well dispersed two-dimensional ^1H-^{15}N HSQC (Fig. 3*A*). Complete backbone assignments were obtained from three-dimensional HNCA, CBCA(CO)NH, HNCACB, and ^{15}N-edited NOESY-HSQC. The C^α, H^α, and C^β secondary chemical shifts (Fig. 3*B*) showed the presence of three short

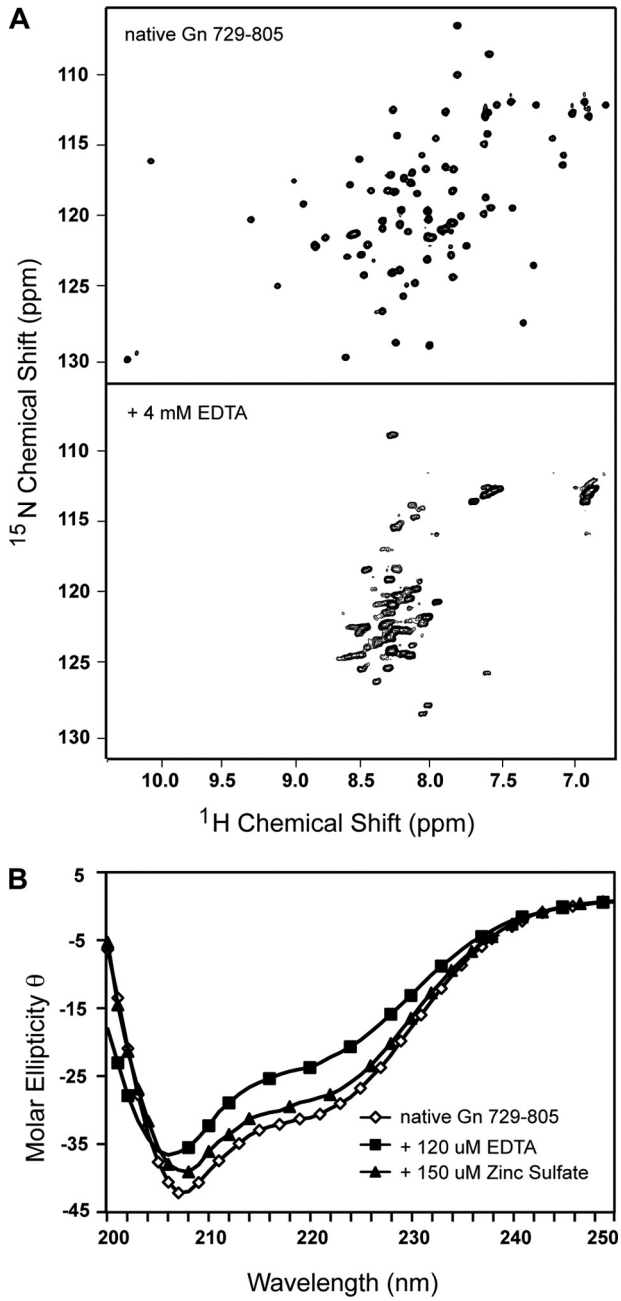

FIGURE 2. **CCHF virus Gn zinc finger domain (residues 729–805) relies on Zn^{2+} for proper folding.** Addition of 4 mM EDTA to a sample of ^{15}N-labeled $Gn^{729-805}$ effectively narrows the HSQC spectrum into a characteristic of an unfolded protein (*A*). Likewise, addition of a metal chelator causes a downward shift at 208 nm in the CD spectra toward random coil (Y axis: molar ellipticity θ per residue, deg·cm^2 dmol^{-1}residue^{-1} $\times$ 10^4) (*B*). Titration of zinc sulfate back into the sample recovers the original CD trace (*B*).

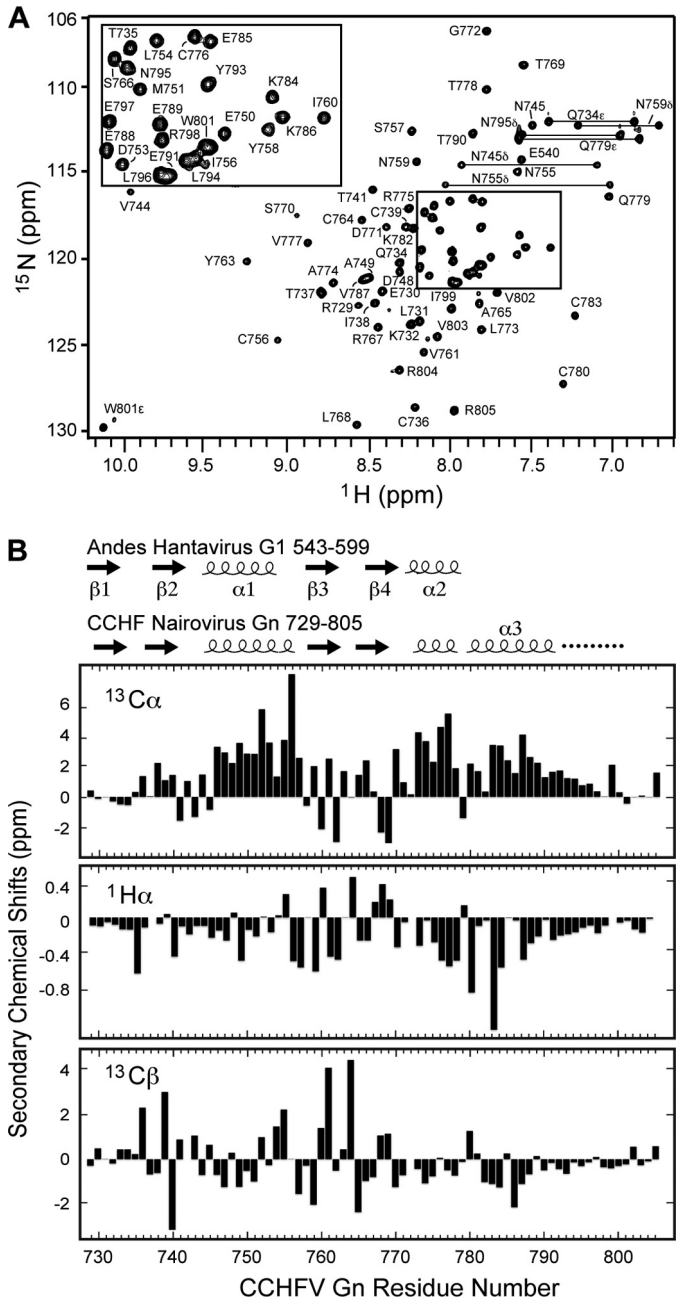

FIGURE 3. The CCHF virus Gn zinc finger yielded a well dispersed two-dimensional ^{1}H-^{15}N HSQC spectrum (*A*). The smaller peak in the tryptophan (*W801*) side-chain suggested a minor conformation of the tryptophan ring possibly due to ring flip-flop. Secondary chemical shifts for $^{13}C\alpha$, $^{1}H\alpha$, and $^{13}C\beta$ suggest the presence of three short α helices interspersed with two short β hairpins (*B*).

α-helices with an intervening random coil region between the second and third helix. Two more regions in random coil orientations flanked the central sequence of the domain as indicated by the heteronuclear {^{1}H}-^{15}N NOE (Fig. 4*C*). Side chain assignments were completed using two-dimensional ^{1}H-^{13}C HMQC, three-dimensional HBHA(CO)NH, and three-dimensional ^{13}C edited HMQC-NOESY. There were six invariant cysteine and two histidine residues (His-752 and His-776) in $Gn^{729-805}$ (Fig. 1), all of which were involved in Zn^{2+} coordination. Long distance NOE's confirmed that His-752 and His-776 were involved in Zn^{2+} coordination. Notably, His-752 Hϵ1 shares an NOE with Cys^{736} Hβ's. Likewise, His-776 Hϵ1 and Hδ2 share NOEs with Cys-761 and Cys-780 Hβ's. A two-dimensional ^{15}N HMQC (23) spectrum showed that His-752 and His-776 coordinated Zn^{2+} through the Nδ1 and Nϵ2 atoms (supplemental Fig. S1), respectively. Manual analysis of three-dimensional ^{15}N- and ^{13}C-edited NOESY spectra identified 1193 unambiguous interproton NOE distance restraints. The NOE restraints together with 26 φ and 26 ψ dihedral angle restraints and zinc coordination restrains (Table 1) were used in structure calculation and refinement in CYANA and

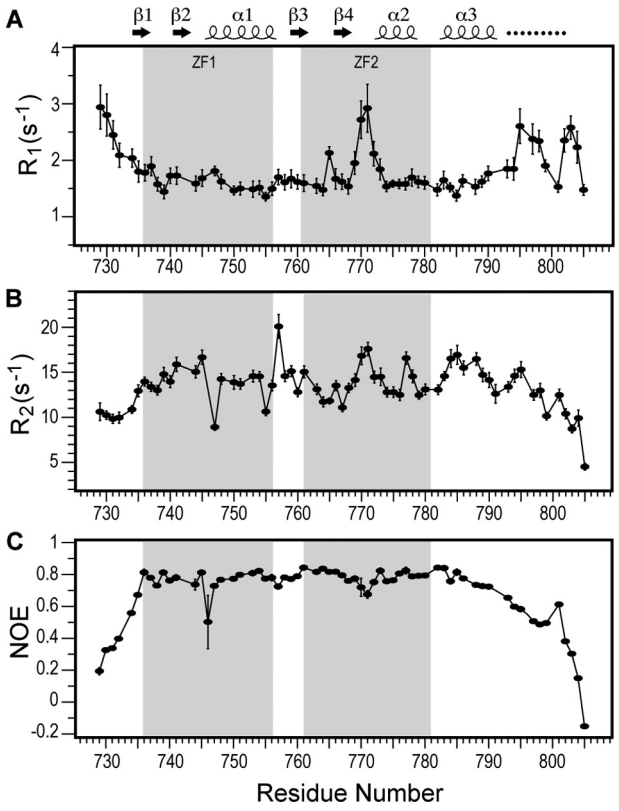

FIGURE 4. **Amide backbone relaxation rates R_1 (*A*), R_2 (*B*), and heteronuclear {^{1}H}-^{15}N NOE (*C*) of the CCHF virus Gn zinc finger.**

TABLE 1

NMR restraints and structural statistics for the 20 refined NMR structures

Total distance restraints	1193
Intraresidue (i, i)	246
Sequential (i, i +1)	407
Long Range (i-j) > 4)	307
Total dihedral angle restraints	52
Phi	26
Psi	26
RMS deviation from mean structure	
Backbone atoms (N, Cα, C′) (Å)	0.25
All heavy atoms (C, N, O) (Å)	0.76
NOE violations	
Max distance violation (Å)	0.47
Max dihedral angle violation (°)	5.3
Energies (kcal/mol)	
Mean GB[a]-AMBER energy	−3359
Mean restraint energy	79
Ramachandran plot	
Most favorable region (%)	79.2
Additionally allowed regions (%)	20.1
Generously allowed regions (%)	0.6
Disallowed regions (%)	0.2

[a] Generalized Born potential.

AMBER. The 20 low energy NMR structures of $Gn^{729-805}$ converged into a family of structures (Fig. 5) with low restraint violations and good Ramachandran plot statistics (Table 1).

Structure of CCHF Virus Zinc Finger—The NMR structure of $Gn^{729-805}$ reveals a rigid, compact three-helix structure with four short β-strands (Fig. 5). The structure contains a pair of tightly associated, back to back ββα zinc fingers connected by a short four residue linker (Ser^{757}-Ile^{760}) (Fig. 5). The first CCHC-zinc finger array (ZF1) consists of a Zn^{2+} ion coordinated to residues Cys-736, Cys-739, His-752, and Cys-756 and forms the classical ββα zinc finger fold. Cys-736 and Cys-739 form part of a short β-hairpin. Thr-737 and Ile-738 form a loop with Cys-736 and Cys-739 on either side of the hairpin. The structure contains helix α1 formed by Ile-747 to Ser-757 that folds back toward the β-hairpin, forming the ββα zinc finger fold. Cys-756 forms the fourth Zn^{2+}-coordinating residue and is located on the same surface of helix α3 with His-752.

Likewise, the second CCHC-zinc finger array (ZF2) consists of a second Zn^{2+} ion coordinated to residues Cys-761, Cys-764, His-776, and Cys-780 into a classical ββα zinc finger fold. Cys-761 and Cys-764 are positioned on either side of a short β-hairpin. Pro-762 and Tyr-763 form a loop between Cys-761 and Cys-764. The structure is followed by a short helix α2 formed by Leu773-Cys-780 and folded back toward the β-hairpin, forming the ββα zinc finger fold. His-776 is located toward the middle of helix α3, and the final coordinating cysteine (Cys-780) is located at the end of helix α3. Although ZF2 also resembles the classical ββα fold, a minor difference exists when compared with ZF1. The helix α2 of ZF2 is shorter than helix α1 by three residues. This is due to the presence of helix breakers Gly-772 and Pro-781 located at either end of helix α2.

Unlike many classical ββα zinc fingers which form independently folded domains like "beads-on-a-string," the two CCHF virus zinc fingers were tightly stuck together, with over 65 NOEs observed between ZF1 and ZF2. These NOEs fix the relative orientation of ZF1 with respect to ZF2. Among these NOEs, the strongest were observed between Met-751 (ZF1) and Tyr-763 (ZF2), Cys-739 (ZF1) and Ala-774 (ZF2), His-752 (ZF1) and Val-777 (ZF2), and Thr-741 (ZF1) and Val-777 (ZF2).

In addition to the two classical ββα fold zinc fingers, the structure contains an additional helix, helix α3, formed by Lys-782 to Glu-791 that packs against the dual zinc finger fold. A hydrophobic interaction between Val-744 of ZF1 and Val-787 keeps helix α3 pinned to the core structure. The orientation of helix α3 to ZF2 is partially determined by the helix breaker Pro-781, the residue immediately following ZF2. Pro-781 is 100% identical among Nairoviruses (Fig. 1) and serves as a kink between helix α2 and α3. Strong Cys Cα to Pro-718 Cδ NOEs indicated a trans proline isomer. The C-terminal 13 residues (Leu-792 to Lys-805) are primarily unstructured. NOEs between Ile-799 Cγ2 and Met-751 Cγ indicate the unstructured tail is pinned to the rest of the structure.

The ^{15}N backbone relaxation rates (R_1 and R_2) as well as the heteronuclear {^{1}H}-^{15}N NOE (Fig. 4) showed that the ZF1, linker (residue 757–760), and ZF2 regions behave with nearly similar amide backbone dynamics. The average R_1 values for ZF1, ZF2 and linker regions were well within each other, with values of 1.60 (+ 0.15), 1.81 (+ 0.40), and 1.65 (+ 0.04) s^{-1}, respectively (Fig. 4*A*). ZF1 and the linker had essentially similar R_1 values, however, within ZF2, the R_1 values increased for residues 770 and 771 of the loop connecting β4 and α2, indicating increased mobility of this region. Likewise, the average R_2 values for ZF1, ZF2, and linker regions were similar to each other, with values of 13.8 (+ 1.8), 13.8 (+ 1.8), and 15.6 (+ 3.1) s^{-1}, respectively. Interestingly, the first linker residue, Ser-756, showed increased R_2 without a corresponding increase in R_1, which suggested chemical exchange on the μs-ms timescale for

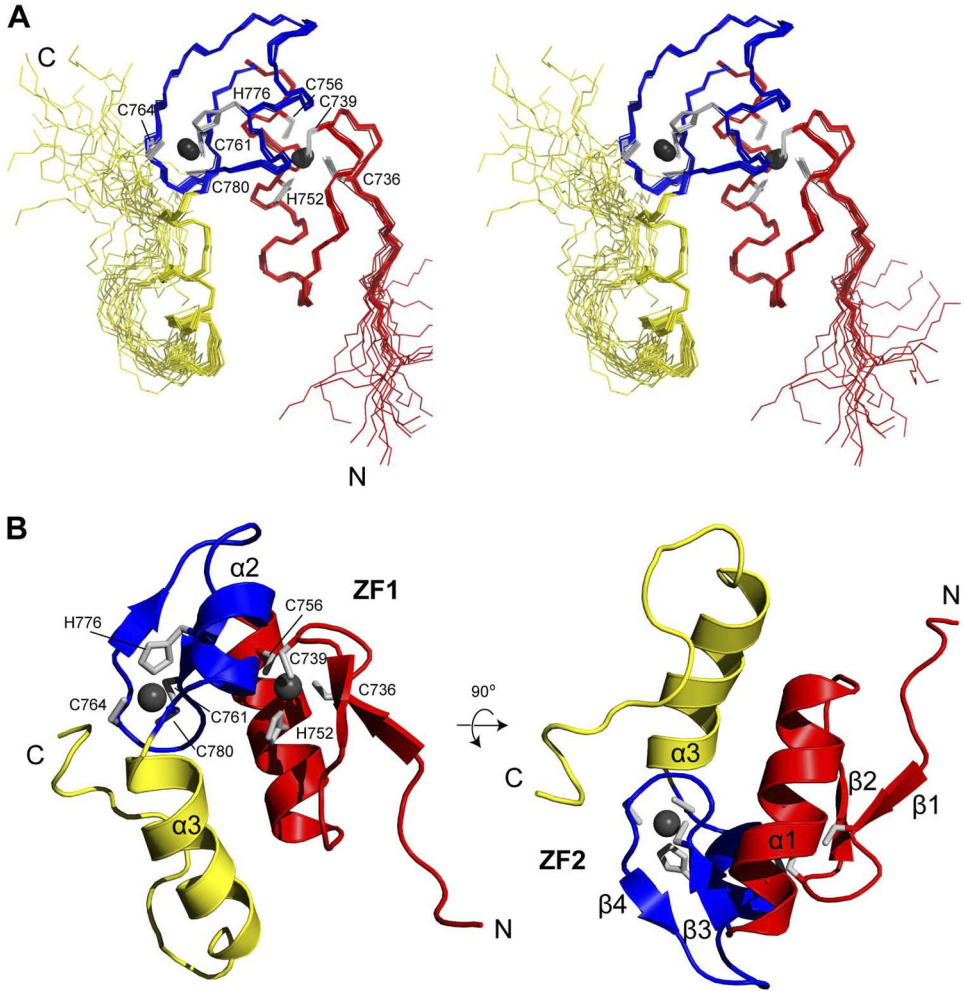

FIGURE 5. **NMR structure of CCHF virus Gn tail zinc finger.** Stereoview of the superposition of 20 lowest energy NMR structures of CCHF virus Gn zinc finger (*A*). CCHF virus Gn zinc finger domain folds into a compact three-helix structure consisting of two back-to-back $\beta\beta\alpha$ zinc fingers with helix α3 pinned underneath the core zinc finger structure (*B*).

Ser-756. The average heteronuclear $\{^1H\}$-^{15}N NOE for the ZF1, linker and ZF2 regions was 0.8 (Fig. 4*C*), indicating reduced flexibility for the dual zinc finger domain including the linker region. In brief, the NMR amide backbone relaxation parameters (Fig. 4) confirmed that the two zinc fingers essentially tumble as one entity, that the linker between the two zinc fingers was rigid and tumble at the same rate as the zinc fingers, and that the loops and tails were flexible.

CCHF Virus Zinc Finger Contains Conserved Electrostatic Surfaces—Analysis of the surface electrostatics of the CCHF virus $Gn^{729-805}$ reveals clustering of positive and negatively charged surfaces on opposite faces of the structure (Fig. 6). Surface residues Glu-740, Glu-750, and Asp-753 of ZF1 converge with surface residues Glu-789 and Glu-791 of helix α3 and Glu-797 of the C-terminal unstructured region to form a large, nearly contiguous negatively charged surface. Similarly, Arg-767 and Arg-775 of ZF2 converge with Lys-782, Lys-784, and Lys-786 of helix α3 and Arg-798 of the C-terminal unstructured region to form a large contiguous positively charged surface. Of the charged surface residues, only Glu-750, Glu-797, Lys-784, and Arg-798 are not conserved in Nairoviruses. Most of the surface electrostatics, therefore, is a conserved feature of the Nairoviruses zinc finger domain.

CCHF Virus Gn Tail Binds RNA—RNA electrophoretic mobility shift binding assays (EMSA) were carried out using two different proteins: the zinc finger domain of CCHF virus consisting of $Gn^{729-805}$, and the zinc finger domain $G1^{534-599}$ of the related Andes hantavirus. The RNA sequences used in the EMSA were a 58-mer RNA of the Andes hantavirus and a 51-mer RNA of the CCHF virus (Fig. 7*A*). Both RNA sequences contain 23–26 nucleotides at the 5′ and 3′ termini of the M genomic segments of the Andes and CCHF viruses, and these 5′ and 3′ strands are complementary to each other and are expected to form into hairpin-like panhandle structures (Fig. 7*A*). On a 12% native acrylamide gel, the Andes hantavirus RNA traveled as a single band consistent with a 58-mer hairpin (Fig. 7*B*), however, the CCHF virus 51-mer RNA migrated as two bands, a higher molecular weight form and a lower band migrating at a size consistent with a 51-mer hairpin (Fig. 7*B*). Incubation of the Andes hantavirus protein with the 58-mer RNA failed to affect the migration of RNA (Fig. 7*B*). The RNA bands in the presence of the Andes hantavirus protein were similar to the free RNA band (Fig. 7*B*). However, incubation of the CCHF virus zinc finger protein notably affected the migration of the CCHF virus RNA, as demonstrated by the appearance of an additional band (marked with *asterisk*, Fig. 7*B*)

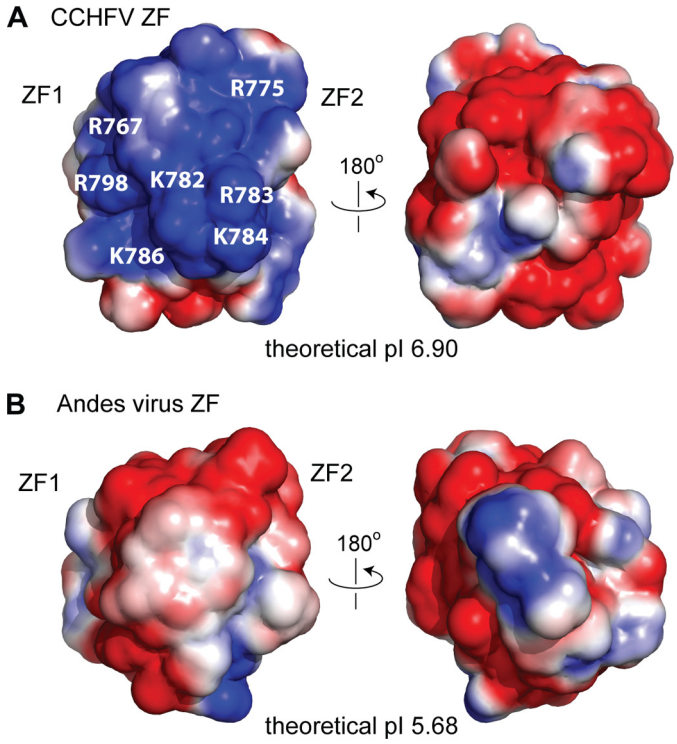

FIGURE 6. **Comparison of the surface electrostatics of the CCHF virus (*A*) and Andes hantavirus (*B*) zinc fingers.** Analysis of the surface electrostatics of the CCHF virus Gn zinc finger reveals a contiguous cluster of basic charges (colored *blue*) that cover the entire half of the structure (*A*). Rotating the structure 180° reveals an equally large cluster of acidic charges (colored *red*) on the opposite face (*A*). Surface electrostatics of the Andes hantavirus zinc fingers (PDB 2K9H) in the same orientation as the CCHF virus structure (*B*). Notably, the clustering of conserved basic surface in Nairovirus (*A*) is absent in Hantavirus (*B*).

migrating between the hairpin and the higher MW form of the CCHF virus RNA. The protein-RNA complex band was most likely formed between the zinc finger protein and the RNA hairpin. Because the 51-mer CCHFV RNA existed in two forms (the hairpin and the higher MW form), the relative amounts of each form must be in equilibrium and binding of the CCHFV zinc finger protein to the hairpin RNA will also affect the amount of the RNA B form as seen in Fig. 7*B*. We are designing new RNA sequences that will only form hairpins for future studies of the protein-RNA interaction of the CCHF virus zinc finger. Nevertheless, the main conclusion from the EMSA results demonstrated that the CCHF Nairovirus Gn tail (residues 729–805) interacted with RNA whereas the Andes hantavirus G1 tail (residues 534–599) did not.

To test our hypothesis that conserved basic residues in the CCHF virus zinc finger protein may be involved in RNA binding, point mutations were introduced in conserved lysine and arginine residues into aspartic acid and the mutant proteins were used in EMSA. The point mutants K783D, K786D, and R767D retained the ability to bind RNA suggesting these basic residues may not be critical in RNA binding (supplemental Fig. S2*A*). However, the K782D mutation disrupted the protein-RNA interaction, as its shift pattern resembles that of RNA alone. Coincidentally, Lys-782 is located at the kink between helix α2 and α3, and is pointed away from the zinc finger domain (supplemental Fig. S2*B*), suggesting that the kink between helix α2 and α3 may be important for RNA binding interaction of the CCHF virus Gn zinc finger.

DISCUSSION

We report here that an envelope glycoprotein of a Nairovirus contains a dual CCHC-type zinc finger (Figs. 2–5) domain that

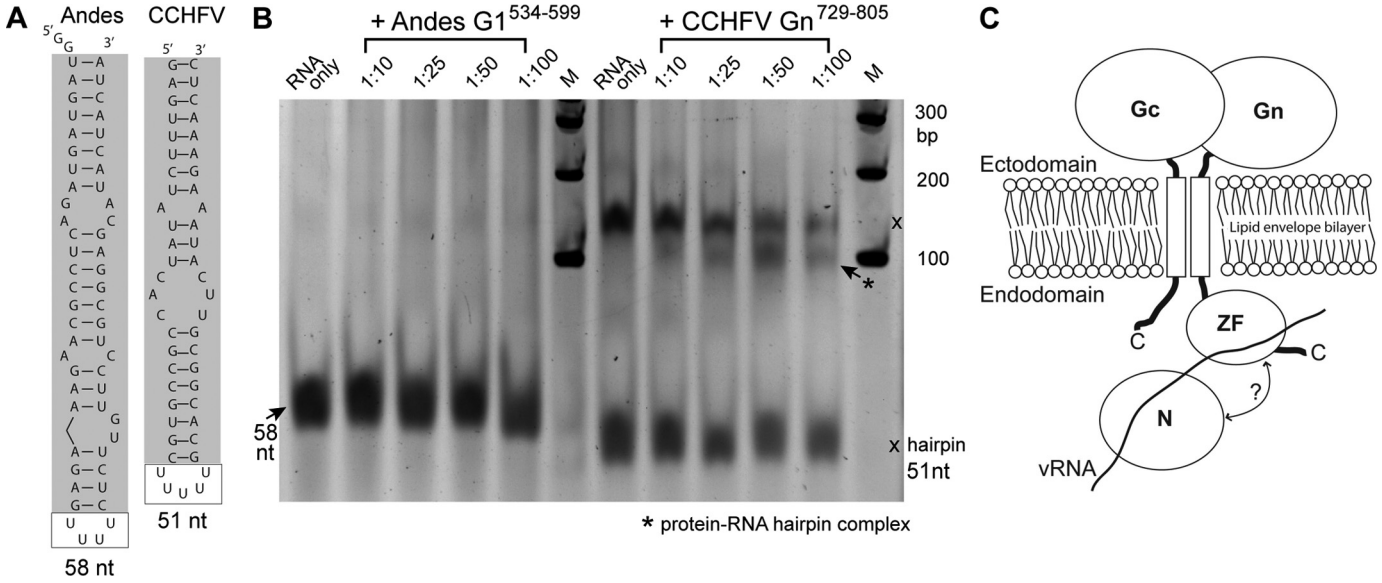

FIGURE 7. **RNA electrophoretic mobility shift assay comparing the Andes Hantavirus and the CCHF virus zinc fingers.** RNA sequences used in the EMSA: Andes Hantavirus RNA (58 nt) and CCHF virus RNA (51 nt) (*A*). CCHF virus RNA traveled in two forms (marked with ×), lower band consistent with a 51-nt hairpin form, and a higher molecular weight band above 100 bp (*B*). While the Andes hantavirus zinc finger fails to alter the mobility of Andes hantavirus RNA, increasing the amount of CCHF virus zinc finger causes the appearance of an additional band (marked with *) for the CCHF virus RNA, thus suggesting a protein-RNA complex (*B*). Each lane contained 0.24 μmol RNA, proteins came from 0.4 mM stock diluted accordingly (*B*). Surface electrostatics combined with EMSA results of the Gn tail provide mechanistic insight into RNP packaging (*C*). We propose a model in which packaging consists of a zinc finger-RNA complex. Studies in related viruses suggest a nucleocapsid, Gn tail interaction, presented here as a possible additional packaging site (*C*).

binds RNA. Although zinc fingers have been known in viruses, in particular, the HIV-1 nucleocapsid protein zinc fingers (33) are critical in RNA packaging and viral assembly, zinc fingers are rarely found in viral envelope glycoproteins. Including the zinc finger in this report, there are currently only three known structures of viral envelope glycoprotein zinc fingers. First is the zinc finger of the Hantavirus G1 envelope glycoprotein (11), and second is the zinc finger of the Junin virus envelope glycoprotein (34) (also an RNA virus, of family *Arenaviridae*). In all three cases, the cytoplasmic tails of the envelope glycoproteins contain the zinc finger domains. The Junin virus zinc fingers (34) form a unique fold that do not show any structural nor sequence similarity with the Nairovirus and Hantavirus zinc fingers. Although the Nairovirus and the Hantavirus zinc fingers (11), which both belong to family *Bunyaviridae* (Fig. 1), show an overall similar global fold (supplemental Fig. S3), they also have major structural differences (Fig. 6) and properties (Fig. 7).

Key Differences between Nairovirus and Hantavirus Gn Zinc Fingers—Examination of the surface electrostatics of the CCHF virus Gn tail reveals key differences when compared with the Andes hantavirus structure (Fig. 6). Whereas the CCHF Gn tail displays sharp clustering of conserved charges that form a large contiguous swath on the protein surface, the Andes hantavirus zinc fingers display charges that are predominately negative and apparently randomly dispersed (Fig. 6). The variation in charge conservation is also evident in the sequence analysis (supplemental Fig. S3). Whereas the spacing of CCHC motif is mostly conserved, the spacing between conserved charges is highly variable. The CCHF virus displays conserved negative charges on ZF1 and conserved positive charges on ZF2. The Andes hantavirus sequence, however, displays clustering of conserved positive charges on the sequences flanking the negatively charged core zinc finger structure.

Moreover, the CCHF virus Gn tail contains a structural motif that is absent in the Andes hantavirus structure. Helices α2 and α3 (Fig. 3*B*), residues Leu-773 to Leu-792, form a helix-kink-helix motif due the positioning of the conserved helix breaker Pro-781. While not an uncommon motif, this structural aspect in the CCHF Gn tail forms the core scaffold for a large positively charged surface partly composed of the conserved charges at Arg-775, Lys-782, and Lys-786 (Fig. 6*A*). By contrast, the Andes hantavirus structure contains neither the corresponding proline nor the charges to support a similar motif (Fig. 1). Instead, it contains the non-conserved helix breaker Gly-598 followed by conserved charges exclusively on the C-terminal end of ZF2 (Fig. 1). In this respect, the surface electrostatics of the CCHF virus Gn tail may more closely resemble that of Orthobunyaviruses, with conserved helix breakers flanked by conserved basic charges (Fig. 1). Perhaps not coincidentally, Nairoviruses and Orthobunyaviruses are both arthropod-borne, whereas Hantaviruses are rodent-borne (3). Overall, the general preservation of the fold indicates that the dual zinc finger motif plays a general but important role in the life cycle of both Nairoviruses and Hantaviruses. However, the major differences in the surface electrostatics of the CCHF virus and Hantavirus cytoplasmic tail structures (Fig. 6) also suggests that while general, the function of the tail may be species specific.

Proposed Role in Viral Assembly—Given the data available regarding the *Bunyaviridae* Gn role in viral assembly (8–10), it is likely that the surface electrostatics play an important role in assembly of the CCHF virus, presumably via direct interaction with some component of the ribonucleoprotein. The large positively charged surface of the Gn tail would suggest RNA binding, as is the traditional role for zinc fingers in retroviruses (33, 35). Our EMSA results (Fig. 7) suggest that this may in fact be the case. Using increasing amounts of $Gn^{729-805}$ revealed the migration of an additional RNA band (Fig. 7*B*), which likely represents a protein-RNA complex consisting of $Gn^{729-805}$ and the hairpin-like M segment panhandle. While the observed complex is weakly bound and therefore likely to be nonspecific, these results suggest RNA interaction mediated by some of the conserved residues in the Gn tail. Additionally, these results suggest the possibility of an interaction between the Gn tail and the RNA component of the ribonucleoproteins (Fig. 7*C*). A reverse genetics system for studying the CCHF virus has only recently been developed (36), thus allowing testing of this model in the future.

In summary, we present the NMR structure of a zinc finger domain in the Gn tail of the CCHF virus. Currently, this is the only available atomic structure for a protein component of the Nairovirus genus. The global fold of this zinc finger is similar to that of the Hantavirus zinc finger, which represents a unique fold of two classical-type $\beta\beta\alpha$-zinc fingers that are stuck together (in contrast, individual classical $\beta\beta\alpha$-zinc fingers behave as independent domains, like beads-on-a-string). We also demonstrated that the CCHF virus Gn tail binds RNA *in vitro*, thus suggesting the possibility of an interaction between the Gn tail with the viral RNA. Taken together, our results contribute novel mechanistic insight toward understanding the CCHF virus life cycle.

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Role of the Stable Signal Peptide and Cytoplasmic Domain of G2 in Regulating Intracellular Transport of the Junín Virus Envelope Glycoprotein Complex

Sudhakar S. Agnihothram,[1,2] Joanne York,[1] and Jack H. Nunberg[1]*

Montana Biotechnology Center[1] and Division of Biological Sciences,[2] The University of Montana, Missoula, Montana 59812

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Enveloped viruses utilize the membranous compartments of the host cell for the assembly and budding of new virion particles. In this report, we have investigated the biogenesis and trafficking of the envelope glycoprotein (GP-C) of the Junín arenavirus. The mature GP-C complex is unusual in that it retains a stable signal peptide (SSP) as an essential component in association with the typical receptor-binding (G1) and transmembrane fusion (G2) subunits. We demonstrate that, in the absence of SSP, the G1-G2 precursor is restricted to the endoplasmic reticulum (ER). This constraint is relieved by coexpression of SSP in *trans*, allowing transit of the assembled GP-C complex through the Golgi and to the cell surface, the site of arenavirus budding. Transport of a chimeric CD4 glycoprotein bearing the transmembrane and cytoplasmic domains of G2 is similarly regulated by SSP association. Truncations to the cytoplasmic domain of G2 abrogate SSP association yet now permit transport of the G1-G2 precursor to the cell surface. Thus, the cytoplasmic domain of G2 is an important determinant for both ER localization and its control through SSP binding. Alanine mutations to either of two dibasic amino acid motifs in the G2 cytoplasmic domain can also mobilize the G1-G2 precursor for transit through the Golgi. Taken together, our results suggest that SSP binding masks endogenous ER localization signals in the cytoplasmic domain of G2 to ensure that only the fully assembled, tripartite GP-C complex is transported for virion assembly. This quality control process points to an important role of SSP in the structure and function of the arenavirus envelope glycoprotein.

Arenaviruses are endemic in rodent populations worldwide (53), and infection can be transmitted to humans to cause severe acute hemorrhagic fevers (44, 51). Recurring outbreaks are common in regions of arenavirus endemicity, and therapeutic options to combat arenavirus infection are limited. Phylogenetic analyses divide the arenaviruses into the Old World species, such as Lassa fever and lymphocytic choriomeningitis (LCM) viruses, and the New World species, such as Junín and Machupo viruses. Up to 300,000 infections with Lassa fever virus occur annually in Africa (45), and outbreaks of New World viruses in the Americas are sporadic but routine (51). Recently, infections by LCM virus in transplant recipients have been reported (8). In the absence of effective prophylaxis and treatment, the hemorrhagic fever arenaviruses remain an urgent public health concern.

The arenaviruses are enveloped viruses whose genomes consist of two single-stranded RNA molecules, each of which encodes the ambisense expression of two of the four viral proteins (5, 9). The viral envelope glycoprotein (GP-C) is translated from a genomic-sense mRNA generated from the short (S) genomic RNA, whereas the nucleocapsid protein is translated from the antigenomic-sense mRNA. Similarly, the viral matrix protein (Z) and RNA-dependent RNA polymerase are encoded in an ambisense orientation by the long (L) RNA. During biogenesis, arenaviral particles assemble and bud at the plasma membrane (49, 60). Viral entry into target cells is initiated by GP-C binding to cell surface receptors followed by endocytosis of the virion into smooth vesicles (2). Although α-dystroglycan serves as a binding receptor for the Old World arenaviruses (6), the receptor utilized by the major New World group of arenaviruses is unknown (59). GP-C-mediated membrane fusion is activated upon acidification of the maturing endosome (2, 7, 13, 14) to deposit the virion core into the cell cytoplasm and initiate replication.

The arenavirus envelope glycoprotein complex consists of three noncovalently associated subunits derived from the GP-C precursor: in addition to the typical receptor-binding (G1) and transmembrane fusion (G2) subunits, the complex contains a stable signal peptide (SSP) subunit (4, 18, 65) (Fig. 1). The 58-amino-acid SSP is generated by the cellular signal peptidase and subsequently myristoylated (65). The mature G1 and G2 subunits are generated upon cleavage by the cellular SKI-1/S1P protease (1, 35, 38) in the early Golgi compartment (3). This proteolytic maturation event is essential for membrane fusion activity. The arenavirus G2 is a member of the class I group of viral fusion proteins (25, 64) that orchestrate membrane fusion through the triggered formation of a stable six-helix bundle core (references 16, 17, 32, and 63 and references therein).

A tripartite envelope glycoprotein complex is unusual among viral envelope glycoproteins, and the role of the unique arenavirus SSP subunit has not been fully defined. In the GP-C complex, SSP exists as a transmembrane protein, likely in a type II topology with an extended luminal C terminus (19, 23). The N terminus is modified by myristoylation, which is important for efficient membrane fusion activity (65). Recombinant GP-C constructs in which SSP is replaced by a conventional

* Corresponding author. Mailing address: Montana Biotechnology Center, The University of Montana, Science Complex, Room 221, Missoula, MT 59812. Phone: (406) 243-6421. Fax: (406) 243-6425. E-mail: jack.nunberg@umontana.edu.

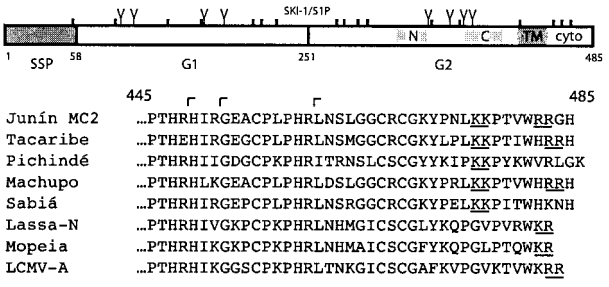

FIG. 1. Schematic representation of the Junín virus GP-C glycoprotein and G2 cytoplasmic domain sequences. Amino acids of the Junín virus envelope glycoprotein are numbered from the initiating methionine, and cysteine residues (|) and potential glycosylation sites (Y) are marked. The SSP and SKI-1/S1P cleavage sites and the resulting SSP, G1, and G2 subunits are indicated. Within G2, the C-terminal transmembrane (TM) and cytoplasmic (cyto) domains are shown, as are the N- and C-terminal heptad repeat regions (light-gray shading). A comparison of G2 cytoplasmic domain sequences among arenavirus species is detailed below the schematic. Sequences include the New World isolates Junín (D10072), Tacaribe (M20304), Pichindé (*U*77601), Machupo (AY129248), and Sabiá (YP_089665) and Old World isolates Lassa-Nigeria (X52400), Mopeia (M33879), and LCMV-Armstrong (M20869). The sites used to generate truncations in the Junín virus cytoplasmic tail are indicated by angle brackets and dibasic amino acid sequences are underlined.

signal peptide do not undergo significant proteolytic maturation by the SKI-1/S1P protease (18, 65). In the Old World Lassa fever arenavirus, this defect can be rescued by coexpression of SSP in *trans* (18).

In the present report, we examine the biogenesis of the GP-C complex of the Junín virus, a member of the New World Tacaribe complex of arenaviruses that is responsible for recurring outbreaks of hemorrhagic fever in the pampas grasslands of Argentina. We show that SSP association is required for transport of the G1-G2 precursor from the endoplasmic reticulum (ER) and thereby for proteolytic maturation in the Golgi. In the absence of SSP, the G1-G2 precursor is constrained to the ER by dibasic amino acid sequences in the cytoplasmic domain of G2. Association with SSP overcomes this block to permit transit of the fully assembled complex through the Golgi and to the cell surface. Moreover, our studies suggest that, in addition to modulating trafficking of GP-C, SSP association may also be important for the membrane fusion activity of the GP-C complex. The unique roles for SSP in the arenavirus life cycle may suggest novel strategies towards the prevention and treatment of arenaviral disease.

MATERIALS AND METHODS

Molecular reagents, recombinant vaccinia viruses, and monoclonal antibodies. The GP-C coding region from the pathogenic Junín virus strain MC2 (28) was provided by Victor Romanowski (Universidad Nacional de La Plata, Argentina) and introduced into the mammalian expression vector pcDNA3.1+ as described previously (65). For *trans*-complementation studies (18), the CD4sp-GPC construct in which SSP was replaced by the conventional signal peptide of CD4 (65) was coexpressed with an SSP construct in which a stop codon was introduced following the C-terminal SSP amino acid T58 (SSP-term). A chimeric glycoprotein (CD4ecto) bearing the CD4 signal peptide and ectodomain fused to the transmembrane and cytoplasmic domains of G2 was constructed using the human CD4 cDNA (41) obtained through the National Institutes of Health (NIH) AIDS Research and Reference Reagent Program. Mutations were introduced by QuikChange mutagenesis (Stratagene), and PCR was used to generate truncations and chimeric plasmids. For the cytoplasmic-domain truncation series and in a control cleavage-defective GP-C plasmid (cd-GPC) (65), a C-terminal 15-amino-acid S-peptide (Spep) affinity tag (34) was introduced to facilitate biochemical analysis (65). All constructs were verified by DNA sequencing, and three independent clones typically were tested to ensure consistent phenotypes.

Optimal expression of the Junín virus GP-C gene and its derivatives in Vero 76 cells was achieved using the bacteriophage T7 promoter of the pcDNA3.1 vector and infection by a recombinant vaccinia virus expressing the T7 polymerase (vTF7-3) (24). The vaccinia virus vCB21R-lacZ expressing the β-galactosidase gene under the control of the T7 promoter was used in our analysis of cell-cell fusion (47). These recombinant vaccinia virus reagents were provided by T. Fuerst and B. Moss and C. Broder, P. Kennedy, and E. Berger, respectively, through the NIH AIDS Research and Reference Reagent Program.

Mouse monoclonal antibodies (MAbs) QC03-BF11 (BF11) and GB03-BE08 (BE08) (54), directed against the G1 subunit of GP-C, were kindly provided by Tom Ksiasek and Tony Sanchez (Special Pathogens Branch, CDC, Atlanta, Georgia). The anti-CD4 ectodomain MAb SIM.2 (43, 48) was obtained through the NIH AIDS Research and Reference Reagent Program.

Expression of GP-C and its derivatives. The glycoproteins were expressed and characterized as previously described (64, 65). Briefly, Vero 76 cells were infected with the recombinant vaccinia virus vTF7-3 (24) at a multiplicity of 2 in Dulbecco's minimal essential medium containing 2% fetal bovine serum (FBS) and 10 μM cytosine arabinoside (araC) (31). After 30 min, the cells were washed and transfected with the GP-C expression plasmid using Lipofectamine 2000 reagent (Invitrogen). Metabolic labeling using 32 to 50 μCi/ml of ^{35}S-ProMix (Amersham Pharmacia Biotech) was initiated 6 h posttransfection in methionine- and cysteine-free medium containing 10% dialyzed FBS and 10 μM araC and was continued for 12 to 16 h. Cultures were then washed in physiological buffered saline (PBS) and lysed using cold Tris-saline buffer (50 mM Tris-HCl and 150 mM NaCl, pH 7.5) containing 1% Triton X-100 nonionic detergent and protease inhibitors (1 μg/ml each of aprotinin, leupeptin, and pepstatin). The expressed glycoproteins were isolated from cleared lysates by immunoprecipitation using either the G1-directed MAbs or the CD4-directed MAb SIM.2 and protein A-Sepharose (Sigma). In some experiments, glycoproteins containing the C-terminal Spep affinity tag were isolated using S-protein agarose (Novagen). Isolated glycoproteins were deglycosylated using peptide:*N*-glycosidase F (PNGase F; New England Biolabs). Proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using NuPAGE 4 to 12% bis-Tris gels (Invitrogen) and the recommended sample buffer containing lithium dodecyl sulfate and reducing agent. Molecular size markers included ^{14}C-methylated Rainbow proteins (Amersham Pharmacia Biotech). Radiolabeled proteins were imaged using a Fuji FLA-3000G imager and analyzed using ImageGauge software (Fuji).

For immunoprecipitation of cell surface glycoproteins, monolayers of metabolically labeled cells were incubated with MAb BE08 or SIM.2 in ice-cold PBS containing 2% FBS and 0.1% NaN_3 for 2 h. Following extensive washing, cells were resuspended by scraping in PBS and lysed as described above. Immune complexes were isolated from cleared lysates using protein A-Sepharose.

Flow cytometry. Vero 76 cells expressing GP-C or its derivatives were labeled using the G1-specific MAb BE08 (54) and a secondary fluorescein isothiocyanate-conjugated goat anti-mouse antibody (Jackson ImmunoResearch). CD4 was detected using a fluorescein isothiocyanate-conjugated mouse anti-CD4 MAb (BD Biosciences). Cells were subsequently stained using propidium iodide (1 μg/ml) and then fixed in 2% formaldehyde (64). Populations were analyzed using a FACSCalibur flow cytometer and CellQuest software (BD Biosciences).

GP-C-mediated cell-cell fusion. The β-galactosidase fusion reporter assay (47) was used to characterize the ability of the envelope glycoproteins to mediate pH-dependent cell-cell fusion (64, 65). Briefly, Vero cells infected with vTF7-3 and expressing the envelope glycoprotein were cocultured with reporter cells infected with vCB21R-lacZ, a recombinant vaccinia virus expressing β-galactosidase under the control of the T7 promoter. The reporter cells were obtained by incubating Vero 76 cells with vCB21R-lacZ at a multiplicity of 2 and allowing the infection to proceed overnight in the presence of 100 μg/ml rifampin (31). The GP-C-expressing cells and reporter cells were cocultured in medium containing both araC and rifampin for 5 h and then subjected to a 30-min pulse of neutral or acidic (pH 5.0) medium. β-Galactosidase expression is induced upon fusion of the effector and reporter cells and was detected, after 5 h of continued cultivation at neutral pH, in cell lysates (Tropix) using the chemiluminescent substrate GalactoLite Plus (Tropix). Cell-cell fusion was quantified using a Tropix TR717 microplate luminometer.

Confocal microscopy. Cells expressing GP-C glycoproteins were harvested by trypsinization 6 h after transfection and reseeded to 8-well chambered cover glasses (Lab Tek II) in medium containing 10 μM araC. After 18 h, cultures were washed in PBS and fixed with 4% formaldehyde for 10 min at room temperature. Following washing and quenching with 50 mM Tris (pH 7.4) in PBS, cultures

were either permeabilized in PBS containing 0.1% Triton X-100 and blocked in the same buffer containing 5% FBS (for intracellular staining) or simply blocked in the absence of detergent (for cell surface staining). GP-C glycoproteins were detected using the G1-directed MAb BF11 and an Alexa Fluor 488-conjugated anti-mouse antibody (Molecular Probes) in the appropriate blocking buffer. The Golgi marker giantin was detected using a rabbit polyclonal antiserum (Covance Research Products) and an Alexa Fluor 568-conjugated anti-rabbit antibody (Molecular Probes). Chambers were covered with Slow Fade Gold (Molecular Probes) and visualized using an inverted Nikon TE-300 microscope. Fluorescence was examined using a Bio-Rad Radiance 2000 confocal laser scanning microscope and images were merged using Lasersharp software (Bio-Rad).

RESULTS

SSP association is required for proteolytic maturation. The arenavirus SSP is distinct from conventional signal peptides in that it is retained as an essential subunit of the mature GP-C envelope glycoprotein complex and mediates functions beyond translocation of the nascent polypeptide to the ER (18, 20, 65). We previously showed that a recombinant Junín virus GP-C glycoprotein in which SSP was replaced by the conventional signal peptide of human CD4 (CD4sp-GPC) was unable to undergo efficient maturation by the SKI-1/S1P protease (65), extending similar observations with GP-C of the Old World Lassa fever virus (18). In this Old World virus, the deficiency in proteolytic cleavage in the absence of SSP was reversed by coexpression of SSP in *trans* (18).

To investigate the role of SSP in the proteolytic maturation of the Junín virus GP-C, we determined whether the coexpression of SSP in *trans* could likewise rescue cleavage. In these studies, the Junín virus CD4sp-GPC construct was cotransfected with the SSP-term plasmid encoding the 58-amino-acid SSP. Optimal expression in Vero cells was dependent on T7 RNA polymerase provided by the recombinant vaccinia virus vTF7-3 (24). Cells were metabolically labeled, and GP-C glycoproteins were immunoprecipitated using the G1-directed MAb BE08 (54). Baseline studies were performed using the native GP-C glycoprotein that included its endogenous SSP. Expression of the native glycoprotein resulted in the isolation of a 60-kDa G1-G2 precursor glycoprotein and a heterodisperse smear of G1 and G2 subunits (30 to 35 kDa) (Fig. 2A, top panel). These mature subunits are best resolved following deglycosylation by PNGase F to yield 22- and 27-kDa polypeptides, respectively (bottom panel). The G1 and G2 subunits were absent upon expression of an SKI-1/S1P cleavage-defective glycoprotein (cd-GPC) (65). A GP-C precursor glycoprotein bearing SSP is often detected as a minor species, suggesting incomplete signal peptidase cleavage in transfected cells (20, 23, 65). As previously reported (18, 20, 23, 65), SSP was coprecipitated as part of the wild-type and cleavage-defective GP-C complexes (Fig. 2A, top panel).

Expression of the CD4sp-GPC glycoprotein in the absence of SSP generated the 60-kDa G1-G2 precursor (Fig. 2A, top panel, −SSP) and considerably lesser amounts of the cleaved glycoproteins (bottom panel). By contrast, expression of SSP in *trans* (+SSP) enabled efficient cleavage of the G1-G2 precursor glycoprotein to produce mature G1 and G2 subunits (bottom panel). The relative efficiency of proteolytic maturation of CD4sp-GPC in *trans* was similar to that of the native GP-C glycoprotein. Furthermore, SSP was coprecipitated with the CD4sp-GPC complex (top panel). Thus, coexpression of SSP

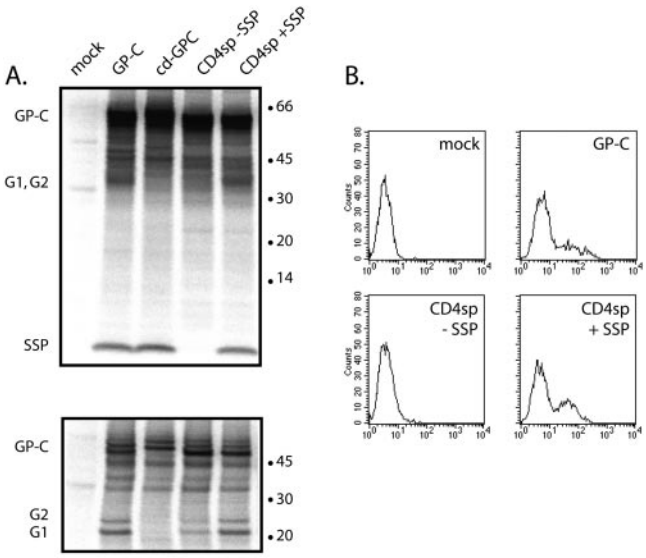

FIG. 2. Coexpression of SSP in *trans* rescues SKI-1/S1P cleavage and cell surface expression of the G1-G2 precursor. (A) Metabolically labeled glycoproteins were immunoprecipitated using the G1-specific MAb BE08 and separated on NuPAGE 4-to-12% bis-Tris gels. The wild-type (GP-C) and SKI-1/S1P cleavage-defective (cd-GPC) glycoproteins are shown for comparison with the CD4sp-GPC construct encoding the conventional signal peptide of human CD4. CD4sp-GPC was expressed alone (−SSP) or with SSP (+SSP). In the bottom panel, the glycoproteins have been treated with PNGase F to resolve G1 and G2 polypeptides. The deglycosylated GP-C polypeptides reveal both the G1-G2 precursor and, in SSP-containing constructs, the pre-GP-C precursor (65); additional species that migrate more slowly than the G1-G2 precursor and with the pre-GP-C precursor are likely products of incomplete deglycosylation. cd-GPC contains a C-terminal S-peptide affinity tag and migrates slightly slower than the other G1-G2 precursors. Known GP-C species are labeled at left; minor unidentified bands are also present. The ^{14}C-labeled protein markers (Amersham Biosciences) are indicated (in kilodaltons). (B) Cell surface expression of GP-C in Vero cells was determined by flow cytometry using the G1-specific MAb BE08 (54). The cell population was subsequently stained using propidium iodide (1 μg/ml) to exclude dead cells. Cells were fixed using 2% formaldehyde and analyzed using a FACSCalibur flow cytometer (BD Biosciences). The histograms plot cell number (counts) versus the fluorescence intensity of MAb binding. Background staining of mock-transfected cells is shown to identify nonexpressing cells in the transfected cell populations.

appears to rescue wild-type assembly and proteolytic processing in the New World Junín virus CD4sp-GPC complex.

SSP rescues cell-cell fusion activity in *trans*. To determine whether the *trans*-complemented complex was also able to mediate pH-dependent membrane fusion, we cocultured cells expressing GP-C glycoproteins with Vero target cells infected with the fusion reporter vaccinia virus vCB21R-LacZ expressing the β-galactosidase gene under control of the T7 promoter (47). In this assay, activation of GP-C-mediated membrane fusion by acidic pH (5.0) results in syncytium formation between the effector and reporter cells and expression of β-galactosidase; the enzymatic activity is then monitored using a chemiluminescent substrate (64). As shown in Fig. 3, pH-dependent cell-cell fusion is readily detected using the native GP-C glycoprotein and absent in the cleavage-defective cd-GPC mutant. Cells expressing the CD4sp-GPC glycoprotein in the absence of SSP were unable to mediate cell-cell fusion

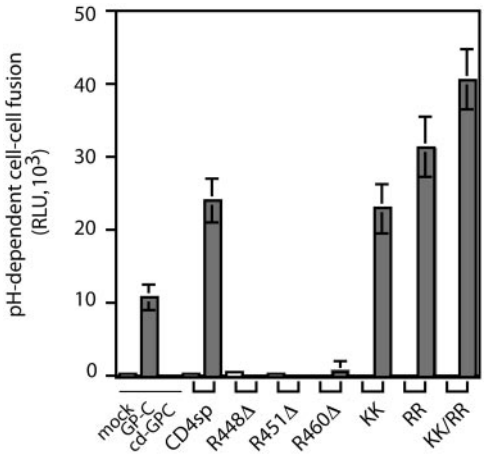

FIG. 3. pH-dependent cell-cell fusion activity. pH-dependent fusion was detected using the recombinant vaccinia virus-based β-galactosidase reporter assay (47) as previously described (64, 65). β-Galactosidase activity was quantitated using the chemiluminescent substrate GalactoLite Plus (Tropix). Relative light unit (RLU) measurements from cultures treated at pH 5.0 are shown after subtraction of background levels from neutral-pH cultures (average background, 1,500 RLU). Control conditions are shown in the underlined bars at left (mock, wild-type GP-C, and cd-GPC). Note that CD4sp-GPC constructs are bracketed in pairs (below the axis) representing the absence (open bars) and presence (gray bars) of SSP. Some bars are not discernible on the scale of the graph. All conclusions were replicated using X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) staining of parallel cocultures.

(Fig. 3, CD4sp, first of the bracketed pairs of bars). By contrast, coexpression of SSP reconstituted pH-dependent cell-cell fusion activity in the *trans*-complemented CD4sp-GPC complex (second of bracketed pairs of bars) to levels greater than those seen with the native GP-C glycoprotein. Thus, expression of SSP in *trans* can fully restore membrane fusion activity to the Junín virus G1-G2 precursor glycoprotein.

SSP association is required for exit from the ER. To investigate the role of SSP in the biogenesis of GP-C, we examined the intracellular localization of the complex by confocal microscopy. In these experiments, Vero cells expressing the wild-type and CD4sp-GPC glycoproteins were fixed, permeabilized, and immunochemically stained using the anti-G1 MAb BF11 (54) and an Alexa Fluor 488-conjugated secondary antibody. Nonpermeabilized cells were similarly stained to detect GP-C accumulation on the cell surface. As shown in Fig. 4, the native GP-C glycoprotein accumulated in the ER and Golgi-like perinuclear structures (GP-C, permeabilized) and on the cell surface (GP-C, surface). Localization to the Golgi apparatus was confirmed using a rabbit polyclonal antibody directed against an integral Golgi membrane protein, giantin (40), and a secondary Alexa Fluor 568-conjugated antibody. Colocalization of GP-C with the Golgi marker is visualized in yellow in the merged images. Expression of CD4sp-GPC in the presence of SSP resulted in a pattern of localization and transport to the cell surface similar to that of native GP-C (Fig. 4, CD4sp, +SSP). These findings highlight the reconstitution of the GP-C complex upon *trans* complementation with SSP.

In the absence of SSP, however, the G1-G2 precursor of CD4sp-GPC exhibited a diffuse reticulate pattern of intracellular expression consistent with retention in the ER (Fig. 4, CD4sp, −SSP). Notably absent was any concentration of GP-C staining to a morphologically defined Golgi apparatus or specific colocalization with the antigiantin MAb (merged image). The orange in the merged image likely reflects the spatial coincidence of green and red fluorescence rather than specific colocalization to a definable Golgi structure. Also absent was any staining of CD4sp-GPC on the cell surface (surface). The lack of transport to the cell surface is not due to the absence of proteolytic cleavage per se, because the cleavage-site-defective cd-GPC mutant is transported to the cell surface as the wild-type glycoprotein (not shown) (1, 35). Nor did we detect punctate staining in the ER that might suggest misfolding of the G1-G2 precursor in the absence of SSP. The difference in trafficking of the G1-G2 precursor to the Golgi in the presence or absence of SSP likely accounts for the effect of *trans* complementation on proteolytic cleavage (Fig. 2A), consistent with the activation of SKI-1/S1P protease in the *cis*-medial Golgi compartment (10, 21).

Next, we examined the role of SSP in the transport of the GP-C complex to the cell surface by using flow cytometry and the G1-specific MAb BE08. In cell cultures transiently expressing the wild-type GP-C glycoprotein, a clear population of GP-C-expressing cells was evident (Fig. 2B, top right). A comparison of cells expressing CD4sp-GPC in the presence or absence of SSP revealed that the GP-C glycoproteins were present on the cell surface only upon coexpression of SSP (bottom panels). Cell surface accumulation of the *trans*-complemented CD4sp-GPC glycoprotein was comparable to that of the native GP-C glycoprotein. Taken together, these results demonstrate that SSP is essential for GP-C transport to the Golgi and the cell surface. In the absence of SSP, the G1-G2 precursor is localized to the ER.

Transit of a CD4 chimera bearing G2 sequences. To further investigate the role of the G2 subunit in ER localization and the role of SSP in regulating transit to the cell surface, we determined whether control by SSP and the G2 subunit might be transferable to a heterologous cell surface protein. Because the ectodomain of human CD4 forms a soluble and secreted protein (11, 58), we fused the CD4 signal peptide and ectodomain to the transmembrane and cytoplasmic regions of G2. In the CD4ecto construct, the C terminus of soluble CD4 (TPV_{372}) (11) was spliced at the G2 ectodomain sequence TPL_{420}, three residues upstream of D_{424}, that nominally defines the junction with the transmembrane domain.

Cells expressing the CD4ecto chimera or native CD4 were metabolically labeled, and cell lysates were immunoprecipitated using the anti-CD4 ectodomain MAb SIM.2. The CD4ecto chimera was expressed as a 55-kDa glycoprotein that comigrated with native CD4 (Fig. 5A, left panel). Upon coexpression, SSP was found to coprecipitate with CD4ecto (Fig. 5A, left panel). This association was specific to G2 sequences in the CD4ecto glycoprotein; SSP did not bind to native CD4 (when coexpressed) (not shown). Thus, the transmembrane and cytoplasmic domains of G2 are sufficient for SSP binding.

Importantly, transport of the CD4ecto chimera through the Golgi apparatus and to the cell surface was dependent on coexpression of SSP. As shown by immunochemical staining using SIM.2 MAb and confocal microscopy (Fig. 4, CD4ecto, permeabilized), the chimeric glycoprotein was largely con-

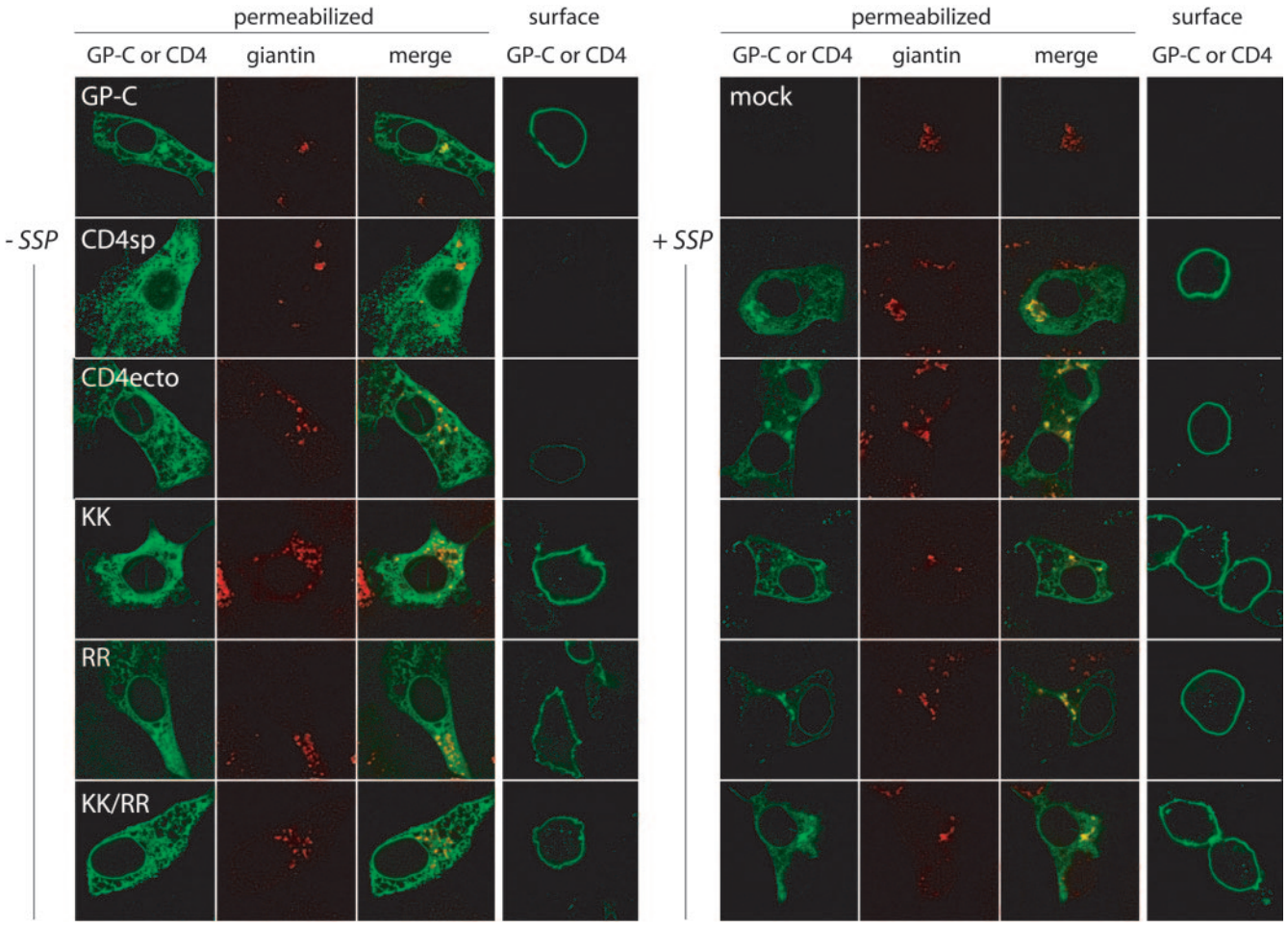

FIG. 4. Intracellular and cell surface visualization of glycoproteins. Confocal images were obtained as described in Materials and Methods. Permeabilized cells were stained in green using either the MAb BF11 (GP-C) or, for CD4ecto, SIM.2 (CD4). Golgi structures were identified using a rabbit polyclonal antiserum and stained in red. Merged images (merge) were created using Lasersharp software. Nonpermeabilized cells (surface) were stained in green using either MAb BF11 or SIM.2. The expressed glycoproteins are indicated in white letters superimposed on the leftmost images. The top row depicts cells expressing native GP-C or mock-transfected cells (all infected with the recombinant vaccinia virus vTF7-3). In subsequent rows, the glycoproteins were expressed either in the absence (−SSP) or presence (+SSP) of SSP. In some images, the Golgi apparatus is vesiculated and dispersed, perhaps due to infection of the cells by vaccinia virus.

strained to the ER in the absence of SSP and failed to colocalize with the Golgi apparatus (−SSP). In addition, only trace amounts of the CD4ecto glycoprotein were detected in the absence of SSP on the cell surface, either through confocal microscopy (Fig. 4, surface) or flow cytometry (Fig. 5B, −SSP). Thus, fusion to the G2 transmembrane and cytoplasmic domains prevented transport of the CD4 ectodomain from the ER.

By contrast, coexpression with SSP resulted in significant localization of CD4ecto in the Golgi (Fig. 4, +SSP) and expression on the cell surface (surface). Mobilization of the chimeric glycoprotein by SSP was confirmed by flow cytometry (Fig. 5B, +SSP). Furthermore, immunoprecipitation studies of CD4ecto expression on the cell surface (Fig. 5A, right panel) identified the surface moiety as the complex of CD4ecto and SSP. Together, these findings demonstrated that the essential elements of ER localization and its control by SSP binding can be recapitulated in a chimeric CD4ecto glycoprotein bearing the transmembrane and cytoplasmic domains of G2.

Analysis of C-terminal truncations in the G2 cytoplasmic domain. Among transmembrane proteins that are retained in the ER, specific localization signals are often encoded within the cytoplasmic domain (references 22, 37, and 62 and references therein). In order to define the determinants in G2 that are required for ER localization, we constructed a series of C-terminal truncations in the cytoplasmic domain of G2. Three arginine residues, spaced 4, 7, and 17 amino acids from the nominal transmembrane domain, were used as endpoints in the truncations (Fig. 1). These positively charged termini were chosen to facilitate anchoring of the truncated CD4sp-GPC glycoprotein in the membrane. The arginine codons were fused to those encoding an S-peptide affinity tag (34) to facilitate analysis of the G2 moiety (65). Metabolically labeled glycoprotein was isolated using the Spep affinity tag and S-protein agarose (Novagen). The truncated CD4sp-GPC glycoproteins (R448Δ, R451Δ, and R460Δ) were well expressed in Vero cells yet failed to coprecipitate significant amounts of SSP (Fig. 6A, top panel). Nonetheless, all three truncated glycoproteins were

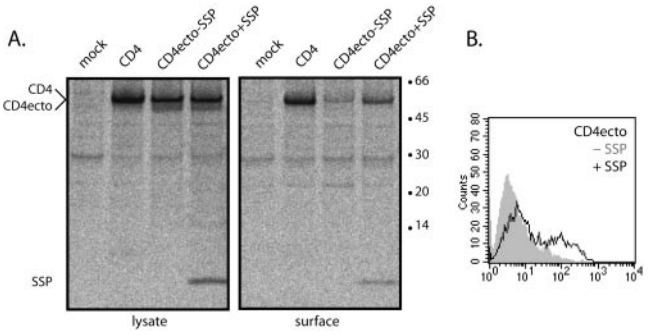

FIG. 5. The chimeric CD4 glycoprotein bearing the transmembrane and cytoplasmic domains of G2 requires SSP for transport to the cell surface. (A) The chimeric CD4ecto construct was expressed alone (−SSP) or with SSP (+SSP) and metabolically labeled. Intact cells were incubated with the anti-CD4 MAb SIM.2 (43, 48) and the cell surface glycoproteins were subsequently isolated from cleared cell lysates using protein A-Sepharose (surface). Intracellular CD4ecto glycoprotein was immunoprecipitated from the post-protein A-Sepharose supernatant using additional SIM.2 MAb (lysate). Mock- and human CD4-transfected cells served as controls. Molecular size markers (in kilodaltons) are as described in the legend to Fig. 2A. (B) Flow cytometry using SIM.2 MAb was otherwise performed as described in the legend to Fig. 2B, and results are plotted similarly. The filled gray (−SSP) and open (+SSP) histograms are overlaid.

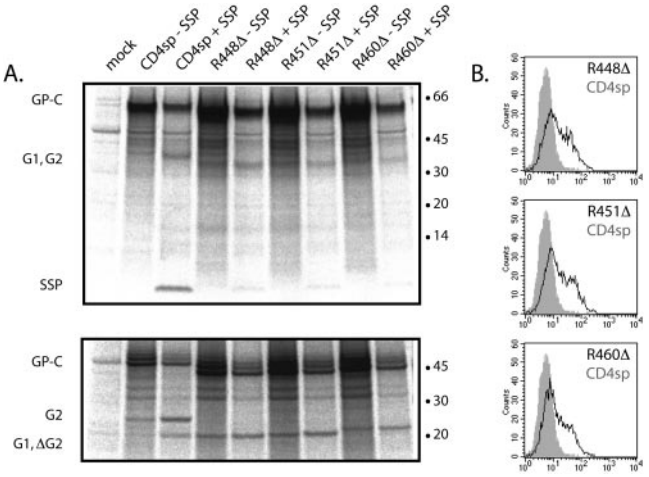

FIG. 6. Truncations to the cytoplasmic domain of G2 ablate SSP binding yet enable transport to the cell surface. (A) The wild-type and truncated CD4sp-GPC glycoproteins (R448Δ, R451Δ, and R460Δ) were expressed alone (−SSP) or with SSP (+SSP). Metabolically labeled glycoproteins were precipitated using the C-terminal Spep affinity tag and S-protein agarose (Novagen) and analyzed as described in the legend to Fig. 2. The G1 and G2 glycoproteins are best resolved following deglycosylation with PNGase F (bottom). Note that the truncated G2 moieties (ΔG2) migrate near the wild-type G1 polypeptide; coassociation between G1 and ΔG2 was formally demonstrated by immunoprecipitation using anti-G1 MAb BF11, which coprecipitated ΔG2 (not shown). Although coprecipitation of SSP was markedly reduced with the truncated glycoproteins, trace amounts could be discerned upon darkening of the image (not shown). This low level of SSP association is judged to be insignificant, as the properties of the truncated glycoproteins are independent of SSP coexpression. Molecular size markers (in kilodaltons) are as described in the legend to Fig. 2A. (B) Cell surface expression of the truncated glycoproteins was determined by flow cytometry as described in the legend to Fig. 2B, and results are plotted similarly. Note that expression of the wild-type CD4sp-GPC glycoprotein (gray histograms in all three panels) is compared with that of the truncations (open histograms), all in the absence of SSP.

subjected to SKI-1/S1P cleavage, in the presence or absence of SSP, to produce truncated and affinity-tagged G2 moieties (Fig. 6A, bottom panel). The relative migrations of the truncated G2 polypeptides correspond to their expected molecular weights but cause them to overlap with the intact G1 polypeptide. The association between G1 and the truncated G2 subunits was separately confirmed by coimmunoprecipitation using a MAb directed to G1 (not shown). By contrast, similar truncations in G2 of the Old World LCM virus were reported to prevent SKI-1/S1P cleavage (35).

Flow cytometry was used to determine whether the truncated Junín virus glycoproteins were also transported to the cell surface without SSP. As shown in Fig. 6B, all three truncation mutants were expressed on the cell surface in the absence of SSP, at levels comparable to the *trans*-complemented CD4sp-GPC glycoprotein (Fig. 2B). Truncations in the context of CD4ecto likewise enabled transport from the ER (not shown). In the LCM virus (35), the truncated GP-C was also expressed on the cell surface. Taken together, these results suggest that amino acid sequences within the cytoplasmic domain of G2 are important in constraining the G1-G2 precursor to the ER. The cytoplasmic region is also important for SSP association.

We have demonstrated that GP-C glycoproteins bearing truncations in the cytoplasmic domain of G2 can be proteolytically processed and transported to the cell surface in the absence of SSP. Surprisingly, however, none of the truncated complexes was able to mediate pH-dependent cell-cell fusion (Fig. 3). It is possible that this failure may be due to insufficient cleavage or transport of the truncated glycoproteins. Alternatively, the failure may reflect a requirement for either SSP or the cytoplasmic domain of G2 for membrane fusion activity.

Dibasic amino acid sequences participate in ER localization. Sequence analysis of the G2 cytoplasmic domain revealed conserved motifs that may be involved in protein trafficking and ER localization. In particular, dibasic amino acid sequences such as the canonical KKXX and RXR motifs are widely utilized in the retrieval of transmembrane proteins to the ER (see references 22, 37, and 62 and references therein). The cytoplasmic domain of Junín virus G2 contains two related dibasic sequences: $KKPT_{479}$ and a C-terminal $RRGH_{485}$. Variants of these sequences appear in other arenavirus G2 proteins (Fig. 1). To assess the potential role of these sequences in ER localization, we mutated the two basic amino acids at each site to alanines, both individually (KK and RR glycoproteins) and as the double mutant (KK/RR).

Immunoprecipitation studies of metabolically labeled whole-cell lysates revealed that all of the mutant CD4sp-GPC glycoproteins were able to associate with SSP (Fig. 7A, top panel). Neither of the dibasic sequences was essential for SSP binding. *trans* complementation with SSP enabled wild-type levels of cell surface expression (Fig. 7B, +SSP) and efficient pH-dependent cell-cell fusion (Fig. 3), arguing against significant adverse effects of the mutations on overall protein folding.

In the absence of SSP, importantly, both the single and double mutants were now capable of transport to the cell

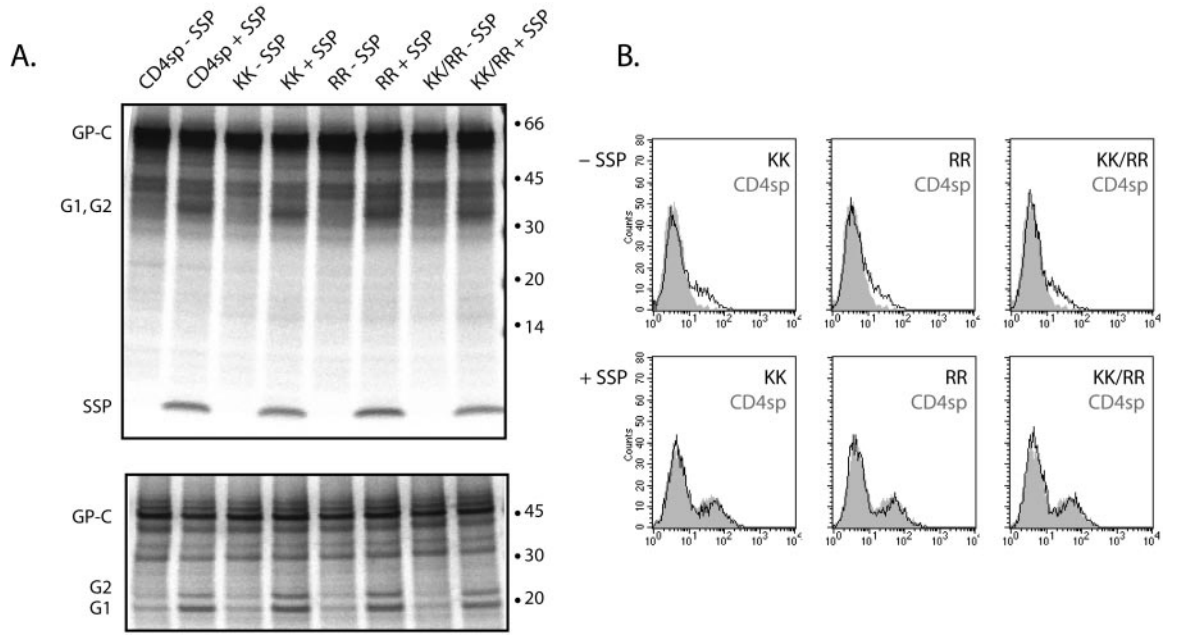

FIG. 7. Alanine mutations to dibasic amino acid motifs enable transport from the ER. (A) CD4sp-GPC constructs containing KK, RR, and double KK/RR mutations were expressed alone (−SSP) or with SSP (+SSP). Metabolically labeled glycoproteins were immunoprecipitated and analyzed as described in the legend to Fig. 2A. Molecular size markers (in kilodaltons) are as described in the legend to Fig. 2A. (B) Flow cytometry was performed as described in the legend to Fig. 2B, and results are plotted similarly. Note that the top panels compare expression of the CD4sp-GPC glycoprotein (gray histograms in all panels) with that of the mutants (open histograms) in the absence of SSP. Expression with SSP is shown in the bottom panels.

surface. This phenotype was evident upon confocal microscopic analysis of nonpermeabilized cells (Fig. 4, surface), although specific localization in the Golgi was difficult to discern (green and merged images). Flow-cytometric studies of cell surface expression indicated that both the single and double mutations provided modest, albeit significant, relief of ER retention (Fig. 7B, −SSP). Evidence for enhanced SKI-1/S1P cleavage of the mutant glycoproteins was, however, difficult to discern in whole-cell lysates, above the residual level of cleavage in the wild-type glycoprotein (Fig. 7A, bottom panel). It is possible that the cleaved species in the wild-type G1-G2 glycoprotein reflect transient residence in the Golgi, prior to retrieval to the ER. In the Old World Lassa fever virus glycoprotein, where cleaved products are not observed in the absence of SSP (18), retrieval of the G1-G2 precursor may be more rapid. Nonetheless, mobilization of the mutant glycoproteins to the cell surface was consistently observed and distinct from the strict intracellular retention seen with the wild-type glycoprotein. Both KK and RR mutations appeared to be comparably efficacious, and no synergy was observed in the double KK/RR mutant. However, none of the mutant glycoproteins was able to mediate cell-cell fusion in the absence of SSP (Fig. 3). This defect is not attributable to the amino acid substitutions per se, as wild-type levels of fusion were restored upon *trans* complementation with SSP.

To confirm that the mutations are sufficient for significant mobilization of the G1-G2 precursor in the absence of SSP, we examined the glycoprotein by immunoprecipitation from the cell surface (Fig. 8). These experiments confirmed significant expression of the dibasic sequence mutants on the cell surface and demonstrated a preponderance of the proteolytically processed G1-G2 complex, reflecting access to the SKI-1/S1P protease in the Golgi. The efficiency of cleavage in the mutant glycoproteins was relatively unaffected by the presence or absence of SSP (60% cleaved versus 40% cleaved, respectively).

Taken together, these studies identify the two dibasic amino

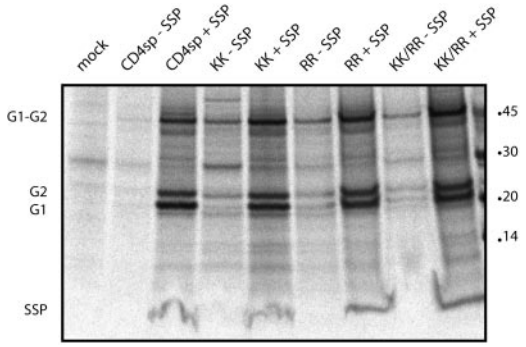

FIG. 8. Cell surface expression of dibasic amino acid motif CD4sp-GPC mutants. Intact cells expressing the constructs shown in Fig. 7 were incubated with the G1-specific MAb BE08, and the cell surface GP-C glycoproteins were isolated from cleared cell lysates using protein A-Sepharose and deglycosylated. The relative amounts of G1, G2, and G1-G2 precursor in each lane were quantitated from the phosphorimage using Image Gauge software (Fuji), and the efficiency of cleavage was determined as the sum of G1 plus G2 relative to total of all forms. Distortion of the SSP band is due to the detergents used in PNGase F treatment. Molecular size markers (in kilodaltons) are as described in the legend to Fig. 2A.

acid sequences ($KKPT_{479}$ and $RRGH_{485}$) as important determinants of ER localization in the absence of SSP. Alanine mutations at either or both of these sites result in partial relief from ER retention and enable transport to the cell surface in the absence of SSP. On the other hand, these mutations do not completely obviate the requirement for SSP association in transport of the G1-G2 precursor (Fig. 8). Quantitative analysis of the glycoproteins indicated that, whereas the mutations were able to increase cell surface expression at least 10-fold, coexpression of SSP resulted in an additional 10-fold increase in all mutants, to the levels of the wild-type glycoprotein. These findings are consistent with our results from confocal microscopy and flow cytometry studies (Fig. 4 and 7). Thus, constraints on the trafficking of the G1-G2 precursor include the dibasic sequence motifs in the cytoplasmic domain of G2 but also involve additional structural elements provided upon full assembly with SSP.

DISCUSSION

The regulation of trafficking through intracellular membranous compartments is central to the biogenesis of membrane glycoproteins (15, 22). Quality control mechanisms for protein folding and assembly are proposed to operate through checkpoints on exit from the ER and through bidirectional transport to and from the Golgi apparatus. Viruses make use of these cellular pathways in the biosynthesis, assembly, and release of new virion particles (15). In our studies, we have characterized the biogenesis of the arenavirus envelope glycoprotein and the requirement for tripartite assembly to enable transport of the GP-C complex from the ER. Without the association of SSP, the wild-type G1-G2 precursor remains localized to the ER. We show that localization is mediated by the cytoplasmic domain of G2 and that the control of trafficking by SSP association is transferable to a chimeric CD4 molecule bearing the G2 transmembrane and cytoplasmic domains. Conversely, regulation of intracellular transport of the GP-C complex does not require G1 or the ectodomain of G2.

Our studies demonstrate that ER localization is mediated in part through dibasic amino acid sequences in the cytoplasmic domain of G2. Alanine mutations to either of two dibasic motifs provide partial relief from ER localization and enable expression of the proteolytically cleaved G1-G2 complex on the cell surface. Upon exit from the ER and transit through the Golgi, the mutant G1-G2 precursor is now fully susceptible to proteolytic maturation by SKI-1/S1P protease. Thus, absent ER localization signals, the arenavirus GP-C precursor can undergo proteolytic maturation much as do the precursor glycoproteins of other class I viral fusion proteins.

Dibasic amino acid sequences are known to mediate ER localization through retrograde transport (retrieval) from the Golgi (references 22, 37, and 62 and references therein). The specific dibasic sequences we have identified as important for ER localization in the Junín virus G2 glycoprotein do not match precisely either of the canonical ER retrieval motifs: the C-terminal KKXX or internal RXR sequences. Although the internal KK sequence studied here is conserved among the New World arenaviruses, the C-terminal RRXX sequence shows considerable variation (Fig. 1). Among the Old World viruses, only the C-terminal motif is identifiable. However, variants to the canonical motifs are also common in other ER-localized transmembrane proteins (46, 55, 62) and the efficiency of retention by these sequences is often highly context dependent (26, 57, 66). Many details regarding the mechanisms and molecular determinants involved in ER-Golgi trafficking remain unresolved.

It is noteworthy that a viral envelope glycoprotein destined for the cell surface should encode an ER localization signal. For cellular transmembrane proteins that traverse the Golgi and beyond, dibasic ER localization motifs are commonly found to control the assembly and trafficking of heteromultimeric membrane protein complexes (12, 33, 39, 42, 67; reviewed in references 22 and 46). These endogenous signals prevent transport of the individual subunits and are overcome upon assembly of the multimeric complex. This quality control mechanism ensures that only the fully and properly assembled complex is transported from the ER. In the biogenesis of the Junín virus GP-C complex, we propose an analogous role for SSP association—namely, to mask endogenous ER localization signals in the cytoplasmic domain of G2 and thus enable transport of only the fully assembled tripartite complex.

This strategy for assembly-dependent control of viral envelope glycoprotein trafficking is likely not unique to the arenaviruses. The bunyavirus G_C glycoprotein also contains a noncanonical basic amino acid cluster that may be involved in ER localization (29). In these viruses, transport of G_C from the ER requires association with a second envelope glycoprotein, G_N (30, 36), which in turn retains the G_C-G_N complex in the Golgi (27, 29, 56), the site of virus budding. Together, these observations highlight the use of cellular ER-Golgi trafficking mechanisms during the viral life cycle to control the assembly and transport of multimeric envelope glycoprotein complexes.

Despite mutations that enable the transport of the G1-G2 complex in the absence of SSP, wild-type levels of trafficking were not restored by point mutations to the dibasic amino acid sequences or by truncations in the cytoplasmic domain (not shown). It is possible that additional constraints on GP-C transport lie within the transmembrane domain of G2. Moreover, it is likely that the association with SSP remains essential for the integrity of the GP-C complex. The SSP subunit has uniquely evolved within the arenaviruses for purposes other than simply to relieve ER retention of an envelope glycoprotein precursor. It is telling, then, that despite the accumulation of cleaved G1-G2 complex on the cell surface, none of the glycoproteins lacking SSP is able to mediate membrane fusion (Fig. 3). Notably, GP-C glycoproteins bearing mutations at the dibasic amino acid motifs are unable to promote fusion in the absence of SSP yet are restored to full activity by coexpression of SSP. This defect in fusion is likely not due to the lower levels of cell surface glycoprotein in the absence of SSP, as robust fusion is observed with comparably low levels of cleaved wild-type glycoprotein (see Fig. 6 of reference 64). Rather, we suggest that SSP may be directly involved in modulating pH-dependent membrane fusion by the GP-C complex.

In addition, the G1-G2 complex lacking SSP is not myristoylated. GP-C complexes in which myristoylation is blocked by a G2A mutation are less able to mediate cell-cell fusion than the wild-type glycoprotein (65), perhaps due to alterations in trafficking to specific membrane microdomains (52, 61). The G2A glycoprotein, however, retains 30% of the wild-type

fusion activity, significantly more than the present G1-G2 complexes in the absence of SSP. This comparison suggests defects beyond the lack of acylation in G1-G2 complexes lacking SSP. Separately, myristoylation may also be important during virion assembly in facilitating the colocalization of GP-C with the myristoylated Z matrix protein (50).

Further studies will no doubt delineate the additional roles of the unique SSP subunit in the arenavirus life cycle. Unique solutions embodied in the assembly, trafficking, and membrane fusion activity of the arenavirus GP-C complex may suggest novel approaches for intervention towards the prevention and treatment of arenavirus hemorrhagic fevers.

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Synergistic Attenuation of Vesicular Stomatitis Virus by Combination of Specific G Gene Truncations and N Gene Translocations▽

David K. Clarke,* Farooq Nasar, Margaret Lee, J. Erik Johnson, Kevin Wright, Priscilla Calderon, Min Guo, Robert Natuk, David Cooper, R. Michael Hendry, and Stephen A. Udem†

Wyeth Vaccines Discovery Research, 401 N. Middletown Road, Pearl River, New York 10965

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A variety of rational approaches to attenuate growth and virulence of vesicular stomatitis virus (VSV) have been described previously. These include gene shuffling, truncation of the cytoplasmic tail of the G protein, and generation of noncytopathic M gene mutants. When separately introduced into recombinant VSV (rVSV), these mutations gave rise to viruses distinguished from their "wild-type" progenitor by diminished reproductive capacity in cell culture and/or reduced cytopathology and decreased pathogenicity in vivo. However, histopathology data from an exploratory nonhuman primate neurovirulence study indicated that some of these attenuated viruses could still cause significant levels of neurological injury. In this study, additional attenuated rVSV variants were generated by combination of the above-named three distinct classes of mutation. The resulting combination mutants were characterized by plaque size and growth kinetics in cell culture, and virulence was assessed by determination of the intracranial (IC) 50% lethal dose (LD_{50}) in mice. Compared to virus having only one type of attenuating mutation, all of the mutation combinations examined gave rise to virus with smaller plaque phenotypes, delayed growth kinetics, and 10- to 500-fold-lower peak titers in cell culture. A similar pattern of attenuation was also observed following IC inoculation of mice, where differences in LD_{50} of many orders of magnitude between viruses containing one and two types of attenuating mutation were sometimes seen. The results show synergistic rather than cumulative increases in attenuation and demonstrate a new approach to the attenuation of VSV and possibly other viruses.

Vesicular stomatitis virus (VSV) is a member of the *Vesiculovirus* genus of the family *Rhabdoviridae*. The negative-sense virus genome is 11,162 nucleotides long and contains five genes in the order 3′ N-P-M-G-L 5′, encoding the five major viral proteins (1, 3). The bullet-shaped VSV particle (160 nm by 80 nm) contains a ribonucleoprotein core (nucleocapsid) composed of genomic RNA closely associated with N protein and a RNA polymerase composed of a complex of L and P proteins enveloped in a host cell-derived plasma membrane (4, 18, 19, 44, 53, 56). Following uptake of the virus particle by susceptible cells, nucleocapsid and viral RNA polymerase are released into the cytoplasm and viral mRNA transcription ensues. A 3′-5′ gradient of viral mRNA transcription leads to abundant N protein expression and successively decreasing levels of P, M, G, and L proteins (1, 3, 15, 19, 27, 57). This gene expression gradient provides virus proteins in a suitable ratio for subsequent viral genome replication and assembly of mature virus particles. Virus replication in cell culture is rapid, and virus progeny are detectable 5 to 6 h postinfection.

Since the initial recovery of infectious recombinant VSV (rVSV) from genomic cDNA (39, 61), effort has been directed towards the development of rVSV as a vaccine vector targeting a variety of different human pathogens, including human immunodeficiency virus type 1 (HIV-1) (25, 31–34, 48–51). The major advantages of rVSV vaccine vectors and their immunogenicity and protective efficacy in animal models have been described in detail previously (12). However, VSV is both neurotropic and neurovirulent in mice (54, 58, 60) and can cause neurological disease when injected directly into the brain of cows and horses (24). The original rVSV Indiana serotype vector ($rVSV_{IN}$) developed by J. Rose and colleagues was less pathogenic following intranasal inoculation in mice than the cell culture-adapted virus from which it was derived, but the neurovirulence (NV) potential of this vector following direct intracranial (IC) inoculation was not known. To address this question, an exploratory nonhuman primate (NHP) NV study based on the methodology used for NV testing of mumps vaccine seed lots was carried out. In that pilot study, wild-type (wt) VSV_{IN} and $rVSV_{IN}$ caused clinical signs of severe neurological disease following intrathalamic inoculation of animals; two additional rVSV vectors expressing the HIV-1 Gag protein did not cause any clinical signs of disease, but histological examination of the central nervous system (CNS) in these animals revealed evidence of necrotic and inflammatory lesions (30). These findings indicated that $rVSV_{IN}$ vectors would require further attenuation before being considered suitable for clinical evaluation.

When it became possible to recover infectious VSV from genomic cDNA (39, 61), directed approaches to study rVSV attenuation were adopted. One attenuation strategy known as gene shuffling involves rearranging the natural gene order of VSV, which alters normal levels of gene expression (2, 60). Viruses modified by gene rearrangement often grow poorly in vitro and are typically less virulent in vivo (21–23, 42, 60).

A different attenuation strategy involves truncation of the

* Corresponding author. Mailing address: Department of Vaccines Discovery Research, Wyeth, 401 N. Middletown Road, Bldg. 180/267, Pearl River, NY 10965. Phone: (845) 602-3465. Fax: (845) 602-4941. E-mail: clarked3@wyeth.com.

† Present address: IAVI, 110 William Street, Floor 27, New York, NY 10038-3901.

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29-amino-acid cytoplasmic tail (CT) region of the virus G protein (50, 55). Viruses with shortened CTs have slower growth rates, reach lower peak titers in vitro, and are less pathogenic in mice than unaltered viruses (49). Because N gene shuffles and G protein CT truncations involve gene translocations and deletion of part of the G gene, respectively, mutants generated by these strategies have a stable attenuation phenotype-genotype (23, 55).

A third attenuation strategy relies on nucleotide substitutions within the M gene that ablate expression of two in-frame overlapping polypeptides initiated downstream from the M protein translation start codon (29). Viruses that do not express these polypeptides demonstrate reduced cytopathology in a variety of cell lines and are highly attenuated in mice. Consequently, mutants that do not express these polypeptides have been called noncytopathic M mutants (M_{NCP}).

In this study, we sought to explore and define strategies that would allow step-wise increases in $rVSV_{IN}$ vector attenuation to levels beyond those previously described, thereby increasing the range of attenuated vectors from which to generate an ideal $rVSV_{IN}$–HIV-1 vaccine vector for future clinical evaluation. To achieve this we combined G protein CT truncations with either N gene shuffles or M_{NCP} gene mutations. Growth characteristics of the resulting $rVSV_{IN}$ combination mutants were studied in vitro, and their neurovirulence was assessed in a mouse IC 50% lethal dose (LD_{50}) model to determine degree and relative order of vector attenuation.

MATERIALS AND METHODS

Cells and virus. Vero and baby hamster kidney (BHK) cell lines were obtained from the American Type Culture Collection and propagated under conditions of 37°C and 5% CO_2 in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, sodium pyruvate (20 mM), and gentamicin (50 μg/ml). The tissue culture-adapted San Juan strain of the VSV Indiana serotype (VSV_{IN}), a recombinant form of VSV_{IN} ($rVSV_{IN}$) (39), $rVSV_{IN}$ expressing HIV-1 Gag protein ($rVSV_{IN}$ gag5), and two attenuated forms of $rVSV_{IN}$ gag5 ($rVSV_{IN}$CT1 gag5 and $rVSV_{IN}$CT9 gag5) were kindly provided by J. Rose (Yale University, New Haven, CT). A modified form of vaccinia virus Ankara (MVA) that expressed bacteriophage T7 RNA polymerase (MVA-T7) (62) was obtained from Bernard Moss (National Institutes of Health, Bethesda, Maryland) and further modified to express T7 RNA polymerase under the control of an early transcription promoter (38).

Virus propagation, purification, and titration. Virus was routinely amplified on BHK cell monolayers and titrated on Vero cell monolayers. For virus amplification BHK cells were infected at a multiplicity of infection (MOI) of 0.001 to 0.05 PFU/cell. Virus inoculum was adsorbed for 15 min at room temperature (RT) followed by 30 min at 37°C. Additional growth medium was then added, and cells were incubated at 37°C until they became rounded and detached from the flask. Infected cell supernatant was clarified by centrifugation for 10 min at 3,000 × *g*. The virus suspension was then flash frozen in an ethanol-dry ice bath and stored at −80°C prior to titration. Where necessary, virus was further purified from infected cell supernatant by centrifugation through 10% (wt/vol) sucrose in 1× phosphate-buffered saline (PBS). Briefly, ~20 ml of clarified cell supernatant was underlaid with 12 to 15 ml of 10% (wt/vol) sucrose in a Beckmann Ultraclear tube followed by centrifugation at 28,000 rpm in a Beckmann SW-28 rotor for 1.5 h at 4°C. Following centrifugation, supernatant was aspirated, the virus pellet was resuspended in PBS, and the virus suspension was flash frozen and stored at −80°C prior to plaque assay.

For virus titration by plaque assay, freshly confluent Vero cell monolayers in six-well plates were infected with 0.1-ml aliquots from serial 10-fold dilutions of rVSV in growth medium. An additional 0.4 ml of medium was added to each well to prevent cell desiccation, and virus was adsorbed for 15 min at RT followed by 30 min at 37°C. The virus inoculum was then removed, and cell monolayers were overlaid with 3 ml of 0.8% (wt/vol) agarose (SeaPlaque; Cambrex Bio Science Rockland, Inc., Rockland, ME) in growth medium. After 10 min at RT to allow the agarose to solidify, cells were incubated at 37°C in 5% CO_2 for 1 to 4 days for plaque development. The agarose overlay was then removed, monolayers were rinsed once with 2 ml of PBS, and cells were stained and fixed in 0.5 ml of 70% methanol containing 2% crystal violet for 5 min at RT. Plaques were counted after removal of excess stain under running water.

Generation of attenuated $rVSV_{IN}$ genome cDNAs. The generation of both CT1 and CT9 mutants has been previously described in detail (49, 55). The corresponding rVSV genomic cDNAs were generously provided by J. Rose (Yale University, New Haven, CT) and were used in the derivation of the combination mutants described below.

A method for gene translocation within $rVSV_{IN}$ genomic cDNA has been described in detail previously (2, 60). A different method of N gene translocation was used in this study. Briefly, the N gene was first deleted from $rVSV_{IN}$ genomic cDNA by replacing the natural BsaAI-XbaI genome fragment (Fig. 1) with a DNA fragment that was generated by in vitro ligation of two PCR products, one stretching from the BsaAI site in the plasmid vector to the exact 3′ end of the virus leader sequence (positive sense) and the other spanning the region from the transcription start signal of the P gene to the downstream XbaI site. Precise ligation of DNA containing the virus leader sequence, with DNA containing the exact 3′ end of the P gene, was achieved by addition of BsmBI sites to PCR primers. The N gene was then reinserted into the ΔN genome cDNA between the P and M genes (N2), between the M and G genes (N3), and between the G and L genes (N4) by use of a similar approach. For generation of the N2 genome cDNA, a PCR product spanning the entire N gene and 3′ CT intergenic dinucleotide was ligated to flanking PCR fragments in vitro; one DNA fragment stretched from the unique XbaI site to the 3′ end of the P gene and contained the P/M intergenic dinucleotide GT. A second DNA fragment spanned the entire M gene to the unique MluI site in the G gene. Addition of BsmBI sites to the 3′ and 5′ ends of the P and M gene fragments, respectively, and to 3′ and 5′ ends of the N gene fragment allowed all three DNA fragments to be ligated in vitro and then cloned into the XbaI and MluI sites of the ΔN genome cDNA. The N3 cDNA genome was constructed in a similar fashion. A PCR fragment spanning the region from the unique XbaI site in the P gene to the end of the M gene, including the 3′ CT intergenic dinucleotide, was ligated to a PCR fragment spanning the entire N gene, a 3′ CT intergenic dinucleotide, and the first 32 nucleotides of the G gene containing the unique MluI site. Both DNAs were ligated through BsmBI sites at the 3′ end of the P/M fragment and the 5′ end of the N gene fragment. This DNA fragment was then cloned into the unique XbaI and MluI sites of the ΔN cDNA genome. For generation of the N4 genome cDNA, a PCR product spanning the entire N gene was joined with flanking PCR products, one stretching from the unique MluI site to the end of the G gene, including the 3′ CT intergenic dinucleotide, and the other containing the G/L intergenic dinucleotide CT and the region from the 5′ end of the L gene to the unique HpaI site. All three fragments were joined by the addition of BsmBI sites to the 3′ and 5′ ends of the G and L gene fragments, respectively, and to the 3′ and 5′ ends of the N gene DNA fragment. The resulting contiguous DNA fragment was then cloned into the MluI and HpaI sites of the ΔN cDNA genome.

A plasmid cDNA containing the M_{NCP} gene in the $rVSV_{IN}$ backbone was generously provided by Michael Whitt (University of Tennessee, Nashville) (29). The M_{NCP} gag5 and M_{NCP}CT1 gag5 vectors were generated by cloning a DNA fragment that spanned the mutant M_{NCP} gene and part of the P gene into the unique XbaI-MluI sites of $rVSV_{IN}$ cDNAs (generously provided by J. Rose, Yale University, New Haven, CT) containing either the HIV-1 Gag gene inserted between the G and L genes ($rVSV_{IN}$ gag5) or the HIV-1 Gag gene inserted between a truncated (CT1) form of the G gene and the L gene ($rVSV_{IN}$CT1 gag5).

Four N gene shuffle-CT combination mutants (N2CT9, N2CT1, N3CT9, and N3CT1) were generated by swapping the G genes from the N2 and N3 cDNAs with the CT1 and CT9 truncated forms of the G gene via unique flanking MluI and HpaI sites.

Recovery of $rVSV_{IN}$ from cDNA. Infectious virus was recovered from genomic cDNA following transfection of BHK cells with a mixture of plasmids expressing VSV N, P, and L proteins and full-length positive-sense genomic RNA, all under the control of the bacteriophage T7 RNA polymerase transcription promoter (39). For transfection, 95% to 100% confluent BHK cell monolayers in six-well dishes were incubated for 4 h in 3% CO_2 at 32°C in 4.5 ml/well of fresh growth medium. Meanwhile, a plasmid DNA-$CaPO_4$ precipitate was prepared for each cell monolayer by mixing 2 to 4 μg of plasmid containing full-length genomic cDNA, 1.0 μg of N plasmid, 0.5 μg of P plasmid, 0.15 μg of L plasmid, 25 μl of $CaCl_2$ (2.5 M), and water to achieve a 250-μl final volume. The DNA-$CaPO_4$ precipitate was then formed by dropwise addition of 250 μl of 2× BBS (280 mM NaCl, 50 mM BES, 1.5 mM Na_2HPO_4, pH 6.95 to 6.98) with gentle vortexing. The mixture was incubated at RT for 20 min to allow precipitate formation and then added dropwise to cells with gentle swirling. To provide a source of T7 RNA

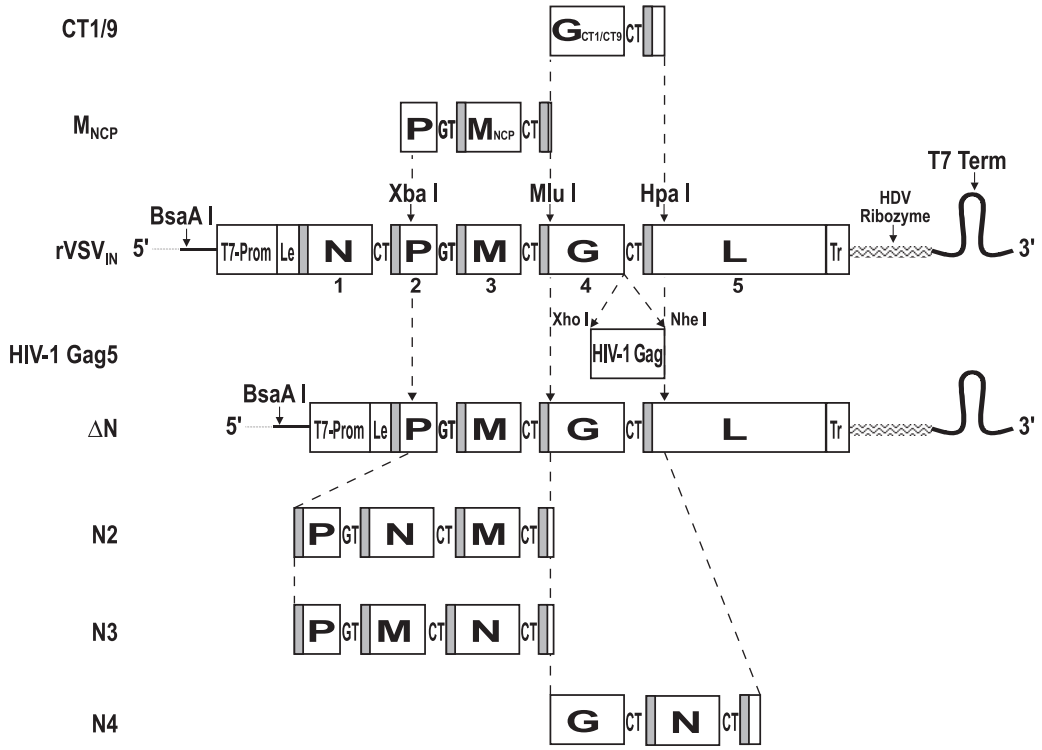

FIG. 1. Construction of $rVSV_{IN}$ mutant cDNA. The BsaAI, XbaI, MluI, and HpaI endonuclease sites used for construction of N gene shuffles and insertion of G genes containing CT truncations and M_{NCP} mutations are indicated with arrows. Virus leader (Le), trailer (Tr), GT, and CT intergenic dinucleotides and transcriptional start signals (shaded boxes) at the beginning of each gene are shown. Synthesis of positive-sense genomic RNA was under control of the T7 RNA polymerase transcription promoter (T7-Prom) and was terminated by a T7 transcription terminator (T7 Term). Hepatitis delta virus ribozyme (HDV Ribozyme) was used to generate the precise viral 3′ end on the positive-sense genomic RNA transcript.

polymerase, MVA-T7-GK16 (38) was then added to each well at an MOI of 3 to 4 PFU/cell along with 20 μg/ml cytosine arabinoside to inhibit amplification of MVA-T7. Cells were then incubated at 32°C in 3% CO_2 for 3 h followed by a 2-h heat shock at 43°C in 3% CO_2 (43). Following heat shock, cells were incubated at 32°C in 3% CO_2 for 18 to 24 h. Transfection medium was then replaced with 2 ml of fresh growth medium containing cytosine arabinoside, and cells were further incubated at 37°C in 5% CO_2 for 48 to 72 h. Transfected cells were then scraped into suspension, gently pipetted repeatedly to reduce cell clumping, and transferred to 95% to 100% confluent Vero cell monolayers in six-well dishes. The following day, cocultures were supplemented with 1 ml of fresh growth medium and incubation was continued for a further 3 to 5 days, during which time VSV cytopathic effect (CPE) became apparent. Rescued virus was then triple plaque purified and further amplified prior to in vitro and in vivo analysis.

In vitro growth studies. For comparison of $rVSV_{IN}$ mutant plaque sizes, plaque assays were performed in duplicate on replicate Vero cell monolayers as described above. For growth kinetics studies, replicate Vero cell monolayers in 25 cm^2 flasks were infected in duplicate at an MOI of 5 PFU/cell. Virus was adsorbed in 0.5 ml of growth medium for 15 min at RT followed by 30 min at 37°C with occasional rocking to prevent cell desiccation. After removal of the inoculum, monolayers were rinsed three times with 5 ml of PBS to remove unbound virus; 5 ml of growth medium was then added to each monolayer, and a 0.5-ml aliquot was immediately removed as a "time h 0" (T_0) sample and replaced with 0.5 ml of fresh medium. Incubation was continued at 37°C in 5% CO_2 for 48 to 72 h, and further samples were taken at T_3 to T_{48}. All samples were flash frozen in ethanol-dry ice and stored at −80°C for titration.

Mouse IC LD_{50} studies. Five-week-old female Swiss Webster mice (Taconic Laboratory Animals and Services, Germantown, NY) were anesthetized and injected IC with $\log_{10}$-fold dilutions of virus in 30 μl PBS (10 mice per dilution, with dilutions adjusted to range around the anticipated LD_{50}). Weight and health status were recorded daily for 2 weeks. Mice becoming either bilaterally paralyzed or showing significant signs of distress or severe illness were sacrificed and recorded as succumbing to VSV disease. The LD_{50} and the 50% paralyzing dose (PD_{50}) were determined by the method of Reed and Muench (45) based on the number of mice that became paralyzed. All animal care and procedures conformed to Institutional Animal Care and Use Committee guidelines. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

RESULTS

Recovery of attenuated $rVSV_{IN}$ vectors from genomic cDNA. The complete spectra of $rVSV_{IN}$ vectors recovered from genomic cDNA and subsequently used for in vitro and in vivo attenuation studies are shown in Fig. 2A. Attenuated $rVSV_{IN}$ mutants were generated using three different attenuation strategies and combinations thereof. In one strategy the N gene was translocated (shuffled) to the second, third, and fourth gene positions (N2, N3, and N4, respectively) in the $rVSV_{IN}$ genome. In another strategy, the G protein CT was truncated to either nine (CT9) amino acids or one (CT1) amino acid. A third attenuation strategy abolished expression of two overlapping polypeptides encoded within the M gene open reading frame, generating the M_{NCP} gag5 mutant containing the HIV-1 gag gene at position 5 in the genome. Both the CT9 and N4 mutants contain an additional "empty" transcriptional unit (TU) at the fifth position in the genome. This TU contains an XhoI-NheI cassette flanked by transcription start and stop signals to facilitate insertion and expression of foreign genes (50, 51). Because results from previous murine NV studies (60) and an exploratory NHP NV study (30) indicated that N gene shuffles and G protein CT truncations on their own might not

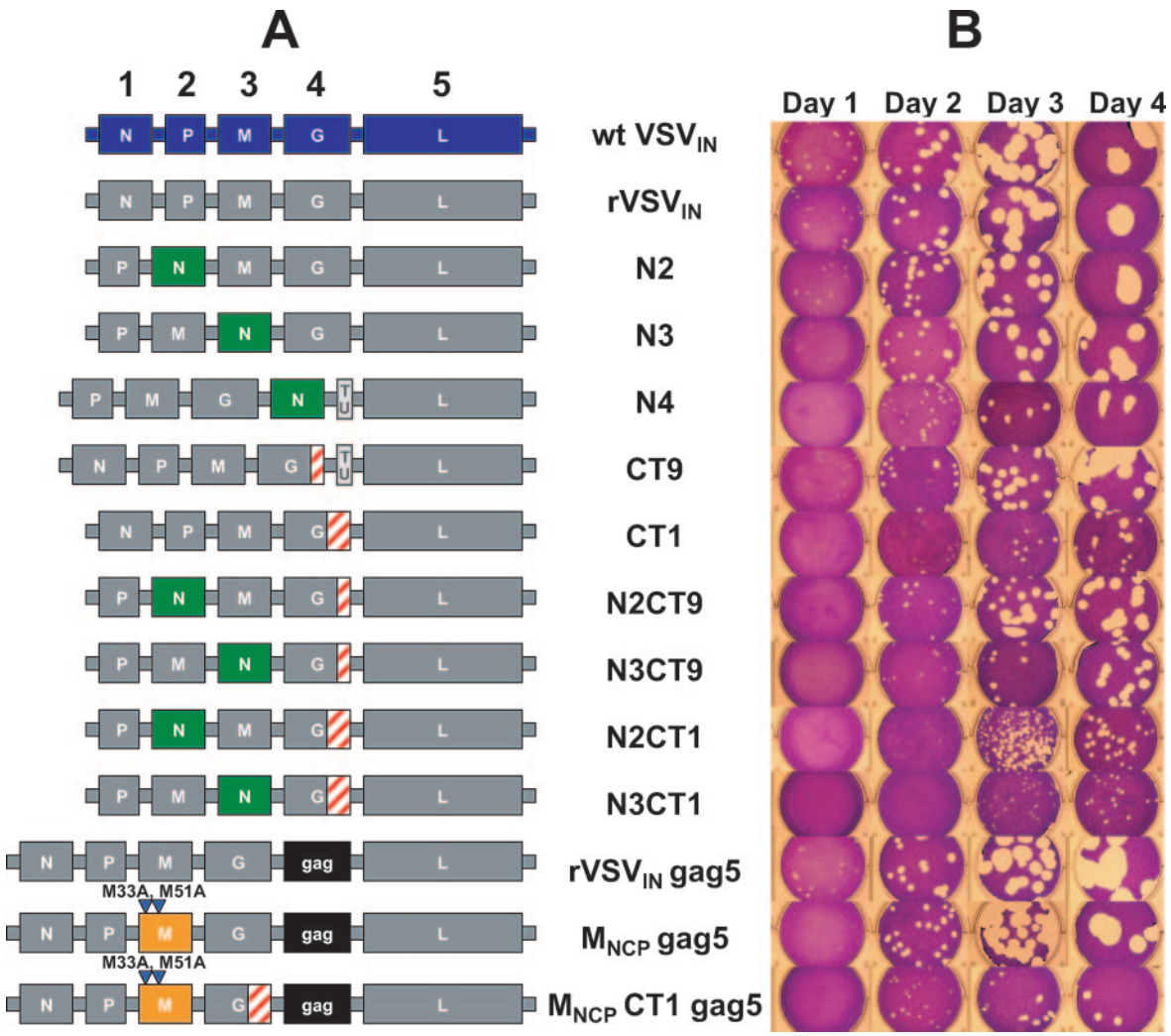

FIG. 2. Genetic organization of rVSV$_{IN}$ mutants and plaque size comparison. (A) Mutants were named to reflect genomic organization and attenuating mutations. The N gene shuffle mutants N2, N3, and N4 were named according to the position of the N gene relative to that of wt VSV$_{IN}$. The G protein CT truncation mutants CT1 and CT9 were named according to the number of amino acids retained in the cytoplasmic tail region of the G protein. Vectors containing noncytopathic M gene mutations (M33A and M51A [triangles]) were named M$_{NCP}$ mutants. Combination mutants were named N2CT1, N3CT1, N2CT9, N3CT9, and M$_{NCP}$CT1 to reflect contributing mutations. An additional empty TU containing transcription start and stop signals but no additional gene was present in N4 and CT9 mutants. The HIV-1 gag gene was present in the fifth position of virus genomes as indicated. (B) Representative plaques produced by wt VSV$_{IN}$ and rVSV$_{IN}$ variants following plaque assay on replicate Vero cell monolayers at 37°C for 1 to 4 days.

attenuate rVSV$_{IN}$ sufficiently for use as a vaccine vector in humans, these mutations were also combined in different configurations in an effort to produce more highly attenuated variants. Most double mutants were generated by combining N gene shuffles with G protein CT9 and CT1 truncations (shuffle-CT mutants), giving rise to N2CT9, N3CT9, N2CT1, and N3CT1 vectors; another double mutant containing the HIV-1 gag gene at position 5 in the genome was generated by combining the G protein CT1 truncation with the M$_{NCP}$ mutations (M$_{NCP}$ CT1 gag5). Even though it was anticipated that the mutant vectors would be more growth attenuated in vitro than the prototype rVSV$_{IN}$ vector developed by J. Rose and colleagues, all single and combination mutants were recoverable from cDNA.

Comparison of rVSV$_{IN}$ mutant plaque size. To gain a first impression on the relative attenuation levels of rVSV$_{IN}$ mutants, plaque sizes on Vero cell monolayers were compared at different times postinfection (Fig. 2B). From this analysis a number of trends emerged. Plaques produced by rVSV$_{IN}$ and wt VSV$_{IN}$ were almost the same size at all time points, indicating that rVSV$_{IN}$ growth was little more attenuated than wt VSV$_{IN}$ growth in cell culture. Virus containing only M$_{NCP}$-attenuating mutations (M$_{NCP}$ gag5) produced a delayed cell CPE, as previously observed (29), that resulted in very small plaques by day 1. At later time points, plaques were only slightly smaller than those made by rVSV$_{IN}$, indicating efficient growth and spread of this mutant in vitro. However, it should be noted that although M$_{NCP}$ plaques were similar in size to

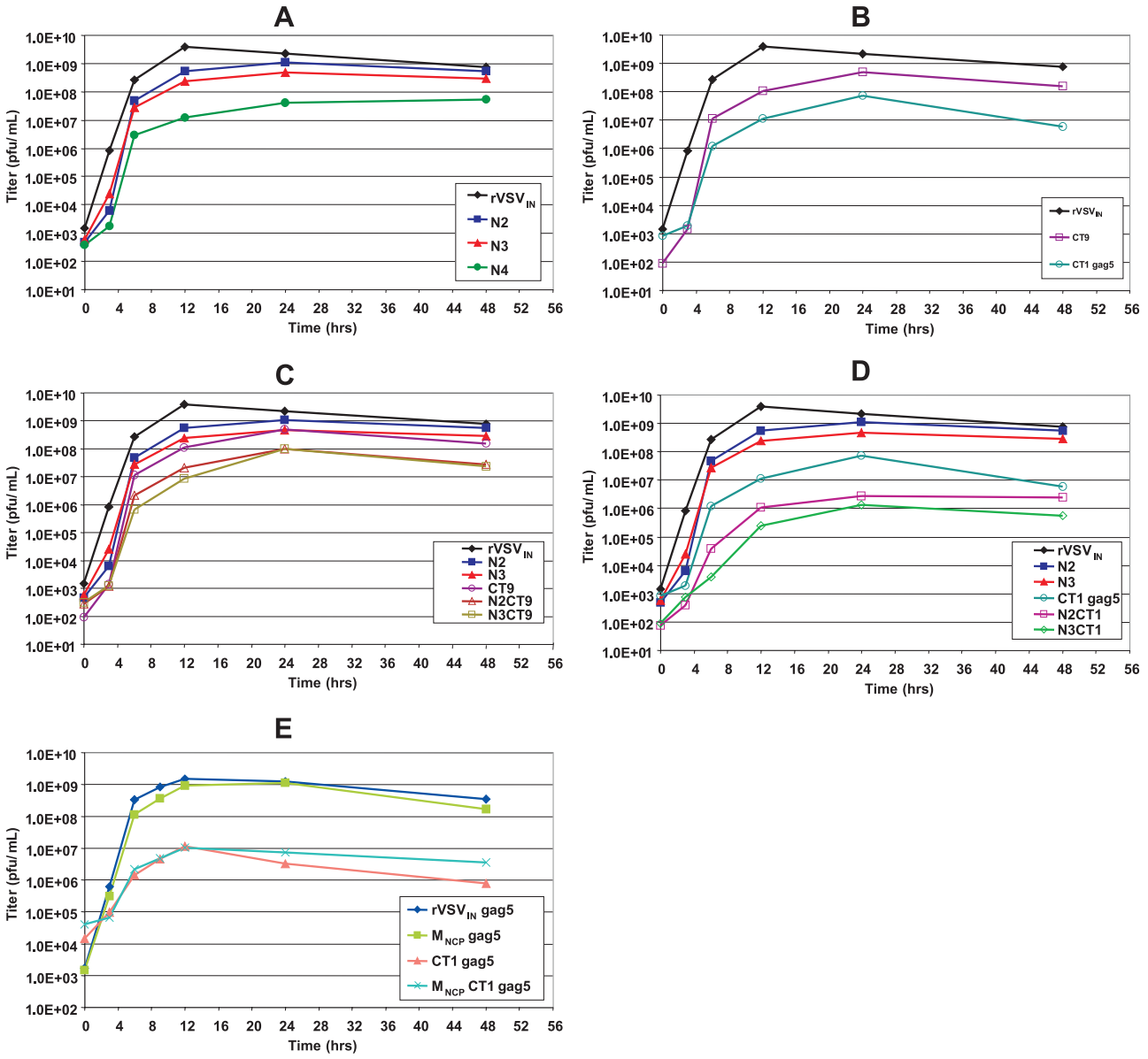

FIG. 3. Growth kinetics of $rVSV_{IN}$ mutants on Vero cell monolayers. Replicate Vero cell monolayers in 25 cm^2 flasks were infected in duplicate at an MOI of 5 PFU/cell. Infected-cell supernatants were collected at intervals postinfection and titrated on Vero cell monolayers. All datum points represent the average titers of samples taken from duplicate infections. Growth curves are shown for mutants containing N gene shuffles (A), CT truncations (B), N gene shuffle-CT truncation combinations (C and D), and M_{NCP} mutations (E).

those made by $rVSV_{IN}$ and $rVSV_{IN}$ gag5 at days 2 to 4, cells within the M_{NCP} gag5 plaques displayed a reduced CPE at all time points. The M_{NCP} CT1 gag5 combination mutant produced plaques that were much smaller than M_{NCP} gag5 plaques and similar in size to those produced by the CT1 virus, indicating that the CT1 truncation was the dominant attenuating mutation affecting virus growth and spread in vitro. Importantly, both CT and N gene shuffle mutants had plaque sizes commensurate with degrees of genetic alteration. For example, CT1 mutants produced smaller plaques than CT9 mutants, and there was a gradient of decreasing plaque size as the N gene was moved further away from the 3′ transcription promoter (N2 to N4), as previously reported (60). When N gene shuffle and CT mutations were combined, plaque size was decreased relative to the results seen with mutants having only one of the two mutations. For example, N2CT1 and N3CT1 produced plaques that were on average smaller than plaques produced by N2, N3, or CT1 mutants at all time intervals. This effect was also seen for N3CT9 but was less notable for N2CT9 except at day 1. Plaque sizes for N shuffle-CT combination mutants also varied incrementally with degree of genetic alteration, and a gradient of decreasing plaque size (N2CT9→N3CT9→N2CT1→N3CT1) was seen. Overall observations of plaque size indicated that the combination of N gene shuffles and CT truncations can attenuate virus incrementally and to a greater degree than either single form of mutation.

In vitro growth kinetics of $rVSV_{IN}$ mutants. We next performed a series of growth kinetic studies measuring the rate and extent of virus growth to further compare relative in vitro attenuation levels among $rVSV_{IN}$ mutants (Fig. 3). As shown in Fig. 3A, the rate of virus growth was reduced in relation to the position (N2 to N4) of the N gene, with a reduction in peak virus titer of 5-fold for N2, 10-fold for N3, and 100-fold for N4 compared to $rVSV_{IN}$ results. The CT9 and CT1 gag5 mutants (Fig. 3B) also had reduced growth rates and reached 10-fold and 100-fold-lower peak titer respectively than $rVSV_{IN}$, in general agreement with previous reports (49, 55). It should be noted that the addition of the HIV-1 gag gene between the G and L genes of $rVSV_{IN}$ did not significantly reduce growth of virus in cell culture and that the CT1 and CT1 gag5 mutants displayed almost identical growth kinetics in vitro (data not shown). More importantly, when N gene shuffles were combined with G protein CT truncations a series of virus mutants was generated that had reduced growth rates and a reduction in peak infectious particle production compared to virus containing either form of mutation alone (Fig. 3C and 3D). Overall, the growth kinetic studies indicated a gradient of increasing virus attenuation (N2CT9→N3CT9→N2CT1→N3CT1) identical to that observed in plaque size comparisons. In this combinatorial approach to virus attenuation, N3CT1 had 1,000-fold-lower peak virus titer than $rVSV_{IN}$ and 50- and 500-fold-lower peak titer than CT1 and N3 mutants, respectively (Fig. 3D). A separate series of growth kinetic studies were performed for the M_{NCP} mutants, as they were originally generated with the HIV-1 gag gene inserted between the G and L genes of the genome(s). As previously described, virus containing the M_{NCP} mutations replicated to a nearly normal peak titer in cell culture (Fig. 3E) but with a delayed onset of CPE in most cell types (29). Unlike the synergistic attenuation of virus growth seen with N gene-CT combination mutants, combining M_{NCP} mutations with the CT1 truncation did not significantly alter growth in cell culture compared to the results seen with the CT1 mutant alone, indicating that the CT1 mutation was the dominant attenuating mutation in vitro.

Assessment of $rVSV_{IN}$ vector neurovirulence in mice. Young mice are much more sensitive to infection with VSV following IC inoculation than following intranasal inoculation (54, 60). Moreover, unlike wt VSV_{IN}, attenuated $rVSV_{IN}$ mutants containing either cytoplasmic tail truncations (CT1 and CT9) or N gene shuffles (N2, N3, and N4) do not cause death following intranasal inoculation (25, 60). Therefore, to measure differences in virulence among the attenuated $rVSV_{IN}$ mutants, mice were inoculated IC, and the cumulative animal deaths, time until death, and frequency and severity of paralysis were measured. The LD_{50}s and the PD_{50}s were calculated based on the method of Reed and Muench and are shown in Fig. 4A. The time to death in animals receiving a lethal dose is shown in Fig. 4B.

Mice receiving wt VSV_{IN} reproducibly died 2 to 4 days postinoculation, and the LD_{50} was only 1 to 2 PFU. In agreement with plaque size comparisons and growth kinetics studies, $rVSV_{IN}$, with and without HIV gag inserted between the G and L genes, was only marginally more attenuated than wt VSV, with an LD_{50} of approximately 2 to 5 PFU and a slightly delayed onset of death at 2 to 5 days. Viruses containing either CT truncations or N gene shuffles alone were slightly more attenuated than $rVSV_{IN}$, with LD_{50} values of 12 to 21 PFU for CT9, CT1, N2, N3, and N4. Although the LD_{50}s of N2, N3, and N4 mutants were similar, there was a respective incremental increase in time to onset of death. However, a dramatic decrease in virulence was seen when the CT1 mutation was combined with N gene shuffles. Most notably, for N3CT1 the LD_{50} increased to $>10^5$ PFU compared to 15 PFU and 12 PFU for CT1 and N3 viruses, respectively, and the LD_{50} for N2CT1 was 1.1×10^4 PFU, demonstrating powerful synergistic attenuation of virulence for these combinations of mutations. Moreover, when animals died at higher doses, onset to death was delayed to 4 to 11 days postinoculation for N2CT1 and 6 to 14 days for N3CT1. To a lesser extent, and consistent with the order of attenuation observed in vitro, synergistic attenuation was also observed for N3CT9, with an LD_{50} of 524 PFU and delayed onset of death. As the least attenuated among the combination mutants in vitro, the LD_{50} dose for N2CT9 was very similar to the LD_{50}s for N2 and CT9; however, time to onset of death was delayed compared to that seen with the N2 and CT9 variants.

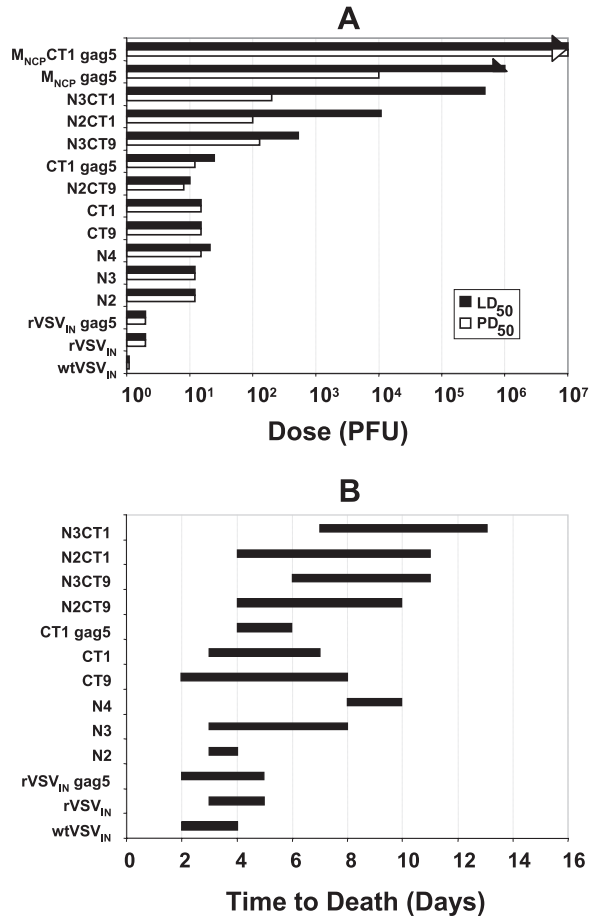

FIG. 4. Neurovirulence properties of $rVSV_{IN}$ mutants in mice following IC inoculation. In a series of experiments, 5-week-old Swiss Webster mice were inoculated IC with $\log_{10}$-fold dilutions of virus. Mice were monitored for 2 weeks for mortality and morbidity (paralysis). (A) The LD_{50} and PD_{50} values were determined by the method of Reed and Muench. (B) Time to death was recorded for mice in the group receiving the dose immediately above the determined LD_{50}. Arrowheads indicate results in which LD_{50} and PD_{50} were not achieved.

Many of the mice inoculated with the combination mutants, in particular, N3CT1 and N2CT1, displayed morbidity in the form of paresis and unilateral paralysis, from which they started to recover by week 3 postinfection, without mortality. Thus, the PD_{50}s for N3CT1 and N2CT1 were less than their respective LD_{50}s. Mice receiving the more virulent viruses died quickly without a measurable paralytic phase. Therefore, the PD_{50} and LD_{50} values for these viruses were recorded as being identical. Overall, the gradient of attenuation for the N gene shuffle-CT combination mutants observed in vivo was identical to that observed in vitro.

IC infection with M_{NCP} gag5 primarily caused some mild paralysis (PD_{50} of 10^4 PFU) but not death at up to the highest dose (10^6 PFU) tested, and an LD_{50} dose was not achieved. However, combining the M_{NCP} and CT1 mutations reduced the amount of paralysis compared to the results seen with M_{NCP} gag5 alone such that neither a LD_{50} nor a PD_{50} could be calculated for M_{NCP}CT1 gag5. The very high level of attenuation observed for the M_{NCP} gag and M_{NCP}CT1 gag5 mutants in vivo and the absence of a measurable LD_{50} for both mutants at input levels that were approaching a practical limit for M_{NCP}CT1 gag5 prevented any clear conclusions concerning the synergistic effect of combining the M_{NCP} and CT1 mutations. However, the M_{NCP} mutation was clearly the dominant attenuating mutation in vivo, while the CT1 mutation was clearly dominant in vitro.

DISCUSSION

An exploratory NV study of NHPs indicated that the $rVSV_{IN}$–HIV-1 vaccine vectors pioneered by J. Rose and colleagues retained significant levels of virulence and might be insufficiently attenuated for clinical evaluation (30). The present study was undertaken to investigate strategies for further attenuation of $rVSV_{IN}$ and to identify less-virulent variants that might be more suitable as vaccine vectors for HIV-1 and other pathogens.

Variants containing only a single form of attenuating mutation were more growth attenuated than the prototypic $rVSV_{IN}$ vector in vitro but, except for the M_{NCP} mutant, were still highly neurovirulent when tested in the murine IC NV model. Virus containing only the CT1 mutation also caused significant neuropathology in an exploratory NHP NV study (30). In an effort to further increase $rVSV_{IN}$ vector attenuation, CT truncations were combined with either N gene shuffles or M_{NCP} mutations. Most of the resulting combination mutants were more growth attenuated in vitro than vectors containing either single form of mutation. Growth kinetics studies showed that N2CT1 and N3CT1 reached approximately 500- to 1,000-fold-lower peak titers than $rVSV_{IN}$ and approximately 50- to 500-fold-lower peak titers than N2, N3, or CT1 mutants. Furthermore, the degree of vector attenuation could be altered incrementally depending on the pairing of specific N gene shuffle and CT mutations. For example, the N3CT1 mutant was more growth attenuated than N2CT1, which was more attenuated than N3CT9 and N2CT9 mutants. The gradient of increasing virus attenuation for these combination mutants was N2CT9→N3CT9→N2CT1→N3CT1. The same order of attenuation was also observed in vivo, but differences in attenuation between combination mutants and virus with only one type of attenuating mutation were even more dramatic. The N2, N3, CT9, and CT1 mutants still retained high levels of virulence following IC inoculation of mice (LD_{50}s of 12 to 21 PFU) consistent with previously published data for N gene shuffles (60). In contrast, N gene shuffle-CT combination mutants had incrementally increasing LD_{50}s ranging from 10 PFU for N2CT9 to $>10^5$ PFU for N3CT1. Specifically, attenuation synergy appeared to be greater when the CT1 truncation was combined with N2 and N3 gene shuffles.

The differences in relative attenuation between single and combination mutants observed in vitro probably reflect predominantly virus-specific growth attenuation factors. It is thought that the length of the CT tail of VSV G protein may affect the efficiency of virus budding from the cytoplasmic membrane of infected cells. Shorter CTs reduce the rate of particle formation and peak virus titer produced in vitro, possibly due to impaired CT interaction with viral core proteins (16, 28, 50, 55). The N gene shuffles attenuate virus by a different mechanism. During virus replication, N protein is essential for the encapsidation of nascent genomic RNA, and the resulting nucleocapsid structure is the functional template for mRNA transcription and further genome replication. When the N gene is translocated further away from the single 3′ transcription promoter, N protein expression decreases (60). Consequently, limiting N protein reduces the level of nucleocapsid available for transcription, replication, and subsequent incorporation into virus progeny. When transcription is reduced, all virus proteins are expressed less abundantly, placing additional constraints on the availability of all the components needed for assembly and morphogenesis of virus progeny. When both attenuation strategies are combined, not only are viral nucleocapsid and truncated G protein, along with other virus proteins, limiting for viral morphogenesis but impaired interactions between viral nucleocapsid core and the truncated G protein CT likely also further constrain the efficiency of mature particle formation. In vivo, innate and cellular immune responses are additionally superimposed on these growth-attenuating virus-specific factors and likely contribute to the level of attenuation observed in mice.

Innate immunity is usually rapidly induced in response to viral infection in the periphery and likely also plays an important role in controlling virus replication early (days 1 to 5) following IC inoculation of mice (7, 10, 46). Recently, a role for type I interferon has been proposed for control of attenuated but not pathogenic strains of rabies virus, a relative of VSV, following IC inoculation of mice (59), and it is possible that differences in virulence between attenuated and pathogenic strains of VSV can also be explained by differential stimulation of alpha/beta interferon in the CNS. VSV growth in the brain may also be controlled by the induction of nitric oxide, which can inhibit VSV replication in vitro and in neurons (5, 8, 11, 13, 35–37, 47). Acquired cellular immunity likely also plays an important role in killing some types of infected cells and clearance of virus in the CNS early (days 1 to 8) in the infection (11, 46). In contrast, the humoral immune response does not appear to have a significant role in the control and clearance of VSV from the CNS following direct IC inoculation of mice (7, 11).

In view of the host-specific responses to VSV infection of the CNS described above, it is possible that the more slowly rep-

licating, highly attenuated N gene shuffle-CT truncation combination mutants less efficiently down-regulate innate immune responses, leading to a more potent antiviral state (14). For example, reduced expression of the VSV M protein and associated polypeptides can diminish the efficiency of host cell protein shutoff, allowing more efficient induction of innate immune responses (9, 20, 26, 29). Since the N gene shuffle-CT truncation combination mutants also down-regulate viral gene expression, including the M gene, the innate immune response may be better able to control these viruses. Similar reasoning likely also explains the observed differences between in vitro and in vivo attenuation of M_{NCP} mutants described here. In vitro, the M_{NCP} gag5 and M_{NCP}CT1 gag5 mutants are not subject to innate immune responses, and peak titer is close to that of rVSV gag5 and CT1 gag5, respectively, indicating that the CT1 mutation was the dominant attenuating mutation in vitro. However, both M_{NCP} gag5 and M_{NCP}CT1 gag5 had highly attenuated phenotypes in mice, indicating that the M_{NCP} mutations were dominant in vivo, presumably due to the reduced ability of these viruses to interfere with innate immune responses.

In general, the mouse IC LD_{50} NV model proved to be highly sensitive and capable of discriminating changes in virulence within the range of attenuated rVSV vectors tested in this study. Rodent models have also been used to assess the NV potential of other virus vaccine vectors and some licensed live virus vaccines and vaccine candidates, including smallpox vaccine (40), some yellow fever virus vaccine strains (6), attenuated Venezuelan equine encephalitis virus (41), the Jeryl Lynn strain of mumps virus vaccine (52) and a modified measles virus vaccine strain (17). Interestingly, some of the more highly attenuated rVSV vectors described here produced less morbidity and mortality following IC inoculation than some of the licensed live virus vaccines. However, it should be emphasized that differences in virus biology and the natural susceptibility of different mouse strains to virus infection and replication make direct comparison of attenuation levels among different virus vaccines and candidate vaccines extremely difficult.

In summary, the net effect of combining specific N gene shuffles and G protein CT truncations was a measurable synergistic attenuation of $rVSV_{IN}$ growth in vitro and a dramatic reduction of virulence in the very sensitive mouse IC LD_{50} model. These findings suggest that combining mutations that interfere with viral morphogenesis by impairing interactions between structural proteins with mutations that lead to down-regulation of viral structural protein expression may be a useful general mechanism for synergistic attenuation of $rVSV_{IN}$ and other RNA and DNA viruses. Because of the potential of $rVSV_{IN}$ as a vaccine vector for HIV-1 and other human pathogens, experiments are now under way to confirm attenuation of the combination mutants in NHP NV studies and explore the immunogenicity of these highly attenuated $rVSV_{IN}$ vectors.

ACKNOWLEDGMENTS

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VIRUS-CELL INTERACTIONS

Cytoplasmic Tail Truncation Stabilizes S1-S2 Association and Enhances S Protein Incorporation into SARS-CoV-2 Pseudovirions

Lizhou Zhang,[a] Nancy Hom,[b] Amrita Ojha,[a]* Klaus N. Lovendahl,[b] Huihui Mou,[a]§ Kelly K. Lee,[b] Hyeryun Choe[a]

[a]Department of Immunology and Microbiology, Scripps Biomedical Research at the University of Florida, Jupiter, Florida, USA
[b]Department of Medicinal Chemistry, University of Washington, Seattle, Washington, USA

Lizhou Zhang, Nancy Hom, and Amrita Ojha contributed equally. Author order was determined based on the time spent on the project.

ABSTRACT Truncations of the cytoplasmic tail (CT) of entry proteins of enveloped viruses dramatically increase the infectivity of pseudoviruses (PVs) bearing these proteins. Several mechanisms have been proposed to explain this enhanced entry, including an increase in cell surface expression. However, alternative explanations have also been forwarded, and the underlying mechanisms for the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) S protein remain undetermined. Here, we show that the partial or complete deletion of the CT (residues 19 to 35) does not modify SARS-CoV-2 S protein expression on the cell surface when the S2 subunit is measured, whereas it is significantly increased when the S1 subunit is measured. We also show that the higher level of S1 in these CT-truncated S proteins reflects the decreased dissociation of the S1 subunit from the S2 subunit. In addition, we demonstrate that CT truncation further promotes S protein incorporation into PV particles, as indicated by biochemical analyses and cryo-electron microscopy. Thus, our data show that two distinct mechanisms contribute to the markedly increased infectivity of PVs carrying CT-truncated SARS-CoV-2 S proteins and help clarify the interpretation of the results of studies employing such PVs.

IMPORTANCE Various forms of PVs have been used as tools to evaluate vaccine efficacy and study virus entry steps. When PV infectivity is inherently low, such as that of SARS-CoV-2, a CT-truncated version of the viral entry glycoprotein is widely used to enhance PV infectivity, but the mechanism underlying this enhanced PV infectivity has been unclear. Here, our study identified two mechanisms by which the CT truncation of the SARS-CoV-2 S protein dramatically increases PV infectivity: a reduction of S1 shedding and an increase in S protein incorporation into PV particles. An understanding of these mechanisms can clarify the mechanistic bases for the differences observed among various assays employing such PVs.

KEYWORDS cytoplasmic tail, entry, infectivity, pseudovirus, S protein, S1 shedding, SARS-CoV-2, spike density

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) encodes 15 nonstructural proteins as well as 4 structural proteins. Of these, only the structural proteins, spike (S), membrane (M), envelope (E), and nucleocapsid (N), are incorporated into the virion. The N protein is essential for the encapsidation of the 30 kb positive-sense RNA genome, the M and E proteins contribute to virus assembly and budding via interactions with other viral proteins (1, 2), and the S protein mediates entry into the target cell.

For successful entry, the S protein needs to be activated by two sequential proteolytic cleavages. The first cleavage divides the S protein into the S1 and S2 subunits, of

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Address correspondence to Kelly K. Lee, kklee@uw.edu, or Hyeryun Choe, hchoe@ufl.edu.

*Present address: Amrita Ojha, Department of Medicine, Division of Infectious Diseases and Geographic Medicine, Stanford University, Stanford, California, USA.

§Present address: Huihui Mou, Department of Pharmacology, University of Minnesota, Minneapolis, Minnesota, USA.

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which S1 binds the receptor angiotensin-converting enzyme 2 (ACE2) and S2 mediates membrane fusion (3). In the case of the first SARS-CoV that emerged 2 decades ago, cleavage at the S1-S2 junction is accomplished in target cells by a cell surface protease, TMPRSS2, or lysosomal proteases, cathepsins (4–8). In contrast, for SARS-CoV-2, cleavage is carried out by furin, a Golgi-resident protease, in infected cells during virus maturation. The second cleavage occurs at a site internal to the S2 subunit, termed the S2′ site, and is carried out by TMPRSS2 or cathepsins for both SARS-CoV and SARS-CoV-2 when they reach the target cells. This second cleavage releases the fusion peptide that is required for subsequent fusion between the cellular and viral membranes (3).

Like SARS-CoV-2, the entry glycoproteins of many viruses are cleaved into the surface and transmembrane subunits prior to virus release from infected cells. For most of these viruses, the two subunits remain associated until the receptor-binding domain (RBD) located in the surface subunit binds the receptor. Receptor binding induces conformational changes in the entry glycoprotein and leads to the dissociation of the surface subunit and subsequent membrane fusion mediated by the transmembrane subunit. In the case of the original Wuhan-Hu-1 strain of SARS-CoV-2, the S1-S2 association is weak, and thus, S1 was easily shed from the spikes (9). To overcome this problem, the virus acquired the D614G mutation early in the pandemic, which stabilized the S1-S2 association and increased virus infectivity (9–12).

Another modification of the SARS-CoV-2 S protein that increases virus infectivity is cytoplasmic tail (CT) truncation. The understanding that CT truncation of viral entry glycoproteins enhances pseudovirus (PV) infectivity originated decades ago from intriguing observations that lentiviruses grown in human T cell lines acquired a premature stop codon in their CTs (13–16). Multiple subsequent studies reported that CT truncations of the entry glycoproteins of various viruses enhanced PV infectivity (17–21). We also showed that a 19-amino-acid truncation of the SARS-CoV S protein enhanced PV infectivity (21). Mechanistic studies showed that CT truncation upregulated the cell surface expression of the entry glycoproteins (20, 22). These observations have been interpreted to suggest that the removal of the endoplasmic reticulum (ER) retention signal present at the carboxy-terminal end of the CT was responsible for the elevated expression on the cell surface. However, other studies did not observe increased expression of the CT-truncated glycoproteins on the cell surface (23, 24). In addition, even if an increase was observed, the degree of the increase was modest, and thus, it is difficult to explain the dramatic changes in PV infectivity. An alternative mechanism, a conformational change in the ectodomain, was also proposed for various enveloped viruses to explain the increased fusogenicity upon CT truncation of their entry glycoproteins (20, 23, 25–27).

Here, we show that CT truncation of the SARS-CoV-2 S protein modestly increases cell surface expression when both S1 and S2 are measured, but no increase is observed when only S2 is detected. When only S1 is detected, however, CT-truncated S protein expression on the cell surface is substantially elevated. Further investigation shows that these differences are contributed by reduced S1 shedding in the CT-truncated S protein. We also demonstrate through biochemical and cryo-electron microscopy (cryo-EM) studies that PVs bearing the CT-truncated S protein exhibit much higher spike densities. Together, our studies show that CT truncation of the S protein enhances PV infectivity by decreasing S1 dissociation and increasing S protein incorporation into PV particles.

RESULTS

Cytoplasmic tail truncation of the S protein does not change SARS-CoV-2 PV production but dramatically enhances PV infectivity. The CT of the SARS-CoV-2 S protein consists of 37 amino acids that contain four motifs, as shown in Fig. 1A: a putative ER retention signal (KXHXX) (28, 29), a charged cluster (KFDEDDSE) (30), and two cysteine-rich motifs (CRMs), CCSCGSCC and SCCSC (30–32). To better understand the role of the CT in SARS-CoV-2 infectivity, we made truncation variants of the S protein (S-dCTs) in which these motifs were sequentially deleted, and we compared them to

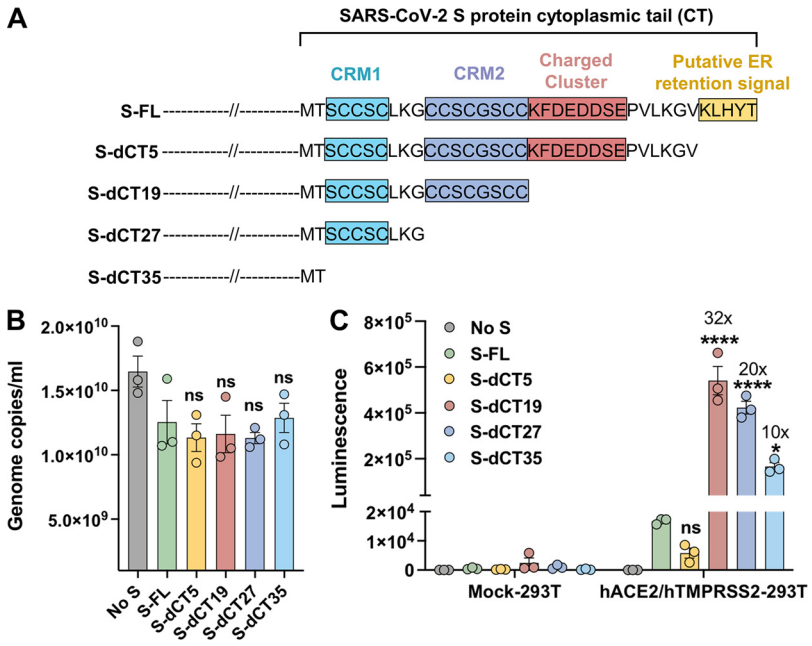

FIG 1 Cytoplasmic tail truncation of the S protein does not change SARS-CoV-2 PV production but dramatically enhances PV infectivity. (A) Diagram representing the CT truncation variants of the S protein used in this study. (B) MLV PVs bearing the full-length S protein (S-FL) or its CT truncation variants (S-dCTs) and expressing firefly luciferase were produced from HEK293T cell transfection, and their titers were quantified by RT-qPCR. (C) Infectivity of the same PVs in parental HEK293T cells or the same cells expressing human ACE2 and TMPRSS2. Cells were infected for 1 h with 5×10^8 genome copies per well in a 48-well plate, and infection levels were assessed by measuring luminescence at 24 h postinfection. Panels B and C show mean values ± SEM from three independent experiments conducted with three independently prepared PVs, and statistical significance was calculated by two-way ANOVA using Sidak's multiple-comparison test (*, $P < 0.05$; ****, $P < 0.0001$; ns, not significant). CRM, cysteine-rich motif.

the full-length S protein (S-FL). PVs expressing firefly luciferase (FLuc) and bearing these S-dCTs (PV-dCTs) were produced from HEK293T cell transfection. PVs bearing S-FL (PV-FL) or no S protein were used as controls. To assess the effect of CT truncation on the PV yield, PV titers were quantified by reverse transcription-quantitative PCR (RT-qPCR). Figure 1B shows that these PVs were produced at comparable levels, indicating that CT truncation did not affect PV production.

Next, we assessed their entry efficiency by infecting HEK293T cells stably expressing human ACE2 and TMPRSS2 (hACE2/hTMPRSS2-293T cells) with the same genome copy numbers. HEK293T cells transduced with empty vectors but selected with drugs in the same way as for hACE2/hTMPRSS2-293T cells were used as negative controls for infection (Mock-293T cells). As shown in Fig. 1C, S-dCT5 unexpectedly decreased PV entry compared to S-FL, indicating that the KLHYT motif, a putative ER retention signal, may play its role only in the context of other motifs. In contrast, S-dCT19 increased PV entry by more than 30-fold, which is consistent with previous reports that a deletion of the last 13 to 21 amino acids from the carboxy terminus of the CT enhances PV infectivity (23, 24, 33, 34). S-dCT27 and S-dCT35, which lack one or both CRMs, respectively, also increased PV entry compared to S-FL but to a lesser degree than did S-dCT19, which contains both CRMs (Fig. 1C). Together, these data confirm that CT truncations, except dCT5, substantially enhance the PV entry efficiency and suggest that although the CRMs may play a role in PV infectivity, they are not essential for either PV production or entry.

Cytoplasmic tail truncation does not increase S2 levels but significantly increases S1 levels on the cell surface. Whereas most viruses that are commonly used as a backbone for pseudotyping systems, including human immunodeficiency virus type 1 (HIV-1), murine leukemia virus (MLV), and vesicular stomatitis virus (VSV), bud from the plasma membrane, SARS-CoV-2 buds from the membranes of the ER or the ER-Golgi intermediate compartment (ERGIC) (35). The S protein is therefore designed

to be expressed in the intracellular compartments, but when its expression level is high, it is also trafficked to the plasma membrane. Because PV production could benefit from increased S protein expressed on the plasma membrane, we investigated whether CT truncation increased S protein expression on the cell surface. We expressed S-FL and S-dCTs on HEK293T cells via transfection and detected them using convalescent-phase plasma samples derived from coronavirus disease 2019 (COVID-19) patients. Note that whereas all COVID-19 convalescent-phase plasma samples efficiently recognize the S2 subunit, they rarely recognize the S1 subunit (Fig. 2A), likely because the original SARS-CoV-2 did not have much S1 remaining on the virion, owing to its shedding (9). When plasma sample 2, which recognizes both S1 and S2, was used for detection, all S-dCTs, except S-dCT5, were expressed at modestly higher levels than S-FL; albeit modest, the difference was statistically significant (Fig. 2B). However, when plasma sample 9, which recognizes only S2, was used, S-FL and all S-dCTs, except S-dCT5, were expressed at comparable levels (Fig. 2C). Because these data suggest that different S1 levels of S-FL and S-dCTs are responsible for the disparate detection profiles of plasma samples 2 and 9, we also detected S1 alone. To detect only S1, we used hACE2-NN-Ig, the human ACE2 ectodomain that contains enzyme activity-null mutations (H374N and H378N) and is fused to the Fc region of human IgG1 (hIgG1) (21). Figure 2D shows that the S1 levels of S-dCTs, except S-dCT5, detected by hACE2-NN-Ig were approximately 4-fold higher than that of S-FL. These data show that the differences between S-FL and S-dCTs in cell surface expression levels result from their differences in S1, but not S2, contents and that S-dCTs have much higher S1 contents in the spike trimers than S-FL. These data also provide an explanation for the conflicting observations made in previous reports on the cell surface expression of CT-truncated glycoproteins (20, 22–24). Our results demonstrate that depending on which component of the glycoprotein is measured, the expression levels of the CT-truncated glycoproteins could appear to be increased or not increased.

The higher S1 levels observed with S-dCTs than with S-FL prompted us to hypothesize that CT truncation may strengthen the S1-S2 association and, consequently, decrease S1 dissociation from S2. However, because higher hACE2-NN-Ig binding to S-dCTs can result from either a decrease in S1 shedding or an increase in the RBD-up conformation, the conformation that binds the receptor (36–38), we attempted to distinguish these two possibilities by comparing the binding of hACE2-NN-Ig to that of an antibody recognizing the N-terminal domain (NTD) of S1. As this antibody binds the NTD, its binding is unlikely to be affected by the change in the RBD conformation. HEK293T cells were transfected with increasing amounts of an S-dCT19 or S-FL plasmid, their cell surface levels of S1 were measured using hACE2-NN-Ig or the NTD antibody, and their S2 levels were measured using plasma sample 9. Both the hACE2-NN-Ig and NTD antibodies detected severalfold-higher levels of S1 in S-dCT19 than in S-FL at all expression levels (Fig. 2E and F), while the S2 levels of S-dCT19 and S-FL were similar when measured using plasma sample 9 (Fig. 2G). The very similar detection profiles with hACE2-NN-Ig and the NTD antibody of S-FL and S-dCT19 suggest that the RBD-up or -down conformation does not significantly contribute to the differences in the S1 levels but that reduced S1 shedding is likely the major source of the observed S1 differences between S-FL and S-dCTs induced by CT truncation.

In contrast to the other S-dCTs, a low level of S-dCT5 was detected on the cell surface when measured using convalescent-phase plasma sample 2 (Fig. 2B). We thus assessed S-dCT5 expression inside the cell. As the relative level of S-dCT5 compared to other S proteins in permeabilized cells was similar to that on the cell surface (Fig. 2H), we further assessed the S-dCT5 level in cell lysates by Western blotting (WB). Because plasma sample 2 does not efficiently detect S protein in cell lysates, while it does in PVs, a polyclonal anti-S antibody was used for blotting. As shown in Fig. 2I, while comparable levels of the uncleaved S band were observed for all S proteins, indicating that S-dCT5 is expressed comparably to the others, much lower levels of the S1 and S2 bands were observed for S-dCT5, suggesting that they may be rapidly degraded once cleaved. Because this antibody detects S1 only very weakly, we used the NTD antibody to better visualize the S1 bands. Figure 2J shows that the S1 bands in all S-dCTs, except

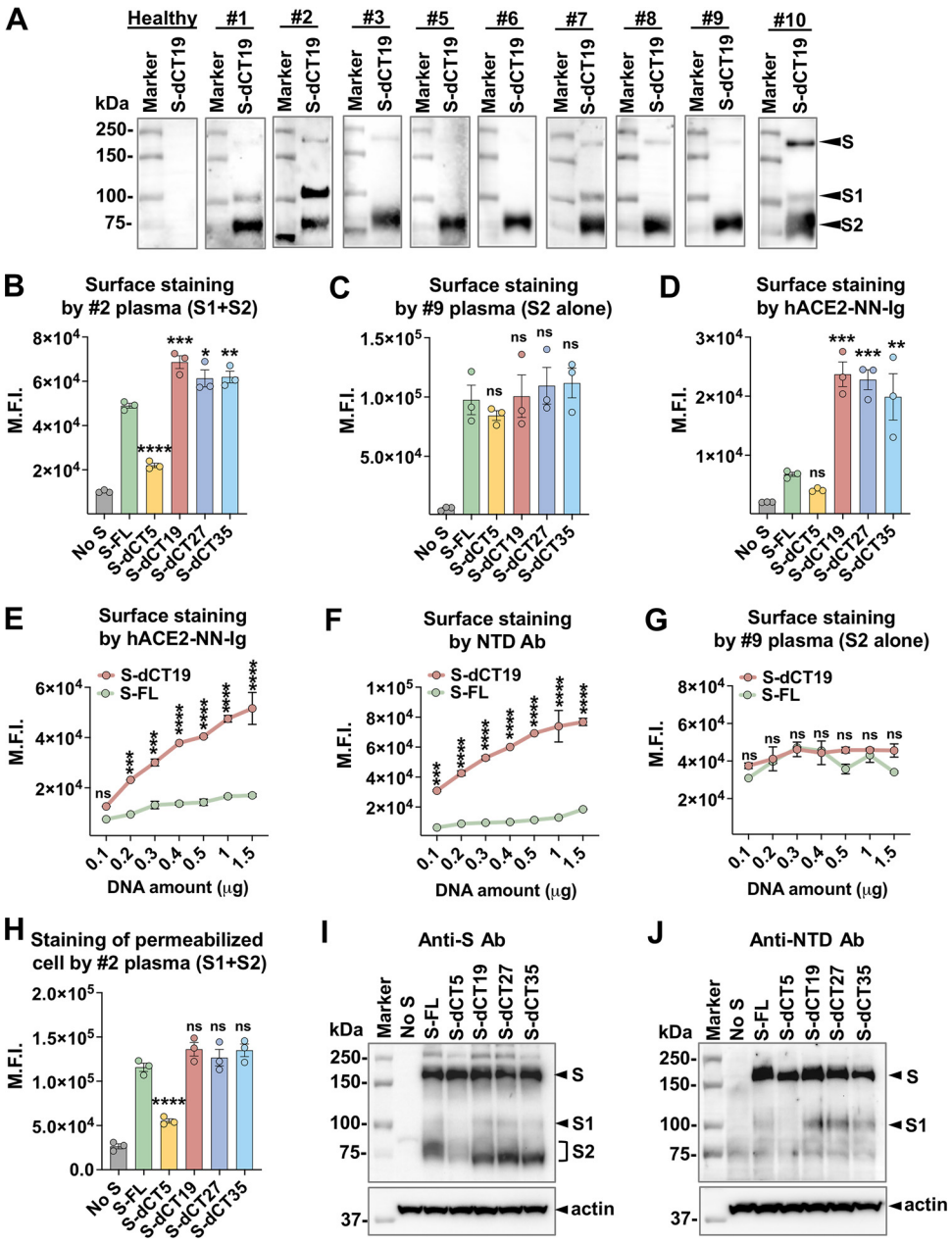

FIG 2 Cytoplasmic tail truncation does not increase S2 levels but significantly increases S1 levels on the cell surface. (A) Ten convalescent-phase plasma samples derived from individuals infected with SARS-CoV-2 early in the pandemic were screened by Western blot analyses for their ability to recognize the S1 and S2 bands of sucrose-pelleted PV-dCT19. (B to D) HEK293T cells grown on 6-well plates were transfected with 0.3 μg of a plasmid encoding the indicated S-FL or S-dCT protein, and their cell surface expression levels were assessed at 42 h posttransfection using plasma sample 2 (at a 1:200 dilution), which recognizes both S1 and S2 (B); plasma sample 9 (at a 1:200 dilution), which recognizes only S2 (C); or 3 μg/mL of hACE2-NN-Ig, which binds only S1 (D). Shown are the mean fluorescence intensity (M.F.I.) values ± SEM from three independent experiments. (E to G) HEK293T cells on 6-well plates were transfected with the indicated amounts of the plasmid encoding S-FL or S-dCT19, and cell surface staining was conducted with 3 μg/mL hACE2-NN-Ig (E), 3 μg/mL NTD antibody (Ab) (F), or plasma sample 9 (at a 1:200 dilution) recognizing only S2 (G). (H) Experiment similar to the one for panel B except that staining was conducted in cells permeabilized with 0.1% saponin. (I and J) S protein expression levels were assessed in cells lysed with dodecyl maltopyranoside and blotted with rabbit anti-S antibody (I) or the same NTD antibody used for panel F (J). The average mean fluorescence intensity values ± SEM from three independent experiments are shown. Statistical significance was analyzed by one-way ANOVA using Dunnett's multiple-comparison test (B to D) or two-way ANOVA using Sidak's multiple-comparison test (E to G) (*, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$; ****, $P < 0.0001$; ns, not significant).

S-dCT5, are stronger than that in S-FL. These data are consistent with our conclusion drawn from cell surface expression and confirm that S-dCTs, except S-dCT5, retain higher levels of S1 than S-FL owing to reduced S1 shedding.

Cytoplasmic tail truncation dramatically enhances functional S protein incorporation into PV particles. As the S protein mediates receptor attachment, the quantity and quality of spike trimers on the virion determine virus and PV infectivity. Thus, we measured the S protein density on PV-FL and PV-dCTs, which were pelleted through a sucrose layer ("sucrose-pelleted PV"), by WB analyses, using plasma sample 2, which recognizes both S1 and S2 (Fig. 2A). When the same numbers of PV particles were analyzed, as supported by the comparable amounts of p30, the MLV Gag protein, the intensities of the S1 and S2 bands of all PV-dCTs, except PV-dCT5, were dramatically increased compared to those of PV-FL (Fig. 3A, left), which were barely detectable only with a much longer exposure of the same blot (Fig. 3A, right). These data demonstrate that CT truncation leads to the much more efficient incorporation of the S protein into PV particles.

To confirm that the increased intensities of the S1 and S2 bands of PV-dCTs assessed by WB analyses (Fig. 3A and B) actually reflect an increased spike density on the virion, we visualized PV-FL and PV-dCT19 by cryo-electron microscopy (cryo-EM). We focused on PV-dCT19 because it exhibits the highest infectivity and virion spike density. Examined by cryo-EM, both PV-FL and PV-dCT19 were quite heterogeneous with respect to their spike densities. Although PV-dCT19 particles generally exhibited higher spike densities than PV-FL particles, to analyze them in a semiquantitative way, we sorted PV-FL and PV-dCT19 cryo-EM particles into high-, medium-, and low (or bald)-spike-coverage groups (Fig. 3C). Three cryo-EM images, each representing the three categories, are shown in Fig. 3C, left. Using these criteria, cryo-EM images of 61 PV-FL and 88 PV-dCT19 particles (see Fig. S1 in the supplemental material) were sorted with four independent counts. Note that we found only one high-density and two medium-density PV-FL particles. Most PV-FL particles belonged to the low-surface-density group. Specifically, 1.3% and 7.1% of the PV-FL particles belonged to the high- and medium-density groups, respectively, while the majority, 91.6%, had low or undetectable levels of spike (Fig. 3D). In contrast, PV-dCT19 virions were more evenly distributed among the high-, medium-, and low-surface-density groups (Fig. 3D): high at 25.4%, medium at 40.6%, and low/bald at 34.0%. These cryo-EM data are consistent with the WB results and clearly demonstrate that PV-FL overall has a much lower virion spike density than does PV-dCT19.

Cytoplasmic tail truncation significantly enhances functional S protein on PV particles. Much higher S1 levels were detected on PV-dCTs (Fig. 3A and B) as well as S-dCTs on the cell surface. To confirm that the higher level of S1 indeed reflects the more efficient retention of S1 on PV-dCTs, we analyzed the S1/S2 ratio of PV-dCTs and PV-FL. Because of the much lower S density of PV-FL, a large amount of PV-FL was compared to a much smaller amount of PV-dCTs in order to detect both the S1 and S2 bands in both PVs. As Fig. 4A and its quantification in Fig. 4B show, the S1/S2 ratio of PV-dCT19 is approximately 2.5-fold higher than that of PV-FL. This result is consistent with the differences that we observed with cell surface-expressed S-FL and S-dCTs (Fig. 2D, E, and F) and again indicates that CT truncation contributes to the greater retention of S1.

To understand the three-dimensional organization of the S protein on the surface of PVs, we performed cryo-EM tomography with PV-FL and PV-dCT19 (Fig. 4 and Fig. S2). On some of the PV surfaces, we were able to distinguish the prefusion trimers, which look like half-blossomed flowers, from the postfusion trimers, which look like thin sticks, as a result of S1 shedding and the conversion of S2 to a helical-bundle conformation (Fig. 4C) (37–39). Sequentially slabbing through the three-dimensional tomogram slices, we observed that most PV-FL particles have extremely low coverage of surface proteins and that the majority of the spike trimers are in the postfusion conformation, with a subset being in the prefusion conformation (Fig. 4D). In contrast, on PV-dCT19, prefusion spikes can be abundantly identified amid closely spaced clusters

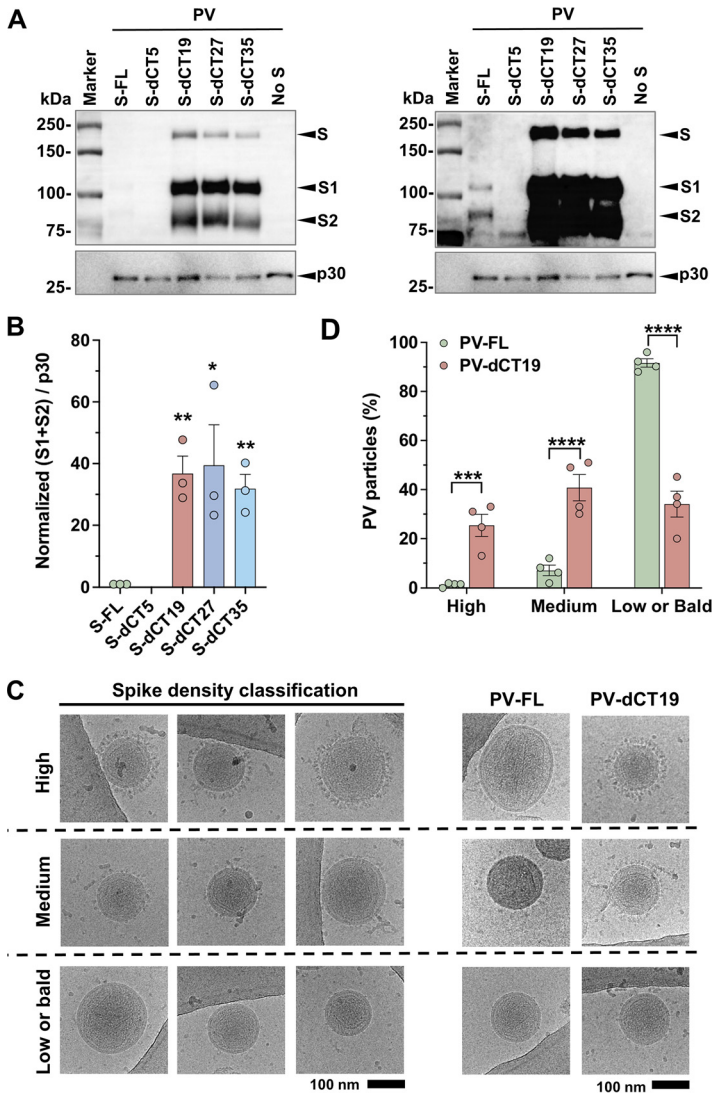

FIG 3 Cytoplasmic tail truncation dramatically enhances S protein incorporation into PV particles. (A) PVs bearing the indicated S variants and produced from HEK293T cell transfection were pelleted through a layer of 30% of sucrose and analyzed by Western blotting. The S, S1, and S2 bands were detected using convalescent-phase plasma sample 2 at a 1:200 dilution. (Left) Blot representative of results from three independent experiments. The S1 and S2 bands of S-FL and S-dCT5 are too weak to be detected. (Right) A much longer exposure of the same blot shown on the left. The S1 and S2 bands of S-FL, but not those of S-dCT5, are visible. (B) Sum of the S1 and S2 band intensities of the Western blot shown in panel A normalized to the intensity of the p30 band in the same blot. Shown are the averages from three independent experiments. (C, left) As both PV-FL and PV-dCT19 exhibit heterogeneous spike densities, to provide an objective guideline for semiquantitative evaluation, three representative cryo-EM images were selected for each of the high-, medium-, and low/bald-spike-density groups. (Right) Images selected separately from PV-FL and PV-dCT19 for each category. Note that there are only one high-density and a few medium-density particles among 61 PV-FL particles. (D) A total of 149 PV particles (61 PV-FL and 88 PV-dCT19 [shown in Fig. S1 in the supplemental material]) were evaluated by four individuals and categorized into the high-, medium-, and low/bald-density groups using the criteria described above for panel C. Mean values ± SEM are shown. Statistical significance was analyzed by an unpaired *t* test (B) and two-way ANOVA using Sidak's multiple-comparison test (D) (*, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.005$; ****, $P < 0.0001$).

of proteins on the PV surface resolved in three dimensions (Fig. 4E). While the high density of protein on the PV-dCT19 particles could mask the presence of some postfusion spikes, and only a portion of the spike conformations are clearly identifiable, many spike trimers appear to be in the prefusion conformation. The higher abundance of prefusion spike trimers on PV-dCT19 is consistent with the presence of intact, functional S-dCTs on the cell surface (Fig. 2) and the high S1/S2 ratios observed in WB

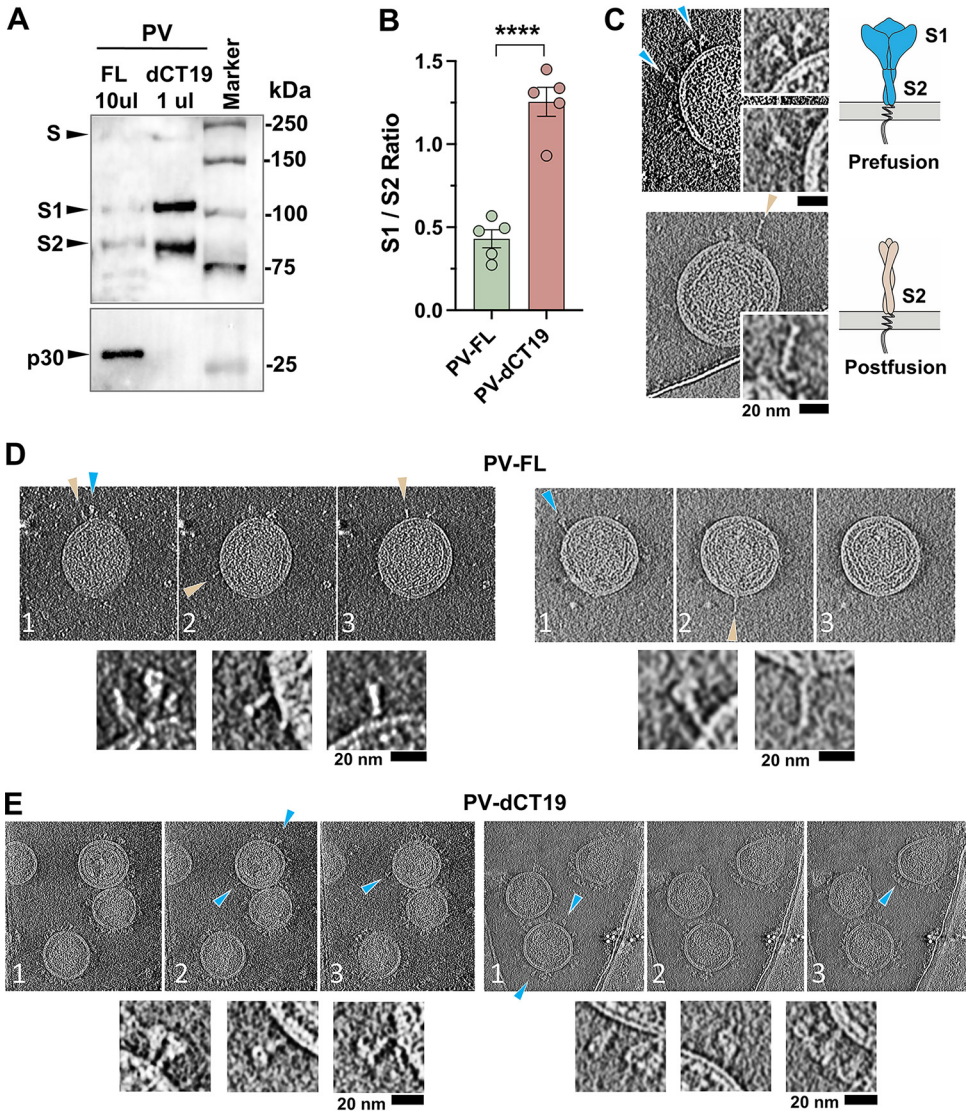

FIG 4 Cytoplasmic tail truncation significantly enhances functional S protein on PV particles. (A) The same PVs shown in Fig. 3A but with PV-FL compared to 10-times-less PV-dCT19 to more accurately measure their S1/S2 ratios. (B) Mean S1/S2 ratios ± SEM of PV-FL and PV-dCT19 analyzed from five Western blots performed with three sets of independently prepared PV batches. Statistical significance was analyzed by an unpaired *t* test (****, $P < 0.0001$). (C) Prefusion (top) (blue arrows) and postfusion (bottom) (light-brown arrow) conformations of spike trimers on sucrose-pelleted PVs examined by cryo-EM tomography. Schematic representations of these two spike conformations are presented at the right. (D) Sequential slices, indicated by the numbers at the bottom left, of PV-FL cryo-electron tomograms, with a mix of prefusion (blue arrows) and postfusion (light-brown arrows) S conformations, enlarged at the bottom. (E) Sequential slices, indicated by the numbers at the bottom left, of PV-dCT19 cryo-electron tomograms, with mostly the prefusion (blue arrows) S conformation, enlarged at the bottom. See also Fig. S2 in the supplemental material.

analyses of PV-dCTs relative to PV-FL (Fig. 4A). Taken together, these data demonstrate that CT truncation enhances S protein incorporation into PVs and S1 retention in the spike, leading to much higher numbers of prefusion spikes on the pseudovirion, thus providing an explanation for the dramatic increase in PV-dCT infectivity.

DISCUSSION

CT truncation of virus entry glycoproteins has been widely used to enhance the infectivity of various PVs. Although several potential explanations were offered, the underlying mechanism for the greatly enhanced PV infectivity is still unclear. Increased cell surface expression of viral glycoproteins upon CT truncation was proposed as an

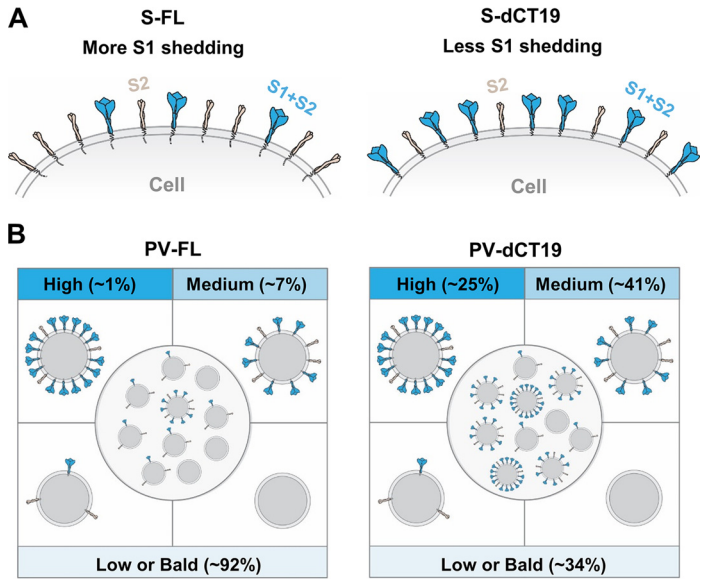

FIG 5 Schematic representation of increased functional spikes on the cell surface and in pseudoviruses upon CT truncation of the S protein. (A) Higher levels of functional S-dCTs are observed on the cell surface, while S2 levels are similar between S-dCTs and S-FL. Functional spikes (in blue), assessed using ACE2-NN-Ig or NTD antibody (Fig. 2D to F), contain both S1 and S2 in the prefusion conformation. Nonfunctional spikes (in light brown), measured using plasma sample 9, which recognizes only S2 (Fig. 2C and G), consist of only S2 in the postfusion conformation. (B) PV-dCTs exhibit much higher levels of functional spikes than PV-FL. The diagrams of four PVs represent those with a high, medium, or low spike density or bald virions. The group of 10 PV particles in the center for PV-FL and PV-dCTs reflects the approximate composition of each PV population with respect to the virion spike density. The proportion of each spike density group in the PV-FL and PV-dCT19 populations was assessed using cryo-EM micrographs (Fig. 3C and D; see also Fig. S1 in the supplemental material).

explanation for the enhanced PV infectivity, but conflicting results were also reported (20, 22–24). Furthermore, even if cell surface expression is increased, the degree of the increase is modest and thus would not be sufficient to explain the dramatic change in PV infectivity. Increased fusogenicity of the glycoproteins induced by CT truncation was also proposed for measles, murine leukemia, vesicular stomatitis, and Nipah viruses (18, 20, 23, 25–27, 33), and a conformational change in the ectodomain induced by inside-out signaling was proposed to explain such increased fusogenicity (20, 25, 27).

Our study here shows that the cell surface expression of CT-truncated SARS-CoV-2 S protein could lead to different outcomes depending on which subunit, S1, S2, or both, is measured (Fig. 5A). If S2 is measured, no significant difference is detected, while a modest increase is observed if both S1 and S2 are measured. However, if only S1 is measured, a much higher S1 level is observed with S-dCT19, -27, and -35 than with S-FL. These data provide an explanation for the conflicting reports on the cell surface expression of the CT-truncated viral entry glycoproteins (20, 22–24).

Our study further identifies two different mechanisms underlying the dramatically increased infectivity of SARS-CoV-2 PVs carrying a CT truncation. Through biochemical and cryo-electron microscopy, we show that CT truncation promotes S protein incorporation into PVs and enhances S1 retention on the S protein. These two events together result in much higher numbers of functional spikes in PV-dCT19, -27, and -35 (Fig. 5B), leading to a dramatic increase in their infectivity. Our results on the increased S1 retention on S-dCTs are consistent with previous reports in which increased fusogenicity upon CT truncation was observed for the entry glycoproteins of various viruses (18, 25–27). Those studies were conducted using syncytium formation assays, and thus, a higher level of the surface subunit of a glycoprotein expressed on the cell surface would have resulted in more efficient syncytium formation. Although further studies are necessary, the increased S1 retention on S-dCTs may arise from the enhanced flexibility of the S2 subunit, which accommodates a more stable S1-S2 interaction.

Our data also show that the differences in spike density induced by CT truncation are much greater in PVs than on the cell surface; while the S2 levels are similar between S-FL and S-dCTs on the cell surface (Fig. 2C), they are much higher in PV-dCTs than in PV-FL (Fig. 3A). A higher spike density in PV-dCTs would be possible if S-dCTs are more enriched in lipid rafts than S-FL because many viruses bud from this microdomain. However, S-dCT27 and S-dCT35 lack some or all of the motifs for palmitoylation, a modification that promotes protein trafficking into lipid rafts (40), and thus, it is unlikely that S-dCTs are enriched in lipid rafts and more efficiently incorporated into the virion. A more likely explanation is that S-dCTs can be more freely incorporated into PVs because they lack the binding motif for Ezrin-Moesin-Radixin proteins that anchor membrane proteins to the cellular cytoskeleton (41). An alternative explanation is that the steric hindrance formed between the structural proteins of PVs (Gag or matrix protein) and the large CT of S-FL makes it difficult to be incorporated into PVs, whereas the smaller CT of S-dCTs allows more efficient incorporation into PVs, as we previously proposed (21).

MATERIALS AND METHODS

Plasmids. The SARS-CoV-2 S protein gene was codon optimized and synthesized by Integrated DNA Technologies based on the protein sequence (Wuhan-Hu-1 strain [GenBank accession number YP_009724390]) and cloned into the pCAGGS vector (42). The genes for cytoplasmic-tail-truncated variants of the SARS-CoV-2 S protein were also synthesized and cloned into the pCAGGS vector. None of these genes contain a tag. The retroviral vector pQCXIX (Clontech), encoding enhanced green fluorescent protein (eGFP) or firefly luciferase (FLuc), and the plasmid expressing the MLV Gag and Pol proteins or the G protein of VSV (VSV-G) were previously described (43). An hTMPRSS2 expressor plasmid was constructed by cloning its residues 1-492 (GenBank accession number NP_005647) into the retroviral vector pQCXIB (44). The hACE2 expressor plasmid was constructed by cloning its residues 20 to 805 (GenBank accession number NM_021804) downstream of the mouse ACE2 signal sequence (MSSSSWLLLSLVAVTTAQS) and the Myc tag sequence into the retroviral vector pQCXIP. The expression plasmid for hACE2-NN-Ig was previously described (45): the hACE2 ectodomain fragment (residues 20 to 615 [GenBank accession number NM_021804]) containing the H374N and H378N mutations was cloned into pcDNA3.1 containing the CD5 signal sequence and the Fc region of human IgG1.

Cells. Human embryonic kidney HEK293T cells were obtained from the ATCC and maintained in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C with 5% CO_2. HEK293T cells transduced to stably express hACE2 and hTMPRSS2 (hACE2/hTMPRSS2-293T cells) or mock transduced (Mock-293T cells) were selected and maintained in medium supplemented with 1 μg/mL puromycin and 10 μg/mL blasticidin (InvivoGen). The transduction vectors for hACE2 and hTMPRSS2 were produced by transfecting pQCXIP-hACE2 or pQCXIB-hTMPRSS2 into HEK293T cells together with the plasmid encoding murine leukemia virus Gag-Pol and the plasmid encoding the G protein of vesicular stomatitis virus. The vector for mock transduction was produced similarly using the empty pQCXIP or pQCXIB plasmid.

MLV PV production and sucrose pelleting. HEK293T cells at ~60% confluence in T75 flasks were transfected using a calcium-phosphate method with 24 μg of total DNA at a ratio of 5:5:1 (by mass) of the retroviral vector pQCXIX encoding eGFP or FLuc, the plasmid expressing MLV Gag and Pol proteins, and the plasmid expressing either the full-length S protein or the truncated version of the S protein of SARS-CoV-2 (Wuhan-Hu-1). Transfected cells were washed at 6 h posttransfection and replenished with 10 mL DMEM supplemented with 10% FBS. The PV-containing culture supernatants were collected at 43 h posttransfection, cleared through 0.45-μm filters, and either immediately aliquoted and stored at −80°C or used for entry experiments.

For sucrose-pelleted PV preparation, 10 mL of the cleared culture supernatants containing PVs precleared by centrifugation in a TX-400 swinging-bucket rotor at 3,000 rpm for 10 min followed by filtration through 0.22-μm filters was loaded onto 2 mL of 30% sucrose (catalog number S7903; Sigma-Aldrich) in NTE buffer (120 mM NaCl, 20 mM Tris, 2 mM EDTA [pH 8.0]) and centrifuged at 50,000 × *g* in an SW41 rotor for 2 h at 10°C. The PV pellets were resuspended in 20 μL NTE buffer with gentle shaking on ice and either used immediately or aliquoted and frozen at −80°C.

PV quantification. PVs were quantified by RT-qPCR using primers and a probe that target the cytomegalovirus (CMV) promoter. Culture supernatants containing PVs were treated with 100 μg/mL RNase A for 1 h at 37°C to degrade RNAs that were not packaged inside the virion, and RNA was extracted with TRIzol and GlycoBlue coprecipitant and digested for 30 min at 37°C with DNase I at 1 IU per 1 μg extracted RNA. DNase I was inactivated by incubation for 10 min at 65°C with EDTA added to a final concentration of 5 mM. DNase-treated RNA was reverse transcribed using a high-capacity cDNA reverse transcription kit (catalog number 4374966; Applied Biosystems). qPCR was performed, using Luna universal probe qPCR master mix (catalog number M3004E; New England BioLabs) with a known quantity of the pQCXIX vector (Clontech), to generate standard curves, and data were collected with CFX Manager 3.1 (Bio-Rad). The primers and probe were synthesized by Integrated DNA Technologies (sense primer 5′-TCACGGGGATTTCCAAGTCTC-3′, antisense primer 5′-AATGGGGCGGAGTTGTTACGAC-3′, and probe 5′-FAM [6-carboxyfluorescein]-AAACAAACT-[ZEN]-CCCATTGACGTCA-IBFQ-3′).

PV entry assay in hACE2-hTMPRSS2-293T cells. A PV entry (transduction) assay was performed by incubating Mock-293T or hACE2/hTMPRSS2-293T cells on 48-well plates with PVs (5×10^8 genome copies in 200 μL per well) for 1 h at 37°C in a CO_2 incubator. Medium was replaced with DMEM containing 10% FBS. The entry levels of PVs expressing firefly luciferase were assessed by measuring luciferase activity using the Luc-Pair firefly luciferase HS assay kit (catalog number LF009; GeneCopoeia) and reading the plates on the SpectraMax paradigm multimode detection platform using SoftMax Pro 6.3 (Molecular Devices).

Screening of human plasma. Deidentified plasma samples were obtained by the Allergy, Asthma, and Immunology Specialists of South Florida, LLC, in mid-2020 for COVID-19 serotyping, and these were exempt (IRB-20-7580) from human subject research under CFR 45.101(b). These plasma samples were screened by Western blotting at a 1:200 dilution for their ability to recognize the S1 and S2 subunits of the SARS-CoV-2 S protein on sucrose-pelleted PV-dCT19. The blot was visualized using 1:10,000-diluted goat anti-human IgG conjugated with horseradish peroxidase (HRP) (catalog number 109-035-098; Jackson ImmunoResearch).

Cell surface and total expression of the spike protein. HEK293T cells at approximately 70% confluence in 6-well plates were transfected with 0.3 μg, unless indicated otherwise, of the plasmid expressing the indicated S protein variant. Cells were detached at 42 h posttransfection in phosphate-buffered saline (PBS) containing 2 mM EDTA. Approximately 1×10^6 cells were fixed with a 2% formaldehyde solution in PBS for 30 min on ice and blocked with PBS containing 2% bovine serum albumin (BSA) (Sigma-Aldrich) and 5% goat serum (Gibco) for 30 min on ice. These cells were then incubated on ice for 90 min in 100 μL of PBS containing either SARS-CoV-2 convalescent-phase plasma at a 1:200 dilution, 3 μg/mL of purified hACE2-NN-Ig (9), or 3 μg/mL of anti-NTD monoclonal antibodies (catalog number SPD-M121; Acro Biosystems), followed by 1:600-diluted anti-hIgG–Alexa Fluor 647 (AF647) (catalog number 109-605-003; Jackson ImmunoResearch). Stained cells were analyzed using an Accuri flow cytometer (BD Biosciences) equipped with the HyperCyt autosampler (IntelliCyt) and ForeCyt 6.2R3 software (IntelliCyt). To measure the total expression levels of the S protein, aliquots of the same cells were permeabilized with 0.1% saponin (Alfa Aesar) in PBS for 10 min at room temperature and subjected to staining on ice as described above.

Western blot analysis of S protein in cell lysates and on pseudovirions. For the detection of S protein bands in the cell lysates, HEK293T cells in 6-well plates were transfected to express S-FL or S-dCT5 and lysed with 0.2 mL of PBS containing 0.5% dodecyl maltopyranoside (Anatrace) and a protease inhibitor cocktail (catalog number A32955; Thermo Scientific). Fifteen microliters of the lysates was analyzed by WB using rabbit anti-S protein antibody (catalog number NR-52947; BEI Resources) at a 1:5,000 dilution or 1 μg/mL of NTD antibody (catalog number SPD-M121; Acro Biosystems) For the analyses of the S protein density on the virion, sucrose-pelleted PVs were analyzed by SDS-PAGE and WB. Unless indicated otherwise, 5 μL PVs was analyzed in each WB analysis. PV proteins were separated on a 4 to 12% Bis-Tris gel (catalog number NW041122; Life Technologies) and transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with 5% milk in 1$\times$ Tris-buffered saline containing 0.1% Tween 20 for 1 h and blotted with human SARS-CoV-2 convalescent-phase plasma at a 1:200 dilution to detect the S protein bands or 1 μg/mL anti-p30 MLV Gag antibody (catalog number ab130757; Abcam) to detect p30 bands as a PV quantity control. S protein bands were detected using 10 ng/mL mouse anti-human IgG antibody conjugated with polymerized HRP (catalog number 61R-I166AHRP40; Fitzgerald), and p30 bands were detected using 1:10,000-diluted goat anti-mouse IgG–HRP polyclonal antibody (catalog number 115-036-062; Jackson Immuno Laboratory). Bands were visualized using the SuperSignal West Atto ultimate-sensitivity substrate (catalog number A38555; Thermo Scientific), and the band intensities were measured using Image Lab software (Bio-Rad).

Cryo-EM of full-length and dCT19 spike proteins. For cryo-EM samples, 300-mesh R2/2 copper Quantifoil grids (Electron Microscopy Sciences [EMS]) were negatively glow discharged for 30 s. The PV sample (3.5 μL) was applied onto the grid and incubated for 30 s, followed by plunge-freezing into liquid ethane with a Vitrobot Mark IV instrument (100% humidity, 4°C, and 4.5 s per blot). The grids were imaged on an FEI Tecnai T12 120-kV electron microscope.

For cryo-electron tomography, grids were prepared as described above, with the addition of 10-nm gold beads at a ratio of 14:1 (vol/vol) before plunge-freezing. Dose-symmetric tilt series (to $\pm$48° with 3° increments) were collected on a Titan Krios instrument with a K3 detector (total dose of 100 $e^-/Å^2$; 7 frames per angle) and a 20-eV energy filter. The data set was motion corrected with MotionCor2 (46), reconstructed with IMOD (47), denoised with Topaz (48), and processed using ImageJ software.

Statistical analysis. All of the statistical details of specific experiments, which included the statistical tests used, numbers of samples, mean values, standard errors of the means (SEM), and *P* values derived from the indicated tests, are described in the figure legends and shown in the figures. Statistical analyses were conducted utilizing GraphPad Prism version 8.0 (GraphPad Software Inc.). Triplicate and other replicative data are presented as means $\pm$ SEM. A *P* value of <0.05 was considered to be statistically significant. For comparisons between two treatments, Student's *t* test (unpaired) was used. For comparisons of each group with the mean of every other group within a data set containing more than two groups, either one-way analysis of variance (ANOVA) with Dunnett's multiple-comparison test or two-way ANOVA with Sidak's multiple-comparison test was used.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.

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We declare no competing interests.

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Polybasic KKR Motif in the Cytoplasmic Tail of Nipah Virus Fusion Protein Modulates Membrane Fusion by Inside-Out Signaling▽

Hector C. Aguilar,[1] Kenneth A. Matreyek,[1] Daniel Y. Choi,[1] Claire Marie Filone,[4] Sophia Young,[1] and Benhur Lee[1,2,3]*

Department of Microbiology, Immunology, and Molecular Genetics,[1] Department of Pathology and Laboratory Medicine,[2] and AIDS Institute,[3] David Geffen School of Medicine at UCLA, Los Angeles, California 90095, and Department of Microbiology, University of Pennsylvania, Philadelphia, Pennsylvania 19104[4]

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The cytoplasmic tails of the envelope proteins from multiple viruses are known to contain determinants that affect their fusogenic capacities. Here we report that specific residues in the cytoplasmic tail of the Nipah virus fusion protein (NiV-F) modulate its fusogenic activity. Truncation of the cytoplasmic tail of NiV-F greatly inhibited cell-cell fusion. Deletion and alanine scan analysis identified a tribasic KKR motif in the membrane-adjacent region as important for modulating cell-cell fusion. The K1A mutation increased fusion 5.5-fold, while the K2A and R3A mutations decreased fusion 3- to 5-fold. These results were corroborated in a reverse-pseudotyped viral entry assay, where receptor-pseudotyped reporter virus was used to infect cells expressing wild-type or mutant NiV envelope glycoproteins. Differential monoclonal antibody binding data indicated that hyper- or hypofusogenic mutations in the KKR motif affected the ectodomain conformation of NiV-F, which in turn resulted in faster or slower six-helix bundle formation, respectively. However, we also present evidence that the hypofusogenic phenotypes of the K2A and R3A mutants were effected via distinct mechanisms. Interestingly, the K2A mutant was also markedly excluded from lipid rafts, where ~20% of wild-type F and the other mutants can be found. Finally, we found a strong negative correlation between the relative fusogenic capacities of these cytoplasmic-tail mutants and the avidities of NiV-F and NiV-G interactions ($P = 0.007$, $r^2 = 0.82$). In toto, our data suggest that inside-out signaling by specific residues in the cytoplasmic tail of NiV-F can modulate its fusogenicity by multiple distinct mechanisms.

Nipah virus (NiV) and *Hendra virus* (HeV) are deadly emerging zoonotic viruses belonging to the new *Henipavirus* genus within the family *Paramyxoviridae* (66). NiV infections result in respiratory and neurological symptoms, often leading to fatal encephalitis, the primary reason for death in humans (32, 64). Microvascular endothelial cell syncytium formation is a hallmark of NiV infection, associated with endothelial cell death, vascular inflammation, and necrosis (70). The mortality rate of NiV-infected humans ranges from ~40% in the original outbreaks in Malaysia and Singapore in 1999 to 2000 to ~70% in Bangladesh in 2005 (5, 6). The natural reservoir for NiV has been determined to be fruit bats of the genus *Pteropus* (46), and pigs served as the intermediate amplifying host in the original Malaysian-Singaporean outbreaks. Ominously, even though human-to-human transmission was not documented in the original outbreaks, direct bat-to-human and human-to-human transmissions have been reported in the later outbreaks in Bangladesh (5, 6). NiV is classified as a BSL4 pathogen and has also been designated as a select agent because of its bio- or agroterrorism potential. These characteristics of NiV underscore the need for research and treatment development against this perilous pathogen and the need for understanding of the necessary components and mechanisms of virus-cell and cell-cell fusions in order to inhibit viral infection and spread.

For paramyxoviruses, two separate membrane proteins are involved in the fusion process, the attachment protein (H, HN, or G), which binds to the receptor molecule in the target cell membrane, and the fusion protein (F) that actually carries out membrane fusion. For most paramyxoviruses, both F and its homotypic attachment protein are necessary for membrane fusion, except for rare cases like the hyperfusogenic simian virus 5 (SV5) W3A isolate (27, 48). Activation of F is believed to occur through the following three steps: (i) binding of the attachment protein to the receptor, (ii) interaction of the attachment and F proteins (or changes thereof), and (iii) conformational changes in F that mediate membrane fusion. The fusion (F) and attachment (G) envelope glycoproteins in NiV or HeV are both necessary for cell-cell fusion, syncytium formation, and viral entry. G is responsible for binding to its cognate receptor, ephrinB2 (9, 44), and at least for NiV, ephrinB3 can also be used as an alternative receptor (45). The high expression of ephrinB2 on neurons and endothelial cells and the patterns of expression of ephrinB3 in the central nervous system largely account for the cell tropism of NiV and HeV (9, 44, 45). However, much less is known about the components necessary for the subsequent steps in the activation of NiV fusion (NiV-F) or HeV-F protein.

Paramyxovirus F proteins belong to the class I fusion proteins that share several structural and functional characteristics. The structures of the retroviral Moloney murine leukemia virus (MoMuLV) p15E, lentiviral human immunodeficiency virus type 1 (HIV-1) gp41, Ebola virus GP2, paramyxovirus SV5 F, and influenza virus hemagglutinin (HA) fusion proteins have all been shown to have similar trimeric coiled-coil core

* Corresponding author. Mailing address: Department of MIMG, 3825 MSB, 609 Charles E. Young Drive East, UCLA, Los Angeles, CA 90095. Phone: (310) 206-8792. Fax: (310) 267-2580. E-mail: bleebhL@ucla.edu.

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structures, suggesting similar membrane fusion mechanisms (8, 14, 20, 72). Class I fusion proteins of enveloped viruses are synthesized as precursors that must be cleaved and hence activated into a metastable conformation that is ready for enabling virus-cell membrane fusion. Typically, cleavage generates a new N terminus that contains a hydrophobic fusion peptide motif. Upon activation of the fusion protein through receptor binding and/or endosomal low pH, the fusion peptide gets inserted into the host cell target membrane. Class I fusion proteins also contain two heptad repeat regions (HR1 and HR2); the C-terminal HR2 region is generally thought to be preformed, but the N-terminal HR1 region is formed only upon fusion peptide insertion (14, 35, 72, 73). Class I fusion proteins function as trimers, and the HR1 and HR2 regions have a strong propensity to fold into coiled-coil domains during six-helix bundle (6HB) formation. The free energy released from fusion protein refolding from the metastable prefusion state to the stable postfusion 6HB state likely drives the virus-host cell membranes together, overcoming the electrostatic repulsion intrinsic to the negatively charged phospholipids' head groups of the two membranes (38, 55).

For NiV and HeV, the fusion protein is cleaved within the endosomal compartment from the precursor F_0 to the F_1 and F_2 subunits (18, 39). Such cleavage is likely required for activation of the F protein into the metastable state. For NiV, after activation into the metastable state, not much is known about the subsequent steps in the triggering of the fusion protein that leads to eventual membrane fusion. We and others have recently reported that N-glycans in both the NiV-F and HeV-F proteins have some effects on protein expression and membrane fusion (3, 15, 40). In addition, we identified N-glycans in NiV-F that both reduce fusion and viral entry and protect the virus against neutralizing antibodies (Abs) (3). These results show some uniqueness of the *Henipavirus* genus fusion proteins. However, little is known about other domains in NiV-F or HeV-F that may have an important role in membrane fusion. Triggering of fusion is usually envisioned to involve primarily the ectodomain of the fusion protein. However, accumulating evidence from retroviral (13, 25, 50, 54), lentiviral (41, 42), and other paramyxoviral (65, 67) envelope (Env) proteins suggests that the Env cytoplasmic tail (CT) is involved in regulating the fusion process.

Multiple reports indicate that fusion mediated by the ectodomain of the retrovirus MoMuLV (2), the lentiviruses simian immunodeficiency virus (SIV) (61) and HIV-1 (71), and the paramyxovirus SV5 (67) fusion proteins can be modulated by inside-out signaling from the CT. Truncation of the long CT of lentiviral Env proteins occurs under certain culture conditions, and increased fusogenicity has been reported for truncated versions of SIV, HIV-1, and HIV-2 Env (16, 31, 41, 60, 61, 76). For the paramyxovirus SV5 F protein, isolates with a short (20-residue) CT (W3A and WR) cause extensive cell-cell fusion, whereas isolates with an extended CT (T1 and SER) cause little or no cell-cell fusion, and truncation of the CT restores fusion to levels seen in W3A and WR isolates (28, 65). For MoMuLV, SIV, and SV5, the hyperfusogenicity caused by truncation of the CT is linked to overall conformational changes in the ectodomain of the protein (2, 61, 67). In MoMuLV and the Mason-Pfizer monkey virus, the CT is even protease cleaved during viral maturation to "prime" the fusion protein for fusogenicity (2, 13). Here we investigated the potential role(s) of the CT of the NiV fusion protein in cell surface expression (CSE), processing, membrane fusion, and viral entry and defined specific residues in a polybasic motif in the CT that can affect the conformation of the ectodomain, fusogenicity, and interaction of the fusion protein with the attachment glycoprotein.

MATERIALS AND METHODS

Expression plasmids and codon optimization. The codon-optimized NiV-G and NiV-F genes were tagged at their C termini with HA and AU1 tags, respectively, as previously described (33). The NiV-HR2-Fc construct was made by fusing the heptad repeat region 2 sequence of NiV-F (amino acids 447 to 488) with the human immunoglobulin G1 Fc constant region as previously described (3, 44). The deletion mutants −T, −T1, −T2, −T3, −T4, −T12, and −T234 and point mutants K1A, K2A, R3A, N4A, and T5A were made by deleting or mutating the codon-optimized wild-type (WT) NiV-F plasmid with appropriately designed primers and the QuikChange site-directed mutagenesis kit (Stratagene, Cedar Creek, TX). All mutations and deletions were confirmed by sequencing the entire open reading frame.

Cell culture. Vero cells were cultured in minimal essential medium alpha with 10% fetal bovine serum (FBS). PK13 and 293T cells were cultured in Dulbecco's modified Eagle's medium with 10% FBS. We obtained 293T and Vero cells from the American Type Culture Collection, and PK13 (porcine fibroblasts) cells were a kind gift from Irvin Chen at the University of California Los Angeles.

Quantitation of cell-cell fusion. Codon-optimized NiV-G and codon-optimized WT or mutant NiV-F expression plasmids (1:1 ratio, 1 μg total) (3, 33) were transfected with 1.5 μg pcDNA3.1 plasmid as filler DNA into 293T or Vero cells growing in 12-well plates at 80% confluence, as indicated. At 12 to 18 h posttransfection, cells were stained with 4′,6′-diamidino-2-phenylindole (DAPI) and syncytium formation was quantified by counting the nuclei in syncytia per ×100 field (at least 10 fields were counted per condition). Syncytia were defined as four or more nuclei visualized within a common cell membrane, as indicated previously (3).

Quantification of NiV-F and NiV-G CSE levels by flow cytometry. Production of antisera from genetically immunized rabbits (with NiV-M and -F or -G expression plasmids) was previously described (44). Sera containing anti-F or anti-G specific activities were used for flow cytometry on NiV-F- or -G-transfected cells at a 1:1,000 dilution. Bound Ab was detected with phycoerythrin-conjugated goat anti-rabbit Abs (Caltag, Burlingame, CA). Antisera were also raised in rabbits immunized with peptides corresponding to amino acids 39 to 57 and 331 to 348 of NiV-F_2 and NiV-G, respectively, as previously described (3, 33). These regions were previously shown to be immunogenic (10). For quantitation of binding of the monoclonal Abs (MAbs), flow cytometry was performed with MAb concentrations of 0.03 to 3 μg/ml. For calculating the binding ratios of any given pair of Abs, data obtained from equal concentrations of the respective Abs were used.

Reverse pseudotype viral entry assay. The ephrinB2 NiV receptor protein was pseudotyped onto a reporter virus, vesicular stomatitis virus (VSV), by transfecting an ephrinB2 expression plasmid into 293T cells and subsequently infecting these cells with recombinant VSV expressing the *Renilla* Luc reporter gene (VSV-ΔG-rLuc), similarly to the procedure described previously for preparation of NiV-F- and -G-pseudotyped VSVrLuc virions (3, 44, 45). ephrinB2 reverse-pseudotyped virions were purified over a 20% sucrose cushion as for NiV-F- and -G-pseudotyped viruses. 293T cells plated in 96-well plates were transfected with NiV-G and WT or mutant NiV-F and, 10 to 12 h later, infected with reverse-pseudotyped virions in phosphate-buffered saline–1% FBS for 2 h at 37°C over a 5-log viral dilution range (10^{-2} to 10^{-6}). After 2 h, cells were washed and 293T cell growth medium was added. At 24 h postinfection, cells were lysed and luciferase activity was measured as relative light units (RLU) with a *Renilla* luciferase detection system (Promega, Madison, WI) and a Veritas microplate luminometer (Turner Biosystems, Sunnyvale, CA). Quantitation of viral genome copies for the ephrinB2 VSV-pseudotyped viral prep was performed exactly as previously described (3). For quantitation of neutralization of viral entry, the reverse-pseudotyped viral entry assay was performed as described above, except in the presence of the indicated amounts of the specified Abs. For the mixed heterotrimer experiments with the K1A and K2A or R3A mutant proteins, the indicated DNA ratios of the expression plasmids for the indicated proteins were transfected into 293T cells 18 h before infection with the reverse-pseudotyped virions.

Western blot analysis of surface NiV-F and NiV-G proteins. Codon-optimized NiV-F and/or NiV-G expression plasmids (1:1 ratios when in combination) were transfected into 293T cells plated in six-well plates (total of 2 μg F and/or G plasmids with 3 μg PCDNA3.1 plasmid as filler DNA/well), as indicated. Cells were either cell surface biotinylated or not (EZ link Sulfo-NHS-LC-Biotin; Pierce, Rockford, IL), as specified, and biotinylated proteins were precipitated with streptavidin-agarose beads (Pierce, Rockford, IL). Twenty percent of the biotinylated cell lysate was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and subsequently detected by Western blotting with anti-tag (HA or AU1), anti-F_2, or anti-G peptide Abs, as indicated. Primary and secondary Abs were used at 1:1,000 and 1:20,000 dilutions, respectively, followed by ECL Plus detection (Amersham Biosciences, Piscataway, NJ). For quantification of relative processing levels for the various NiV-F proteins, the ratio of the densitometric units of the F_1 subunit over those of the sum of the precursor F_0 and the F_1 subunits was calculated.

Lipid raft association of NiV-F proteins. 293T cells transfected with WT or mutant NiV-F proteins (as described above) were washed with phosphate-buffered saline and resuspended in TNE buffer (25 mM Tris [pH 7.5], 150 mM NaCl, 5 mM EDTA) containing a protease inhibitor cocktail (Roche, Indianapolis, IN). Cells were Dounce homogenized, and their nuclei were isolated and discarded. Postnuclear supernatants were treated with 1% Triton X-100 for 30 min at 4°C. Cell lysates were then brought up to a 40% OptiPrep (Sigma-Aldrich, St. Louis MO) concentration in 1.2 ml, placed at the bottom of an ultracentrifuge tube, and layered sequentially with 30% (3 ml) and 5% (0.8 ml) OptiPrep layers (in TNE buffer plus protease inhibitors). These discontinuous gradients were centrifuged at 45,000 rpm for 16 h at 4°C in an SW50.1 rotor. After centrifugation, 12 equal fractions were manually collected from the top, protein from 200 μl of each fraction was precipitated by a methanol-chloroform extraction method (69), and each fraction was analyzed by Western blotting. The overall lipid raft domain isolation procedure was similar to that used by Fleming et al. (22).

Production of NiV-HR2-Fc immunoadhesin and fusion inhibition. The NiV-HR2-Fc expression plasmid was transfected into 293T cells, and at 24 h posttransfection, supernatants were collected and concentrated with a Centriplus YM-10 filter (Millipore, Bedford, MA). Protein concentrations were measured by an Fc-specific enzyme-linked immunosorbent assay as previously described (44). For NiV fusion inhibition, the indicated amounts of NiV-HR2-Fc were added to 293T cells transfected with NiV-G and WT or N-glycan mutant NiV-F expression plasmids. Fusion was quantified after overnight incubation as described above.

Fusion kinetics of WT or mutant NiV-F proteins. The fusion kinetics of WT and mutant NiV-F proteins were determined in a β-lactamase reporter cell-cell fusion assay as previously described (3, 34, 53). For better sensitivity, the β-lactamase gene was also codon optimized for mammalian cell expression (Geneart, Inc., Toronto, Ontario, Canada). Fusion-nonpermissive PK13 effector cells were cotransfected with β-lactamase, NiV-G, and WT or mutant NiV-F expression constructs with Lipofectamine 2000. These were then added to 293T target cells labeled with CCF2-AM dye. Effector and target cells were mixed and incubated at 37°C, and cell-cell fusion was detected by analyzing the shift from green to blue fluorescence, indicating β-lactamase cleavage of CCF2. Fluorescence was quantified every 3 min with a CytoFluor Series 4000 Fluorescence multiwell plate reader (PerSeptive Biosystems, Framingham, MA). The results are expressed as the ratio of blue to green fluorescence obtained with NiV-G- and NiV-F-transfected effectors minus the background blue and green fluorescence obtained with empty-vector-transfected cells.

NiV-F–NiV-G coimmunoprecipitation. 293T cells in 10-cm plates were transfected with 20 μg of the indicated NiV-F-G plasmids at a 1:1 ratio with Lipofectamine 2000. At 24 h posttransfection, cells were lysed and cell lysates were subjected to immunoprecipitation as previously described (3, 33), with a 1:100 dilution of anti-NiV-G peptide serum. Coimmunoprecipitated (co-IP) proteins were analyzed by Western blotting with the appropriate anti-tag Ab as described above and then quantified by densitometry with a VersaDoc Imaging System (Bio-Rad, Hercules, CA).

RESULTS

The membrane-proximal region in the CT of the NiV fusion protein plays a role in membrane fusion. The CT of NiV-F can be conveniently divided into four distinct regions, i.e., a membrane-proximal polybasic region (T1), a functional tyrosine-based endocytic motif (YSRL) (18, 39), a highly charged region (T3), and a C-terminal region that is rich in polar residues (T4). To investigate the potential roles that these cytoplasmic regions may play in membrane fusion, protein expression, processing, and transport, we made a series of deletion mutants that lack various regions of the CT, as illustrated in Fig. 1A. The first amino acid of the CT is a glutamic acid and is likely required to demarcate the membrane-spanning domain. Thus, we kept this amino acid in every deletion mutant in order to maximize the likelihood of correct protein folding and expression. We then analyzed the relative levels of CSE and processing of such deletion mutants and compared them to those of WT NiV-F.

Briefly, 293T cells transfected with expression plasmids for WT NiV-F or the indicated mutant proteins were cell surface biotinylated and lysed and cell surface proteins were precipitated with streptavidin beads and then NiV-F detected by Western blotting with the specified Abs (Fig. 1B). Alternatively, we performed flow cytometric analysis on parallel samples of 293T cells expressing WT NiV-F or the deletion mutants with polyclonal anti-NiV-F antiserum 834, which was previously described (3, 33, 45) (Fig. 1D). Both biotinylation and flow cytometric CSE analyses indicated that the deletion mutants were expressed to at least 50% of the WT level and some were even expressed at levels higher than that of the WT. The cell surface biotinylation experiments also showed that the deletion mutants were cleaved and processed more or less at WT levels, with the exception of the deletion mutant missing the entire CT (Fig. 1B, bottom). Interestingly, although this deletion mutant (−T) did not include removal of the C-terminal AU1 tag, the anti-AU1 MAb was not able to detect this protein by Western blotting, perhaps because of the close proximity of the AU1 tag to the detergent-lipid micelles (Fig. 1B, top left part). However, the −T mutant was clearly expressed, as shown by blotting with an anti-F_2 peptide antiserum previously described (3, 33) (Fig. 1B, right part), as well as by flow cytometry (Fig. 1D). Notably, the −T mutant was also not processed efficiently despite being expressed on the cell surface (Fig. 1B, right part).

Cleavage of NiV-F requires active endocytosis and processing by endosomal cathepsin L, which is in part mediated by the YXXΦ endocytic motif in the T2 region (18, 39). Since the AU1 tag contains a putative YXXΦ endocytic motif, we sought to determine if our AU1 tag had any inadvertent effects on the expression or processing of NiV-F. Figure 1C shows that there were no differences in cleavage or processing efficiency between tagged and untagged WT NiV-F (F and F_{NA}, respectively). Interestingly, the untagged version of the T2 mutant ($-T2_{NA}$), which lacks the endogenous YXXΦ motif, also showed no differences from untagged WT NiV-F (F_{NA}), similar to what has been found with the tagged versions (compare Fig. 1B and C).

Next, we asked whether the CT deletion mutations affected the fusogenicity of the NiV-F fusion protein. To normalize for the differences in CSE, we compared the fusion-to-CSE ratios induced by WT NiV-F and the indicated deletion mutants (Fig. 1D). We performed our syncytium-forming assays by transfecting in 0.3 μg of NiV-F and -G per 12-well plate, which was previously determined to result in CSE and fusion with the WT NiV-F protein in the linear range of measurement (3). CSE was measured by flow cytometry as described above, and fusion was determined by counting nuclei inside syncytia (more than

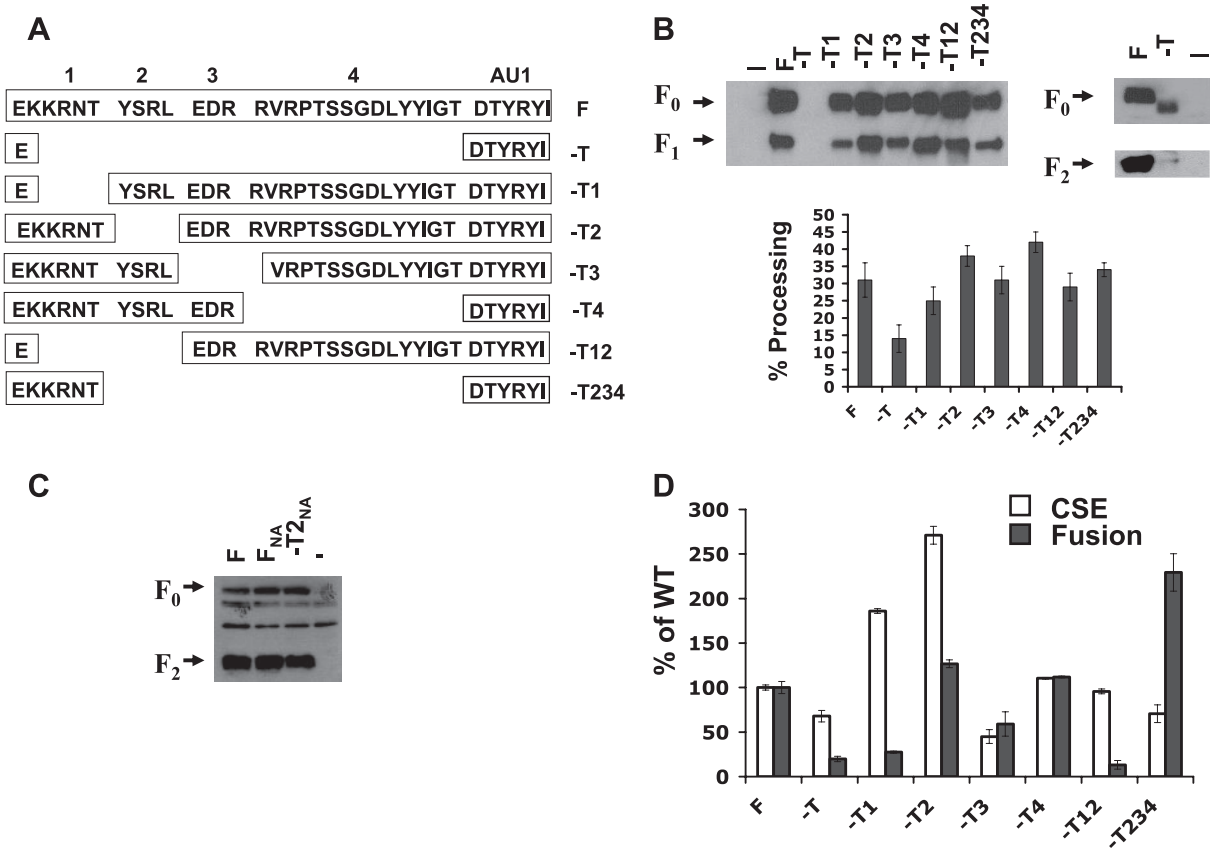


FIG. 1. Analysis of NiV-F CT deletion mutants. (A) Schematic of the NiV-F CT deletion mutants. NiV-F CT was divided into four regions (numbered 1, 2, 3, and 4) as described in the text, and the names of the deletion mutants examined are indicated. (B) Western blot analysis of immunoprecipitated surface WT and mutant NiV-F proteins. Briefly, biotinylated cells were lysed, cell surface biotinylated proteins were precipitated with streptavidin agarose beads, and NiV-F was detected in the biotinylated precipitates by Western blotting with either a monoclonal anti-AU1 tag Ab (left part) or a rabbit anti NiV-F_2 antipeptide Ab (3) (right part). Percent processing was calculated as the densitometric units of the F_1 subunit over those of the sum of the precursor F_0 and the F_1 subunits (bottom part) ($n = 3$). (C) The AU1 tag does not affect cleavage and processing of F. Identical cell surface biotinylation experiments were performed with tagged (F) and untagged versions of WT F (F_{NA}) and the −T2 mutant ($-T2_{NA}$). A rabbit anti NiV-F_2 antipeptide Ab (3) was used to detect NiV-F. (D) Relative levels of CSE and fusion obtained for WT NiV-F and the indicated CT deletion mutants. Fusion was determined by counting nuclei in syncytia per field. At least 10 fields were counted per condition. CSE was determined by flow cytometry with polyclonal anti-NiV-F specific antiserum as described previously (3). Both CSE and fusion levels were separately normalized to levels of WT NiV-F protein, set at 100%. Data shown are averages ± standard errors from three independent experiments.

four nuclei per cell) per microscopic field, respectively. Figure 1D shows the relative CSE and fusogenicity of WT NiV-F and the indicated mutants, and Table 1 (top) shows their corresponding fusion/CSE ratios. Interestingly, all mutants that lacked the membrane-proximal T1 region (−T1 and −T12) were hypofusogenic and had fusion/CSE ratios of less than 0.5 (by definition, that of WT NiV-F is 1.0), while all mutants that retained the T1 region (−T3, −T4, and −T234) had fusion/CSE ratios equal to or higher than that of WT NiV-F, with the exception of the −T2 mutant, which had a fusion/CSE ratio of 0.5. Since the fusion defect in the tailless mutant (−T) was likely due at least partially to its processing defect, it was not included in Table 1 for comparison. These results indicate that the CT, in particular, the membrane-proximal T1 region of the CT, plays an important role in membrane fusion and syncytium formation.

Polybasic KKR motif in the membrane-proximal region of the NiV-F CT modulates NiV-F-induced membrane fusion. To

TABLE 1. Fusion/CSE ratios

Env fusion protein	Fusion/CSE ratio[a]
F	1.0
−T1	0.1
−T2	0.5
−T3	1.3
−T4	1.0
−T12	0.1
−T234	3.2
K1A	5.5
K2A	0.2
R3A	0.3
N4A	0.9
T5A	0.9

[a] The ratio of the normalized fusion and CSE values for each mutant was calculated from the data in Fig. 1D and 2C. By definition, the fusion/CSE ratio for WT NiV-F would be 1.0 (100%/100%). Ratios of >1 indicate increased fusogenicity, while mutants with decreased fusogenicity would have ratios of <1.

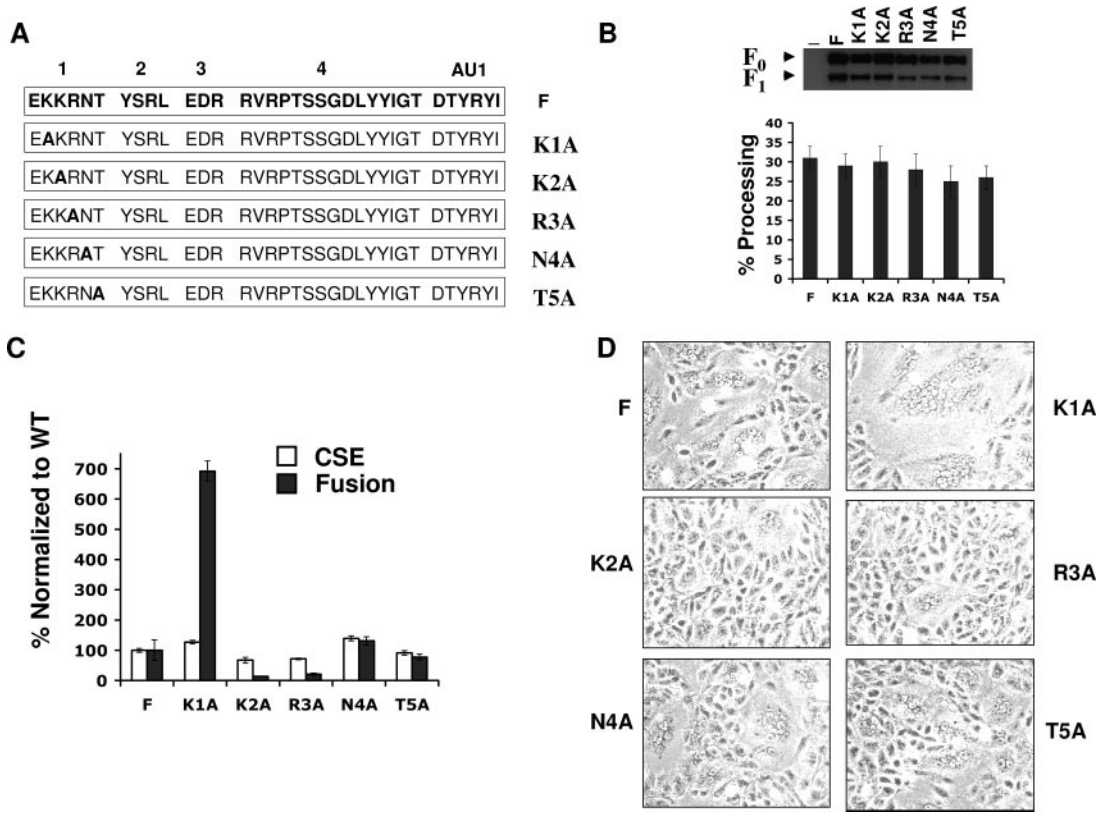


FIG. 2. Analysis of membrane-proximal point mutations in the CTs of NiV-F. (A) Schematic of the NiV-F CT point mutants, showing the sequence of the whole CT, and the positions of the five alanine substitutions in the membrane-proximal region, designated K1A, K2A, R3A, N4A, and T5A. (B) Western blot analysis of immunoprecipitated cell surface biotinylated WT and mutant NiV-F proteins. Surface proteins were analyzed exactly as described in Fig. 1B. Percent processing was also analyzed as described in the legend to Fig. 1B, and the densitometric results are shown graphically. (C) Relative levels of CSE and fusion obtained for WT NiV-F or CT point mutant proteins in 293T cells. Fusion and CSE were determined exactly as for Fig. 1C. Data shown are averages ± standard errors from three independent experiments. (D) Pictures of syncytium formation by the WT NiV-F or the various NiV-F point mutants and WT NiV-G in Vero cells. Representative ×100 fields are shown.

finely map the particular residues within the T1 region that can modulate NiV-F-mediated fusion, we individually mutated each amino acid of the T1 sequence (KKRNT) to an alanine, as depicted in Fig. 2A. 293T cells transfected with an expression plasmid encoding each of the alanine scan mutants were cell surface biotinylated, lysed, precipitated with streptavidin, and subjected to Western blotting to detect NiV-F as described above. All of the alanine scan mutants had levels of CSE and processing similar to those of WT NiV-F (Fig. 2B). Similar levels of CSE of WT and mutant NiV-F proteins were also observed by flow cytometric analyses (Fig. 2C). Next, we determined the fusogenicity of these mutants by quantifying syncytium formation. Representative pictures of syncytia formed by each mutant are shown in Fig. 2D, and the fusion/CSE ratios for WT NiV-F and the indicated mutant were determined (Table 1, bottom). Interestingly, despite WT levels of CSE, mutation of the K1 residue resulted in hyperfusogenicity (fusion/CSE ratio of 5.5) while mutation of the K2 or R3 residue resulted in hypofusogenicity (fusion/CSE ratios of 0.2 and 0.3, respectively) (Table 1, bottom). Mutation of the N4 or T5 residue did not result in any significant change in CSE or fusion (ratio of 0.9) relative to the WT NiV-F protein (Fig. 2C and D and Table 1, bottom). Similar but less dramatic effects on fusogenicity were observed in Vero cells. In summary, these results indicate that the membrane-proximal polybasic KKR sequence in the CT of NiV-F protein can up- or downmodulate its fusogenicity.

Fusion of membrane-proximal NiV-F mutants correlates with entry of ephrinB2-reverse-pseudotyped virus-like particles. Next, we sought to determine if the differences in cell-cell fusion exhibited by the NiV-F CT mutant proteins corresponded to viral entry differences. However, some of these CT mutants were very inefficiently incorporated into our pseudotyped VSV-*Renilla* luciferase (VSVrLuc) reporter viruses, a previously established method for examining NiV envelope-mediated entry (3). To circumvent the problem of variable envelope protein incorporation into VSVrLuc, we developed a novel reverse-pseudotype VSVrLuc entry assay, for which we reverse pseudotyped VSVrLuc with the NiV receptor ephrinB2 (B2-VSVrLuc). We then used these B2-VSVrLuc virions to infect 293T cells previously transfected with equal amounts of mutant or WT NiV-F along with WT NiV-G in a 96-well plate format. Infection of cells expressing HIV Env glycoproteins with viral particles reverse pseudotyped with CD4 and the corresponding coreceptor has been previously reported (36, 56). Figure 3A shows that B2-VSVrLuc viral entry only occurs when cells express both the NiV-F and NiV-G glycoproteins (Fig. 3A). In addition, reverse-

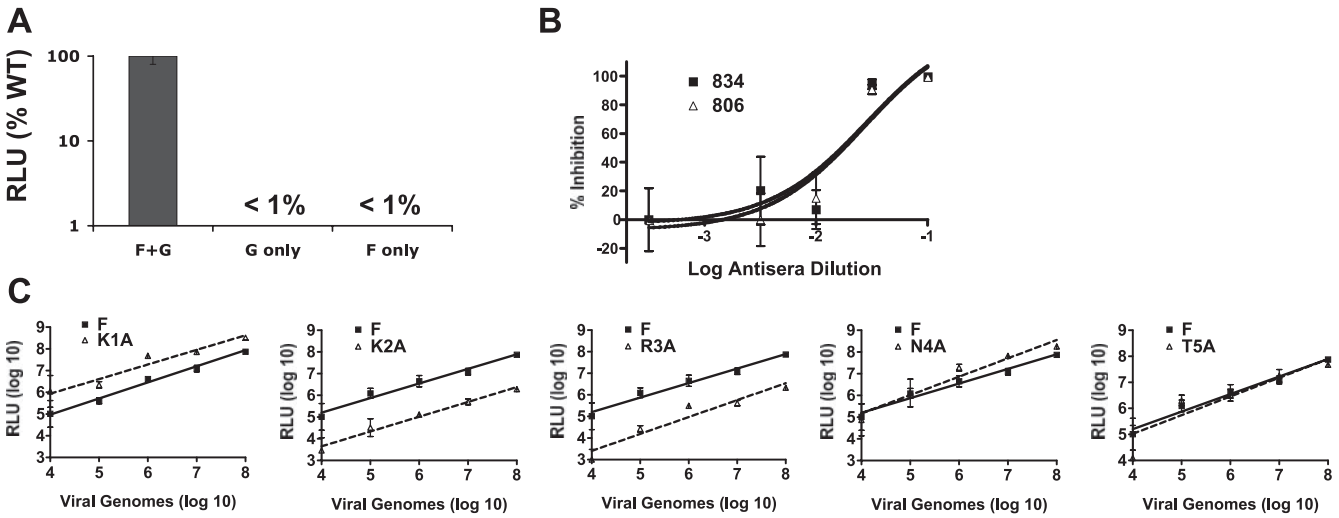


FIG. 3. Reverse-pseudotyped viral entry assay for membrane-proximal CT point mutants. (A) An ephrinB2-pseudotyped VSV-*Renilla* luciferase reporter virus (B2-VSV-rLuc) was used to infect 293T cells previously transfected with expression plasmids for NiV-F–NiV-G, NiV-G alone, or NiV-F alone. Numbers of RLU are shown on a logarithmic scale. (B) Reverse-pseudotyped viral entry into NiV-F- or NiV-G-transfected 293T cells was inhibited by anti-NiV-F and anti-NiV-G specific antisera 834 and 806, respectively. Data are presented as percent inhibition, where 0% represents infection in the absence of any antiserum. The data were normalized as follows. The number of RLU obtained at each serum dilution was calculated as a percentage of the average number of RLU obtained in the absence of any antiserum. Percent inhibition was then calculated as 100% minus the percent infection at each serum dilution. The percent inhibition values were regressed and graphed with GraphPad PRISM. An average of two experiments is shown, with four independent wells per datum point (serum dilution) ± the standard deviation. (C) Relative entry levels of B2-VSV-rLuc virus into 293T cells expressing the WT NiV-G protein and the WT or mutant NiV-F protein. RLU were quantified 24 h postinfection and graphed against the number of viral genomes per milliliter. A single preparation of B2-VSV-rLuc was used for all of the experiments shown. The number of genome copies in the viral preparation was analyzed by reverse transcription-PCR as described in Materials and Methods. The data shown are averages from three independent experiments ± the standard deviations.

pseudotyped viral entry was specifically blocked by previously characterized anti-NiV-F or anti-NiV-G antiserum (3, 33, 44) (Fig. 3B).

We then determined the entry of these B2-VSVrLuc virions into cells expressing WT NiV-G and WT NiV-F or the indicated NiV-F CT point mutants. Entry into K1A-expressing cells was about 8- to 10-fold higher than that of WT NiV-F over several logs of viral input. Conversely, entry into K2A- and R3A-expressing cells was 8- to 30-fold lower than that of WT NiV-F over the same range of viral inputs. Entry levels obtained for the cells expressing the N4A or T5A mutant protein were similar to those expressing WT NiV-F. Thus, our reverse-pseudotype B2-VSVrLuc entry assay results are consistent with our cell-cell fusion results and further demonstrate that the membrane-proximal polybasic KKR motif in the NiV-F CT can modulate virus-cell membrane fusion.

Differential binding and neutralization of hyper- and hypofusogenic NiV-F mutants by distinct novel anti-NiV-F rabbit MAbs. We then asked how specific residues in the KKR region might be modulating fusion. Inside-out signaling from the CT has been reported for other class I enveloped viruses (2, 61), including at least one paramyxovirus (67). We first asked whether any of the KKR alanine mutations affected the overall ectodomain conformation of the NiV-F protein.

We had previously produced conformational polyclonal and monoclonal rabbit Abs by genetically immunizing rabbits with codon-optimized NiV-F and NiV-G and NiV-M expression plasmids (4). We screened a panel of our rabbit MAbs and found two (MAbs 92 and 66) whose epitopes were conformational and distinct. They were conformational because they detected the NiV-F protein in its native state by flow cytometry (Fig. 4A) but not in its denatured form, for example, by Western blot analysis on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (data not shown). They were distinct because Ab 66 bound to NiV-F and HeV-F equivalently while Ab 92 bound to NiV-F approximately10-fold more efficiently than to HeV-F at the same Ab concentrations (Fig. 4A). As a control, polyclonal Ab 834 (3, 33, 44) was used to show that the levels of NiV-F and HeV-F were approximately equally recognized by flow cytometry in the same experiment (Fig. 4A).

Then we measured the relative binding of these Abs to the various WT or mutant NiV-F proteins by flow cytometry. We reasoned that conformational differences in the ectodomain might be revealed by differential binding of these Abs. In order to quantitatively correct for variations in the transfection efficiencies and cell surface protein expression levels of the various mutants from experiment to experiment, we analyzed the binding data obtained by calculating the ratios of the mean fluorescence intensities of pairs of Abs (92-66, 92-834, and 66-834). Figure 4B shows the relative binding ratios of thee Ab combinations for WT NiV-F and the indicated mutants. There was a modest but significant decrease in binding of MAb 92 to the hyperfusogenic K1A mutant, as the 92/834 and 92/66 ratios, but not the 66/834 ratio, were lower than those of the WT NiV-F protein ($P = 0.015$, $P = 0.0005$, and $P = 0.94$, respectively) (Fig. 4B). We also detected an increase in binding of MAb 66 to the hypofusogenic R3A mutant protein, as the 66/834 ratio for this mutant was increased, the 92/66 ratio was decreased, and the 92/834 ratio was unchanged compared to those obtained with the WT NiV-F protein ($P = 0.048$, $P =$

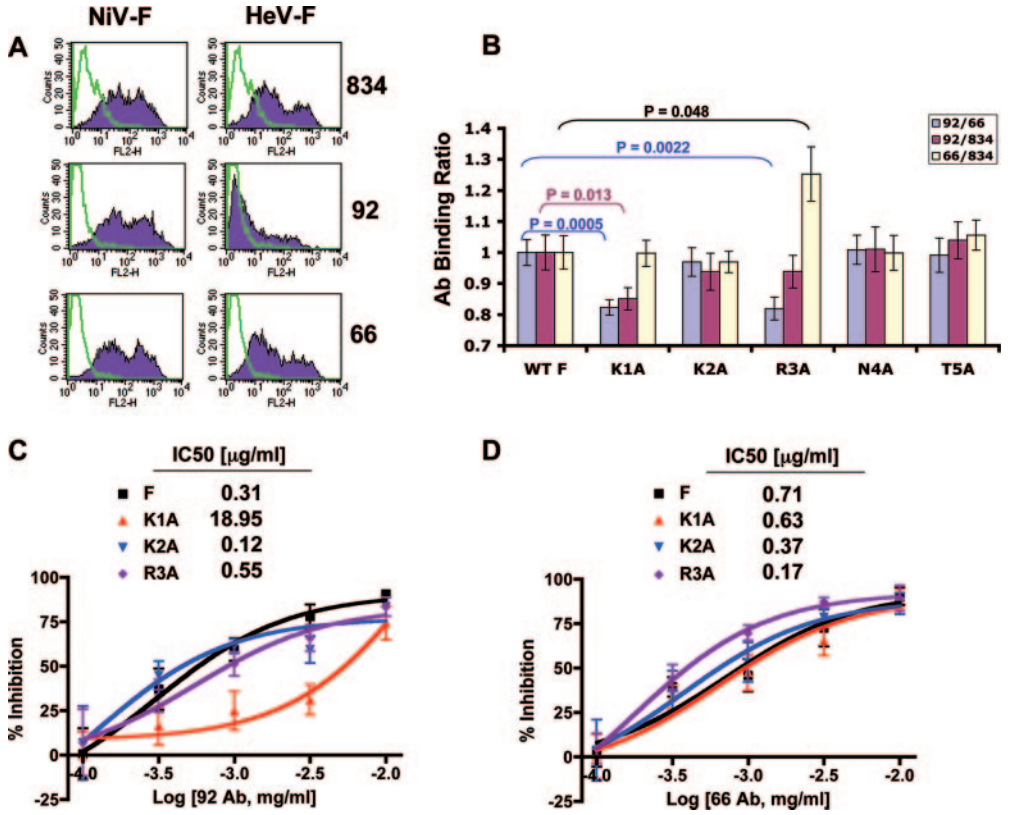


FIG. 4. Specific CT mutants affect the ectodomain conformation as exhibited by differential MAb binding and neutralization. (A) Flow cytometry histograms showing binding of polyclonal anti-NiV-F antiserum 834 or anti-NiV-F MAb 92 or 66 to 293T cells expressing either NiV-F, HeV-F, or neither (pcDNA3 control). Green contours indicate binding of Abs to 293T cells transfected with the pcDNA3.1 backbone only. Overlaid filled purple histograms indicate binding of Abs to NiV-F- or HeV-F-expressing cells, as indicated. (B) MAb binding ratios of pairs of anti-NiV-F Abs. Polyclonal (antiserum 834) or monoclonal (antisera 492 and 66) rabbit Abs were used to stain 293T cells transfected with WT NiV-F or the indicated CT point mutants at a concentration previously determined to be in the linear range of the binding curve. To compare data from repeat experiments and to control for transfection efficiency and differential expression, a set of binding ratios was calculated by dividing the mean fluorescence intensities obtained for the various Abs (92/66, 92/834, and 66/834). The Ab binding ratios for WT NiV-F is necessarily defined as 1. *P* values were calculated with a nonpaired Student *t* test and multiplied by five, which takes into account the Bonferroni correction for the multiple pairwise comparisons (WT versus the five mutants). (C and D) Neutralization of CT mutant proteins by anti-NiV-F Abs. 293T cells expressing the WT NiV-G protein and the WT or mutant NiV-F protein were infected with B2-VSV-rLuc reverse-pseudotyped virus 8 h posttransfection in the presence of increasing amounts of MAb 92 (C) or 66 (D). The amount of viral entry obtained in the absence of anti-NiV-F MAb (artificially represented by the [MAb] = −4.0 datum point) was normalized to 100%, which is equivalent to 0% inhibition. The percent inhibition was then plotted against the logarithm of the Ab concentration. Inhibition curves were regressed, and IC_{50}s were calculated with GraphPad PRISM. The data shown are normalized averages from three separate experiments ± the standard deviations.

0.0022, and $P = 0.84$, respectively) (Fig. 4B). All other point mutant proteins did not display a change in Ab binding relative to that of the WT NiV-F protein (*P* values of >0.5) (Fig. 4B). Similar Ab binding ratios were obtained over an Ab concentration range of 0.03 to 3 μg/ml, and the data shown are for 1 μg/ml.

Next, we measured the neutralization capabilities of MAbs 92 and 66 against the various mutant proteins with our reverse-pseudotyped viral entry assay. In general, our neutralization data were consistent with the above-mentioned binding data. For example, since MAb 92 bound relatively less to mutant K1A, we expected that mutant K1A may also be less sensitive to neutralization by MAb 92, and that was indeed the case. Figure 4C shows that the K1A protein was more than 10-fold less sensitive than WT NiV-F to neutralization by MAb 92 (the 50% inhibitory concentrations [IC_{50}s] for K1A and WT NiV-F were approximately 19 and 0.3 μg/ml, respectively). In addition, since MAb 66 bound more strongly to mutant R3A, we also expected that the R3A mutant might also be more sensitive to neutralization by MAb 66 than the WT NiV-F. Indeed, we observed that the R3A mutant protein was about fourfold more sensitive to neutralization by MAb 66 than was the WT NiV-F protein, as the IC_{50}s for the R3A and WT proteins were approximately 0.17 and 0.71 μg/ml, respectively (Fig. 4D). In toto, our MAb binding and neutralization data show that specific residues in the CT of NiV-F can affect the conformation of its ectodomain.

Association of NiV-F and the hyper- and hypofusogenic mutants with lipid raft domains. Viral envelope glycoproteins are often associated with lipid raft microdomains (22, 26, 43, 47, 62). Such membrane domains are known to have membrane cross-thicknesses greater than those of non-lipid raft cell membrane domains (23, 30) and are enriched in cholesterol and glycosphingolipids. Thus, differential association of WT or mu-

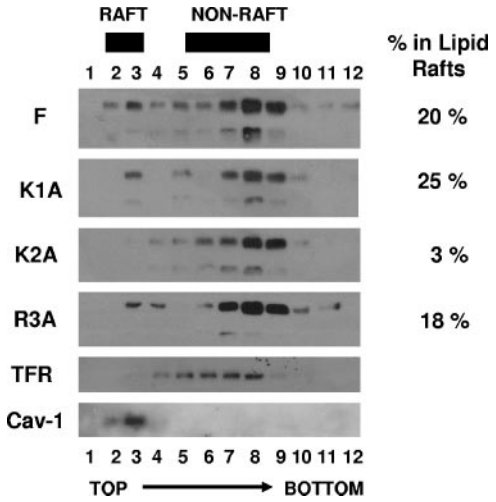


FIG. 5. Association of NiV-F and the hyper- and hypofusogenic mutants with lipid raft domains. Lipid raft fractionations were performed as described in Materials and Methods. Caveolin-1 (Cav-1) and transferrin receptor (TFR) were used as markers for raft (top) and nonraft (bottom) domains, respectively. NiV-F and the indicated mutants were detected by Western blotting with the AU1 Ab. The blots were then stripped and reprobed for Cav-1 and TFR to ensure the integrity of each lipid raft fractionation. Percent NiV-F in lipid rafts was calculated as the percentage of the NiV-F signal observed in the peak Cav-1 fractions (lanes 2 and 3 in most cases) over the sum of signals in the peak Cav-1 and peak TFR fractions (lanes 7 and 8 in most cases) for each sample. This controls for any slight variations between tubes. Representative Cav-1 and TFR blots are shown. The experiment was repeated twice with similar results. Band intensities were quantified by densitometry with a VersaDoc Imaging System (Bio-Rad).

tant NiV-F proteins with lipid raft domains may formally influence the conformation of their ectodomain epitopes, potentially affecting the conformational data in Fig. 4. Therefore, we assessed the relative association of WT and mutant NiV-F proteins with lipid raft domains. First, we observed that a distinct portion (~20%) of the total WT NiV-F protein was associated with lipid raft fractions (Fig. 5), as demonstrated by cofractionation with caveolin-1, a standard marker for lipid raft domains. However, the most of the NiV-F was in nonraft fractions, which were demarcated by the transferrin receptor, a membrane protein known not to be associated with lipid rafts (Fig. 5). With the exception of mutant K2A, all WT and mutant NiV-F proteins were found in both lipid raft and non-lipid raft domains at approximately equal distributions (18 to 25% in lipid raft fractions), indicating that, at least for mutants K1A and R3A, association with lipid rafts did not account for the differences in conformational MAb binding seen in Fig. 4. Interestingly, the hypofusogenic K2A mutant was almost completely absent from the lipid raft fractions, raising the possibility that altered association with lipid raft domains may contribute to its hypofusogenic phenotype and suggesting that mechanistic differences may underlie the hypofusogenic phenotypes of the K2A and R3A mutants.

NiV-F CT fusion mutants are differentially resistant to fusion inhibition by a reagent that prevents 6HB formation and exhibit corresponding rates of fusion kinetics relative to WT NiV-F. Having determined that the specific residues in the CT can affect the ectodomain conformation of NiV-F, we then asked whether the hyper- and hypofusogenic phenotypes exhibited by the NiV-F CT point mutants are mediated by fusion determinants in the ectodomain such as 6HB formation. We have previously shown that a soluble NiV-HR2-Fc protein (HR2 region of NiV-F linked to the Fc constant region of human immunoglobulin G1) inhibits NiV fusion specifically and that the sensitivity of inhibition by this protein inversely correlated with the fusion kinetics of the hyperfusogenic NiV-F N-glycan fusion proteins (3). With the same NiV-HR2-Fc inhibitory reagent, we tested the sensitivity of NiV-F CT mutants or WT NiV-F to fusion inhibition. We observed that the K1A mutant exhibited significantly greater resistance to NiV-HR2-Fc than WT NiV-F for all three concentrations of HR2-Fc tested (Fig. 6A). On the other hand, the K2A mutant exhibited a significantly lower resistance to NiV-HR2-Fc (Fig. 6A), especially when subsaturating amounts of HR2-Fc were

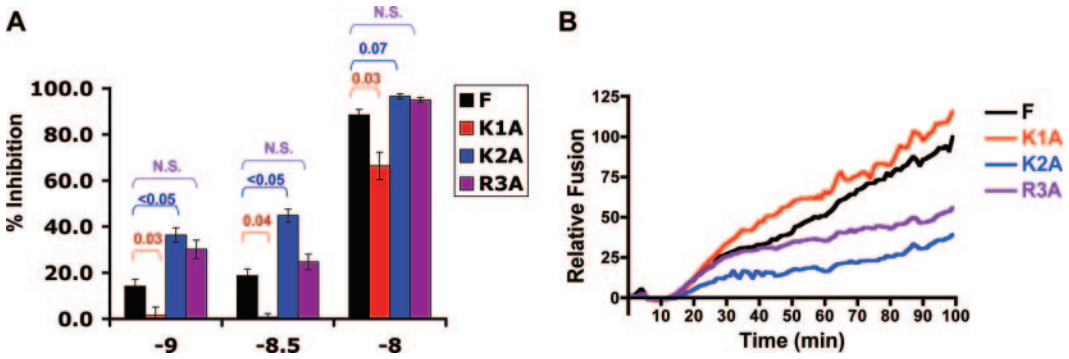
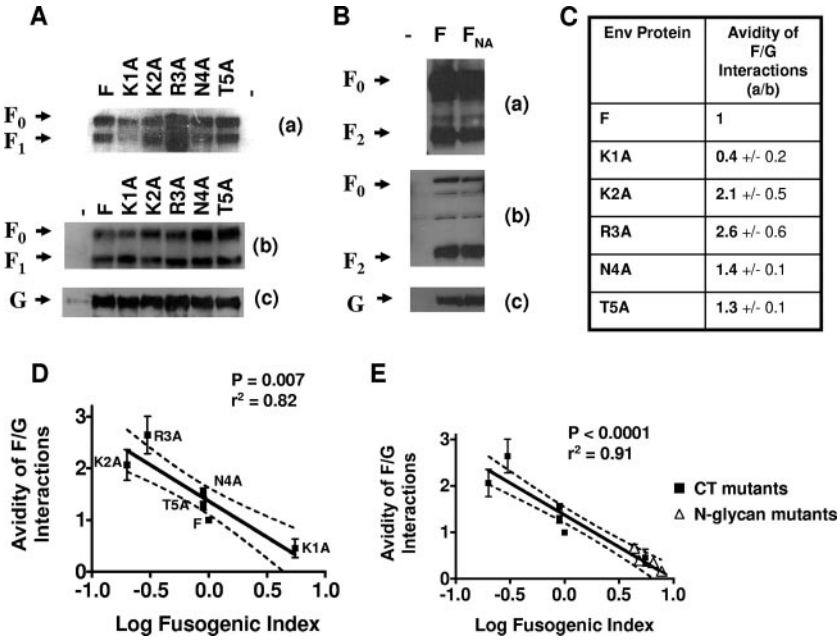


FIG. 6. NiV-F CT fusion mutants are differentially resistant to fusion inhibition by NiV-F HR2-Fc and exhibit corresponding rates of fusion kinetics relative to WT NiV-F. (A) The sensitivity of NiV envelope-mediated fusion to inhibition by NiV-HR2-Fc is shown for WT NiV-F and the indicated CT mutants. For each fusion protein, the amount of fusion in the absence of any inhibitor is set at 0% inhibition. One representative experiment out of two is shown. Error bars indicate standard deviations. *P* values were calculated with the Student *t* test and the Bonferroni correction to account for the multiple pairwise comparisons of significance (F versus K1A, F versus K2A, and F versus R3A). (B) Fusion kinetics of WT or mutant NiV-F protein. NiV-G was expressed with WT NiV-F or the indicated mutants in effector PK13 cells, and the relative rate of fusion was assessed with target 293T cells loaded with CCF2 dye (see Materials and Methods). Relative fusion is the ratio of blue to green fluorescence obtained with NiV-G- and NiV-F-transfected effectors minus the ratio of background blue and green fluorescence obtained with empty-vector (pcDNA3)-transfected cells. Each datum point is an average from three independent experiments.



Env Protein	Avidity of F/G Interactions (a/b)
F	1
K1A	0.4 +/- 0.2
K2A	2.1 +/- 0.5
R3A	2.6 +/- 0.6
N4A	1.4 +/- 0.1
T5A	1.3 +/- 0.1

FIG. 7. Fusogenicity of WT NiV-F and the CT mutants inversely correlates with the avidity of F-G interactions. (A) Western blot analysis of co-IP F_0 and F_1 (top part a), immunoprecipitated G (bottom part c), and the relative amounts of F_0 and F_1 present in total cell lysate (middle part b). Cell lysates of 293T cells transfected with WT NiV-G and NiV-F or the indicated CT mutants were immunoprecipitated with rabbit anti-NiV-G specific antisera. The top and middle parts were blotted with mouse anti-AU1 to detect NiV-F, and the bottom part was blotted with mouse anti-HA to detect NiV-G. (B) A coimmunoprecipitation experiment identical to that in panel A was performed with tagged and untagged NiV-F (F and F_{NA}, respectively) but with a rabbit anti-F_2 peptide Ab for detection. Parts a, b, and c are as in panel A. (C) Relative avidities of NiV-F–NiV-G interactions for WT NiV-F and the indicated CT mutants. The amounts of co-IP NiV fusion proteins in panel A were quantified by densitometry as described in the text, with a VersaDoc Imaging System (Bio-Rad). The avidity of F-G interactions is represented by the ratio of the amount of NiV-F protein co-IP with anti-NiV-G antiserum to the relative amount of NiV-F expressed in cell lysates (parts a and b, respectively). The data presented are averages ± standard errors from three experiments. (D) Avidity of the F-G interactions from panel C plotted against the fusion/CSE ratios from Table 1. Pearson correlation analysis was performed with GraphPad PRISM. (E) The avidities of F-G interactions for the multiple N-glycan mutants previously reported by Aguilar et al. (3) were overlaid with the datum points from panel C and plotted together against their respective fusogenic indexes. CT mutants and N-glycan mutants are represented by closed and open symbols, respectively. Pearson correlation analysis was performed with GraphPad PRISM.

used. These results suggested that the rate of 6HB formation contributed to the hyper- and hypofusogenicity of the K1A and K2A mutants, respectively. Interestingly, mutant R3A did not reveal a significant difference in resistance to inhibition by the NiV-HR2-Fc molecule relative to the WT NiV-F protein, suggesting that the hypofusogenic phenotype of the K2A and R3A mutants may be mediated via distinct mechanisms. This is also consistent with our Ab binding data, which suggest that the K2A and R3A mutants differentially affect ectodomain conformation (Fig. 4B).

In order to determine if sensitivity to NiV-HR2-Fc inhibition is actually due to the rate of 6HB formation and, hence, fusion pore formation, we measured fusion kinetics mediated by NiV-F or the indicated CT mutants and WT NiV-G. Real-time fusion kinetics can be measured and quantified with a β-lactamase reporter cell-cell fusion assay that we previously described for analysis of our hyperfusogenic N-glycan NiV-F mutants (3). We found that cells expressing the hyperfusogenic K1A fusion mutant showed faster fusion kinetics and fused to a greater extent than cells expressing WT NiV-F (Fig. 6B). In contrast, the hypofusogenic K2A mutant showed slower fusion kinetics and fused to a lesser extent than cells expressing the WT NiV-F protein (Fig. 6B). Interestingly, although the cells expressing the R3A mutant fused at the same rate as the WT NiV-F protein for the first 40 min, thereafter, their rates of fusion diverged, with the R3A mutant slowing down significantly such that at 100 min, it had fused at less than 50% of the WT NiV-F level (Fig. 6B). The results in Fig. 6A and B strongly suggest a mechanistic difference between the hypofusogenic phenotypes exhibited by the K2A and R3A mutants.

Fusogenicity of NiV-F inversely correlates to the avidity of F-G interactions for the CT mutant proteins. We had previously provided evidence for the attachment protein displacement model for paramyxoviral entry. At least for NiV, the hyperfusogenic N-glycan mutants appear to have weaker interactions between the NiV-F mutants and NiV-G, allowing greater NiV-F–NiV-G dissociation after receptor binding. Thus, fusogenicity inversely correlated to the avidity of F-G interactions for the hyperfusogenic N-glycan NiV-F mutants (3). Here, we asked if the relative avidity of NiV-F–NiV-G associations correlated with the fusogenicity of the CT mutants.

We coexpressed NiV-G with WT NiV-F or the aforementioned mutants in permissive 293T cells and determined the relative avidity of NiV-F and NiV-G interactions by immunoprecipitating whole cell lysates with anti-NiV-G antiserum and detecting the amount of co-IP NiV-F by Western blotting with an AU1 epitope tag Ab (Fig. 7A, part a). The relative amounts

of WT and mutant NiV-F were also determined in total cell lysates (Fig. 7A, part b). To normalize for the various expression levels of WT or mutant NiV-F in any single experiment, we calculated the ratio of the level of co-IP NiV-F to the corresponding amount of NiV-F in the total cell lysate. For example, if the amount of co-IP NiV-F was densitometrically quantified at 160 U and the corresponding amount of NiV-F in the cell lysate was 100 U, the F-G co-IP ratio would be 1.6. This ratiometric value was arbitrarily set to 1.0 to indicate the relative avidity of the WT NiV-F–NiV-G interactions (Fig. 7C). On this scale, a value of greater or less than 1.0 would indicate a corresponding increased or decreased avidity in F-G interaction relative to the WT proteins, respectively. Also, we note that the AU1 tag did not affect NiV-F's interaction with G, as the same experiment performed with tagged and untagged NiV-F revealed no difference in the amount of F that can be co-IP with G (Fig. 7B).

When we plotted the relative avidity of NiV-G interactions with WT NiV-F or the indicated CT mutants (Fig. 7C) against their fusogenicities (fusion/CSE ratio) as determined in Table 1, we obtained a significant negative correlation ($r^2 = 0.82$, $P = 0.007$) between the avidity of F-G interaction and the fusogenicity of the NiV-F protein (Fig. 7D). Thus, for example, the NiV-F mutant (K1A) with the lowest relative avidity of F-G interaction (0.4) was also the most fusogenic NiV-F CT mutant examined (fusion/CSE ratio of 5.5), and mutants (K2A and R3A) with the highest relative avidities of F-G interaction (2.1 and 2.6) were the least fusogenic (fusion/CSE ratios of 0.2 and 0.3). These results suggest that the effects of the CT mutants on modulating fusogenicity were linked to the increasing or decreasing avidity of F-G interactions and provide further support for the model (3, 63, 75) where dissociation of the attachment protein from the fusion protein is a rate-limiting step required for fusion peptide exposure and subsequent membrane fusion.

DISCUSSION

Our results implicate the cytoplasmic domain of the NiV fusion protein in modulating fusion through its membrane-proximal polybasic KKR motif in an inside-out signaling manner. Our data also shed some light on the mechanisms by which the KKR motif modulates fusion; specific residues within the KKR motif can modulate the conformation of NiV-F's ectodomain and thus have an effect on fusion kinetics by regulating the rate of 6HB formation and the avidity of the F-G interactions.

The CTs of other paramyxovirus fusion proteins are known to be required for various protein functions, including proper surface expression, membrane fusion, fusion pore enlargement, transition from hemifusion to complete fusion, and/or budding (7, 19, 59, 65, 68), although removal of the CT has resulted in quite distinct phenotypes in different paramyxoviruses, ranging from no effect (12) to fusion pore formation (65) to fusion pore enlargement (19) to syncytium formation (59, 65). In this report, we show that relatively large deletions in the NiV-F CT did not significantly compromise conformational integrity or CSE but can either reduce or enhance fusion (Fig. 1). In addition, while point mutations in the membrane-proximal region had no significant effect on conformational integrity, processing, or CSE, they variably affected fusogenicity (Fig. 2). Indeed, we identified a membrane-proximal polybasic KKR patch in the CT of NiV-F as having the ability to up- or downmodulate fusogenicity. Polybasic residues can also be found near the membrane-spanning region in the CTs of most other paramyxoviruses, but to our knowledge, their function in modulating fusion has not been reported.

Our data show that specific CT mutants with changes in the KKR motif mediate their hyper- or hypofusogenic phenotypes through common mechanisms that have been defined for other class I fusion proteins. For example, the hyperfusogenic V3 loop and CT mutants of the HIV-1 envelope glycoprotein also show faster fusion kinetics and display increased resistance to heptad repeat peptide inhibition (1, 52). In the case of NiV, our results suggest that the hyper- and hypofusogenicity phenotypes of the K1A and K2A mutants are governed by the rate of 6HB formation (Fig. 6A) during fusion pore formation, resulting in increased or decreased fusion kinetics, respectively (Fig. 6B). However, since we did not detect any apparent differences between the R3A mutant and WT NiV-F during 6HB formation and its hypofusogenic phenotype was only manifested in slower fusion kinetics at later time points (Fig. 6B), we speculate that a step post 6HB formation, perhaps fusion pore enlargement, may be affected by the hypofusogenic mutation R3A. The CT of at least one other paramyxovirus, SV5, has been implicated in fusion pore enlargement (19). Our lipid raft results also highlight the mechanistic differences observed between the hypofusogenic K2A and R3A mutants observed in Fig. 4 and 6. The K2A, but not the R3A, mutant displayed differences in lipid raft association compared to the WT NiV-F protein. While many hyper- or hypofusogenic phenotypes in class I viral fusion proteins have been identified, it is uncommon to find a contiguous series of residues within a small patch that have such contrasting contributory roles in fusogenicity.

It remains to be determined how these three basic residues in NiV-F CT actually modulate the kinetics of fusion. Do the KKR mutants stabilize or destabilize the metastable prefusogenic conformation of NiV-F, and/or do they affect subsequent steps in the fusion process? It is also possible that the KKR basic motif may interact with cellular proteins that directly or indirectly modulate the actin cortical cytoskeleton, which is intimately involved in membrane dynamics and curvature during fusion and syncytium formation (37, 49, 51). Dutch and colleagues have previously reported that various transdominant Rho-GTPases can up- or down-regulate HeV fusion (57). Since the ERM (ezrin-radixin-monesin) family of proteins is known to connect the CTs of various membrane proteins to the actin cortical cytoskeleton and the ERM proteins themselves are known to be activated and inactivated by distinct Rho GTPases (21, 29), we speculate that the ERM proteins may connect the CT of NiV-F to the actin cortical cytoskeleton and that modulation of the CT's attachment to the cortical cytoskeleton by the Rho GTPases, or by our various KKR mutants, is what accounts for the hyper- or hypofusogenic phenotypes seen. Intriguingly, ERM proteins preferentially bind CTs of membrane proteins that have isoelectric points higher than 9.0 and that have basic amino acid clusters (29, 74). They also prefer to bind CTs that contain phosphorylated serines and tyrosine motifs (17, 58). NiV-F's CT has an isoelectric point of

9.88, contains a C-terminal tyrosine-rich motif, and contains two basic clusters, i.e., the membrane-proximal KKR cluster that we know affects fusion and an RRVR cluster between regions T3 and T4. In addition, preliminary mass spectrometry analysis indicated that the two C-terminal serines in NiV-F are phosphorylated (unpublished observations). Therefore, it seems plausible that cellular factors such as ERM proteins may connect the CT of NiV-F or HeV-F to the actin cortical cytoskeleton of cells, and the strength and stability of this connection may modulate fusogenicity.

Our data also suggest that the KKR motif modulates fusogenicity via an inside-out signaling mechanism. Differential MAb recognition of the ectodomain correlating with differential neutralization (Fig. 4), faster or slower rates of fusion kinetics affected by the rate of 6HB formation (Fig. 6), and differential effects on the avidity of F-G interactions (Fig. 7) all argue that mutations of these cytoplasmic residues can affect the conformation and subsequent fusogenic function of the ectodomain. Interestingly, data from differential MAb binding, rate of 6HB formation, and fusion kinetics experiments also reveal that distinct mechanisms underlie the similar hypofusogenic phenotypes of the K2A and R3A mutants. For example, while MAbs 92 and 66 clearly bound differentially to the R3A mutant, no difference in K2A binding was observed (Fig. 4B). On the other hand, while K2A was significantly more sensitive to inhibition by HR2-Fc compared to WT NiV-F, R3A was similar in sensitivity to WT NiV-F (Fig. 6A). This equivalent sensitivity to HR2-Fc inhibition is consistent with our real-time fusion kinetics data showing that for the first 40 min, R3A fused at the same rate and to the same extent as WT NiV-F, while K2A fused much more slowly from the very beginning (Fig. 6B). However, after 40 min, R3A began to exhibit slower fusion kinetics and eventually fused to a much lesser extent than WT NiV-F at 100 min. As mentioned above, it is likely that the defect in fusion in the R3A mutant is manifested at a stage post 6HB formation, such as fusion pore enlargement.

We previously suggested that a critical parameter that governs NiV envelope-mediated fusion is the avidity of F and G association, which we quantified by a rigorous coimmunoprecipitation assay (3). Our published data showed a strong and significant negative correlation between the degree of hyperfusogenicity exhibited by a variety of ectodomain N-glycan mutants and the avidity of F and G association. We had therefore favored the attachment protein displacement model of paramyxovirus fusion where the dissociation of G from F after receptor engagement better allows for the conformational changes in F that lead to fusion peptide exposure and membrane fusion. We now provide data to further expand and support this model with both hyper- and hypofusogenic mutants (Fig. 7D). Indeed, as shown in Fig. 7E, when we added our present datum points to the datum points from our hyperfusogenic N-glycan mutants (3), the Pearson correlation became even stronger ($r^2 = 0.91$) and more significant ($P < 0.0001$). These data suggest that F and G dissociation can be a common pathway for the triggering of F regardless of the determinants of fusion in F involved. However, since we performed these studies with receptor-containing 293T cells, we are not able to distinguish whether the differences in F-G association between WT and mutant fusion proteins we have observed occur pre or post receptor binding. True avidity measurements would have to be done with truly receptor-negative cells. In addition, it remains to be determined whether the KKR motif affects the interaction of NiV-F with NiV-G directly and/or via modification of the NiV-F ectodomain's overall conformation. At least for one other paramyxovirus F protein (NDV), the ectodomain HR2 region has been implicated in binding to the attachment protein HN (24); therefore, the effects of NiV-F CT mutants on the avidities of NiV-F–NiV-G interactions observed here may be due to inside-out signaling.

We also note that NiV-F processing is usually increased when G is cotransfected (compare Fig. 7A with Fig. 1B and 2B). Since both F and G are encoded by codon-optimized genes, a potential explanation is that expression of G competes for transcriptional or translational resources, resulting in less overexpression of F; overexpression of F in the absence of G can overwhelm the proteolytic machinery required for F cleavage. However, it would be interesting to determine whether the presence of G, and its association with F, can intrinsically affect F processing, either by modulating its endosomal recycling behavior or changing the conformation of F to make it more accessible to cathepsin L cleavage.

Finally, it remains to be determined how the NiV-F CT actually stabilizes or destabilizes F-G interactions and whether the fusion-modulatory role played by the polybasic motif in the CT of NiV-F is unique for NiV (or the henipaviruses). The studies presented in this report point to the many determinants of fusion in NiV-F and underscore the complexities that regulate the "proper" amount of fusion mediated by NiV-F, which has both fusion-promoting and fusion-inhibiting determinants. Further studies of these determinants will enhance our understanding of the pathobiology of this deadly emerging virus and may reveal more targets for therapeutic intervention.

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LETTER

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Single-dose attenuated Vesiculovax vaccines protect primates against Ebola Makona virus

Chad E. Mire[1,2]*, Demetrius Matassov[3]*, Joan B. Geisbert[1,2], Theresa E. Latham[3], Krystle N. Agans[1,2], Rong Xu[4], Ayuko Ota-Setlik[4], Michael A. Egan[4], Karla A. Fenton[1,2], David K. Clarke[3], John H. Eldridge[3,4] & Thomas W. Geisbert[1,2]

The family *Filoviridae* contains three genera, Ebolavirus, Marburgvirus, and Cuevavirus[1]. Some members of the genus, including Zaire ebolavirus (ZEBOV), can cause lethal haemorrhagic fever in humans. During 2014 an unprecedented ZEBOV outbreak occurred in West Africa and is still ongoing, resulting in over 10,000 deaths, and causing global concern of uncontrolled disease. To meet this challenge a rapid-acting vaccine is needed. Many vaccine approaches have shown promise in being able to protect nonhuman primates against ZEBOV[2]. In response to the current ZEBOV outbreak several of these vaccines have been fast tracked for human use. However, it is not known whether any of these vaccines can provide protection against the new outbreak Makona strain of ZEBOV. One of these approaches is a first-generation recombinant vesicular stomatitis virus (rVSV)-based vaccine expressing the ZEBOV glycoprotein (GP) (rVSV/ZEBOV). To address safety concerns associated with this vector, we developed two candidate, further-attenuated rVSV/ZEBOV vaccines. Both attenuated vaccines produced an approximately tenfold lower vaccine-associated viraemia compared to the first-generation vaccine and both provided complete, single-dose protection of macaques from lethal challenge with the Makona outbreak strain of ZEBOV.

Since discovery of the virus in 1976, outbreaks of ZEBOV have been detected sporadically in Africa. With increasing population growth the frequency of human contact with natural virus reservoirs[3] is likely to rise, potentially leading to more catastrophic outbreaks such as the current epidemic in West Africa, thus increasing the need for effective antiviral strategies. A highly effective countermeasure would be a preventive vaccine that can be simply and widely administered to people in regions of virus zoonosis and provide a 'blanket immunity' curtailing any future outbreaks. Also important will be the ability to rapidly combat deliberate misuse of these deadly viruses. Therefore, a preventive vaccine should ideally confer rapid, single-dose protection.

There are currently no licensed filovirus vaccines or post-exposure treatments available for human use. However, there are at least ten different vaccine approaches that have shown the potential to protect nonhuman primates (NHPs) from lethal ZEBOV infection, including platforms based on recombinant adenovirus serotype 5 (rAd5) vectors, combined DNA/rAd5 vectors, combined rAd serotype 26 and 35 vectors, recombinant chimpanzee adenovirus serotype 3 (rChAd3) vectors, combined rChAd3 and modified vaccinia Ankara (MVA) vectors, virus-like particles (VLPs), alphavirus replicons, recombinant human parainfluenza virus 3 (rHPIV3), rabies virus, and recombinant vesicular stomatitis virus (rVSV)[2]. Of the vaccines advancing to phase I trials, the rChAd3 and rVSV vectored vaccines have shown success in single-dose protection of NHPs against ZEBOV challenge; with the caveat that the rChAd3/ZEBOV vaccine requires a boost with an MVA/ZEBOV vector for protection past 6 months[4]. Also, NHPs inoculated with the rChAd3/ZEBOV vaccine were challenged with a ZEBOV seed stock containing a large virus population encoding 8 uridines (U) at a critical transcription editing site in the GP gene[4]. This specific genetic feature typically arises following prolonged passage of ZEBOV in Vero E6 cells and results in higher levels of expression of full-length GP. In contrast, low-passage ZEBOV isolates retain 7U at the GP editing site, resulting in higher levels of secreted GP (sGP) expression, which is associated with greater viral virulence[5–7]. Importantly, studies have shown that rAd-based ZEBOV vaccines that completely protect NHPs against ZEBOV stocks containing high populations of 8U virus are not able to completely protect vaccinated macaques challenged with ZEBOV stocks containing high populations of 7U virus[8].

The first generation rVSV/ZEBOV vaccine that replaces the VSV glycoprotein G with the ZEBOV GP (rVSV/ZEBOVΔG), originally developed by Drs Feldmann and Geisbert[9] and currently licensed by Merck, has demonstrated solid single-dose NHP protection against a low-passage 7U ZEBOV stock[8]. The rVSV/ZEBOVΔG vector has also protected 50% of NHPs when administered shortly after ZEBOV challenge[10], and has demonstrated safety in a NHP neurovirulence model[11]. However, there is a robust post-vaccination viraemia in macaques and a recent phase I trial of the rVSV/ZEBOVΔG vaccine in Geneva was halted due to temporary joint pain in some patients. The level of vaccine-associated viraemia and frequency of adverse events will be more fully documented as data from ongoing phase 3 trials become available for this vector; but the early observation suggest that a further-attenuated rVSV vector may be more desirable for widespread administration in endemic regions of Africa.

To address this possible safety concern we have developed and tested two further-attenuated rVSV/ZEBOV vaccine candidates for efficacy. One of these vaccines is based on an rVSV vector that has advanced through clinical evaluation. It was attenuated by translocating the VSV nucleoprotein (N) gene from position 1 to position 4 in the genome (N4) and truncating the cytoplasmic tail (CT) of the VSV G protein from 29 to 1 amino acids (CT1)[12]. This rVSVN4CT1 vector was modified to maximally express HIV-1 gag from position 1 in the genome (rVSVN4CT1gag1) by positioning the *gag* gene immediately adjacent to the single strong 3′ VSV transcription promoter. The rVSVN4CT1gag1 vector has demonstrated safety in mouse and NHP neurovirulence studies[12,13], and replication is restricted to the IM inoculation site and draining lymph node following vaccination of mice[14]. The rVSVN4CT1gag1 vector has demonstrated safety and immunogenicity in two phase I clinical trials (HVTN 090 and HVTN 087: http://clinicaltrials.gov/) and no post-vaccination viraemia was detected in urine, saliva, and blood of vaccine recipients. The rVSVN4CT1GP1 vector described here (Fig. 1a, N4) is analogous in design to that of the rVSVN4CT1gag1 vaccine and expresses ZEBOV GP from genome position 1. The other attenuated rVSV/ZEBOV

[1]Galveston National Laboratory, University of Texas Medical Branch, Galveston, Texas 77555, USA. [2]Department of Microbiology and Immunology, University of Texas Medical Branch, Galveston, Texas 77555, USA. [3]Department of Virology and Vaccine Vectors, Profectus BioSciences, Inc., Tarrytown, New York 10591, USA. [4]Department of Immunology, Profectus BioSciences, Inc., Tarrytown, New York 10591, USA.

*These authors contributed equally to this work.

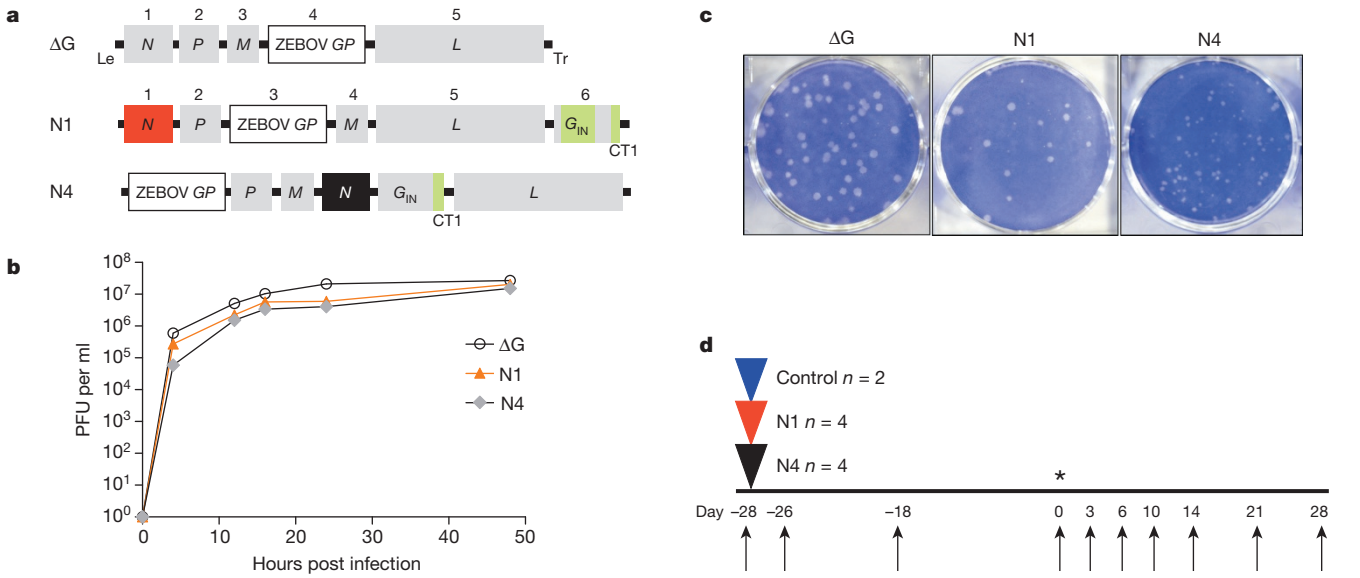

Figure 1 | rVSV/ZEBOV vector design, growth kinetics and vaccine study strategy. **a**, Genome organization comparing ZEBOV GP (Mayinga strain)-expressing rVSV vectors as described in methods. The rVSV/ZEBOVΔG (ΔG) vector had the natural VSV *G* gene replaced with the ZEBOV *GP* at position 4 within the genome. rVSVN1CT1GP3 (N1) vector retained the position of VSV *N* in position 1 (orange box), insertion of ZEBOV *GP* at position 3 and a truncated form of VSV *G* containing the CT1 truncation was inserted at position 6. The rVSVN4CT1GP1 (N4) vector had the insertion of ZEBOV *GP* in position 1, attenuating *N* gene translocation (N4) (black box) and truncated G protein cytoplasmic tail (CT1). Numbers above vector constructs designate genome positions. Virus leader (Le), trailer (Tr), and intergenic regions are shown in black. Shaded regions represent deleted amino acid regions. **b**, Single-cycle growth kinetics comparing the ΔG, N1, and N4 vectors depicted in **a**. Data shown are mean ± s.d. from two biological replicates titrated by plaque assay in triplicate. Titre differences between ΔG and N1 vectors were statistically significant at 4 ($P = 0.0001$), 12 ($P = 0.0055$), and 24 h post infection ($P = 0.0001$). Likewise, ΔG and N4 vector titres were significantly different at 4 ($P = 0.0001$), 12 ($P = 0.0005$), 24 ($P = 0.0001$), and 48 h post infection ($P = 0.0068$). Unpaired *t*-test, $P = 0.05$. **c**, Crystal violet-stained Vero cell monolayers showing plaques generated by the ΔG, N1, and N4 vectors at 48 h post infection. **d**, Flow chart showing the day of vaccination (triangles), days of sampling (arrows), day of challenge (*). Blue triangle, unvaccinated cohort; orange triangle, N1-vaccinated cohort; black triangle, N4-vaccinated cohort.

vaccine described here (rVSVN1CT1GP3), expressing a truncated form of VSV G, was designed to be of intermediate attenuation between rVSVN4CT1GP1 and the first generation rVSV/ZEBOVΔG vaccine (Fig. 1a, N1). Both attenuated rVSV/ZEBOV vectors express GP from the ZEBOV Mayinga strain, as do most other candidate ZEBOV vaccines currently under evaluation. Sequence homology between GPs from the new West African Makona strains analysed to date and the 1976 Mayinga strain is approximately 97%. Although this difference is not likely to affect the protective efficacy of the current ZEBOV vaccines against the heterologous West African strains, it is possible that small changes in sequence could lead to reduced efficacy of a vaccine[15]. It is well established that small variations in sequence and even single amino acid changes in sequence for other viruses including influenza, respiratory syncytial virus, polio, equine infectious anaemia virus, and SIV can reduce vaccine efficacy. Here we assessed the ability of our newly developed next-generation rVSV-based vaccines expressing ZEBOV Mayinga GP to protect against heterologous challenge with the new outbreak Makona strain of ZEBOV in cynomolgus monkeys.

Table 1 | Clinical findings for NHPs challenged with ZEBOV-Guinea

Animal	Vaccine	Day −26*	$PRNT_{50}$†	Clinical signs observed‡	Final outcome
129	N/A	−	0/0	Fever (6), anorexia (5–8), depression (6–8), mild rash (6–8), lymphopenia (3, 6), thrombocytopenia (6), ALT→(6), ALP→→ → (6), AST→→→ (6), GGT→→→ (6), CRP increase (6)	Expired day 8
276	N/A	−	0/0	Fever (6), anorexia (6–7), depression (6–7), mild rash (6–7), thrombocytopenia (6, 10), ALT→(6), ALP→→→ (6), AST→→→ (6), GGT→ (6), CRP increase (6)	Expired day 7
0910078	N1	+	0/40	Ø§	Survived
1001100	N1	−	0/160	CRP increase (6)	Survived
117	N1	−	0/80	Lymphopenia (6), CRP increase (6, 10)	Survived
0907095	N1	−	0/160	Lymphopenia (6, 10), CRP increase (6, 10), ALT→→(6), ALP→(6), AST→→(6)	Survived
0807174	N4	+	0/160	Lymphopenia (6), CRP increase (6, 10)	Survived
0901014	N4	−	0/80	Ø	Survived
119	N4	+	0/80	Ø	Survived
0811013	N4	+	0/20	Ø	Survived

* rVSV viraemia 2 days post vaccination. −, below limit of detection (25 PFU per ml); +, up to 3×10^2 PFU per ml.
† 50% plaque reduction neutralization titre at day of challenge and terminal day presented as day of challenge/terminal day.
‡ Days after ZEBOV challenge are in parentheses. Fever is defined as a temperature more than 1.4 °C above baseline or at least 0.9 °C above baseline and ≥ 39.7 °C. Lymphopenia and thrombocytopenia are defined by a ≥ 35% drop in numbers of lymphocytes and platelets, respectively. ALT, alanine aminotransferase; ALP, alkaline phosphatase; AST, aspartate aminotransferase; GGT, gamma glutamyltransferase. CRP, C-reactive protein: two- to threefold increase,→; 4- to fivefold increase, →→; > 5 fold increase, →→→.
§ No symptoms observed.

Results from an *in vitro* growth kinetics study (Fig. 1b) indicate an approximate tenfold reduction in growth rate early in infection for rVSVN4CT1GP1 relative to rVSV/ZEBOVΔG. Also noted during virus plaque assay were the larger more rapidly forming plaques generated by rVSV/ZEBOVΔG compared to rVSVN4CT1GP1, with rVSVN1CT1GP3 showing intermediate growth and plaque size (Fig. 1c).

We next tested if the further-attenuated rVSV/ZEBOV vaccines could provide NHPs with single-dose protection against challenge with ZEBOV isolated from the current outbreak in Guinea[16]. Groups of four cynomolgus macaques were inoculated intramuscularly with 2×10^7 plaque-forming units (PFU) of either rVSVN4CT1GP1 or rVSVN1CT1GP3; a group of two control macaques were unvaccinated (Fig. 1d, arrow heads). None of the macaques showed any sign of illness or distress following vaccine administration. Consistent with the statistically significant growth differences between rVSV/ZEBOVΔG and the more attenuated vectors seen during *in vitro* growth kinetics studies, levels of both attenuated vaccine viruses detected in the blood of vaccinated macaques (500 PFU per ml) were 10- to 50-fold lower than those detected for the more replication competent rVSV/ZEBOVΔG[9] (Table 1, day −26). The ZEBOV GP-specific humoral immune response was assessed for all animals before vaccination (Fig. 2a, −28) and after vaccination (Fig. 2a, −18 and 0) by IgG capture ELISA and neutralizing antibody titres (Table 1, plaque 50% reduction neutralization test $(PRNT)_{50}$). Results showed neutralizing titres at terminal days for vaccinated cohorts and detectable circulating levels of anti-ZEBOV GP IgG for both vaccine cohorts after vaccination and before challenge with no detectable levels for the unvaccinated control animals (Fig. 2a). A cell-mediated immune response was also detected in all vaccinated animals by ZEBOV GP-specific interferon gamma (IFN-γ) ELISpot assay 10 days after vaccination (Extended Data Fig. 1a and b).

The eight vaccinated and two unvaccinated control macaques were challenged by intramuscular injection with 1,000 PFU of a low passage 100% 7U Makona strain stock of ZEBOV[16] 28 days after the single injection vaccination (Fig. 1d, asterisk). None of the animals vaccinated with either of the two further-attenuated rVSV/ZEBOV vectors showed any severe signs of illness following challenge with ZEBOV (Table 1), whereas the two unvaccinated control macaques succumbed to disease on days 7 and 8 (Fig. 2b). Circulating infectious ZEBOV was isolated from both of the unvaccinated control macaques on days 3 and 6 post challenge (Fig. 2c, blue) but no circulating infectious ZEBOV could be detected in any of the vaccinated animals. Examination of tissues by immunohistochemistry showed abundant ZEBOV antigen in tissues of the unvaccinated control animals (129 and 276) (Fig. 3a–d) whereas ZEBOV antigen was not detected in tissues of the macaques vaccinated with rVSVN1CT1GP3 (1001100) or rVSVN4CT1GP1 (0807174) (Fig. 3e–h).

Here we show protection against a new West African Makona strain of ZEBOV using a novel filovirus vaccine platform. The large reduction in vaccine-associated viraemia indicates a significant increase of *in vivo* attenuation for these next-generation rVSV/ZEBOV vaccine vectors, which should translate into greater safety and reduced adverse

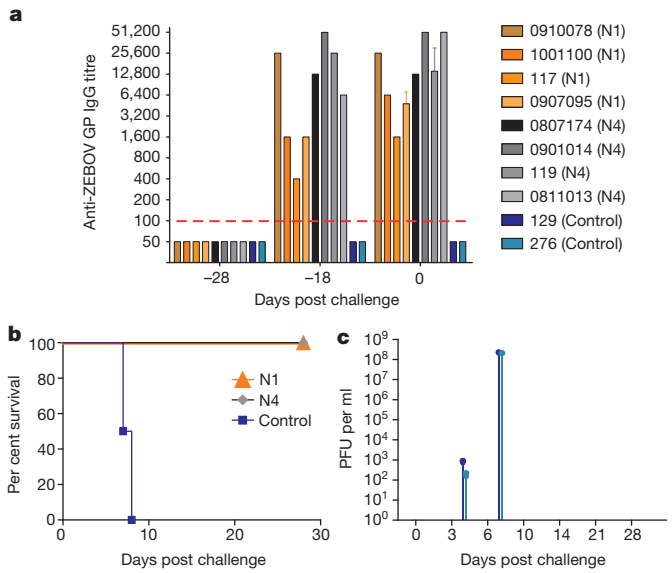

Figure 2 | N1 and N4 vaccination results in circulating anti-ZEBOV GP IgG and protection in cynomolgus macaques. **a**, Reciprocal endpoint dilution titres for circulating IgG against ZEBOV GP for control (blue), N1 (orange), and N4 cohorts (black-grey) on day of vaccination (−28), 10 days post vaccination (−18), and on day of challenge (0). Red dashed line depicts limit of detection for ELISA assay. Error bars represent s.e.m. **b**, Kaplan–Meier survival curve for each cohort post ZEBOV challenge. **c**, Circulating infectious virus load displayed as plaque forming units per ml. Data shown are from individual animals. Lower limit of detection is 25 PFU per ml.

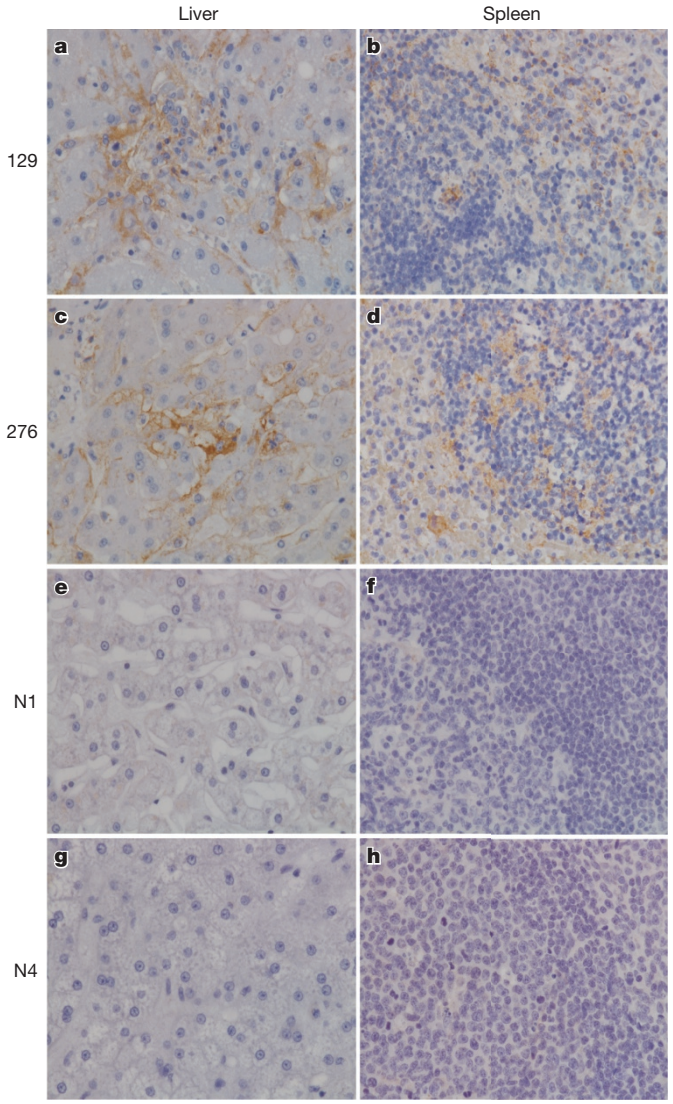

Figure 3 | Comparison of ZEBOV antigen in tissues of cynomolgus macaques either vaccinated or unvaccinated. **a**, **c**, Liver, diffuse cytoplasmic immunolabelling (brown) of sinusoidal lining cells in both ZEBOV-infected control animals. **b**, **d**, Spleen, diffuse cytoplasmic immunolabelling of dendriform mononuclear cells in the red and white pulp of ZEBOV-infected control animals. **e**, **f**, Liver and spleen, respectively, with a lack of immunolabelling from N1 cohort animal 0910078. **g**, **h**, Liver and spleen, respectively, with a lack of immunolabelling from N4 cohort animal 0807174.

events in humans. Importantly, single-dose vaccination of NHPs with highly attenuated forms of rVSV expressing ZEBOV Mayinga GP provides complete protection from heterologous challenge with a highly virulent 7U ZEBOV isolated early during the current West African outbreak[16]. ZEBOV genome sequencing from cases later during the West Africa outbreak has revealed little drift in the GP gene[17,18], suggesting that this vaccine platform could also be efficacious against currently circulating ZEBOV. These findings pave the way for the identification and manufacture of safer, single-dose, high efficacy vaccine(s) to combat current and future filovirus outbreaks in Africa and their potential use as biological weapons.

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Author Contributions D.K.C., D.M. and T.E.L. designed the vaccine vectors and did preparative work. J.H.E. M.A.E., A.O.-S. and R.X. designed, conducted, and analysed the *in vitro* vaccine characterization studies. C.E.M., J.H.E., and T.W.G. conceived and designed the NHP study. C.E.M., J.B.G. and T.W.G. performed the NHP vaccination and challenge experiments, and conducted clinical observations of the animals. J.B.G. and K.N.A. performed the clinical pathology assays. J.B.G. performed the ZEBOV infectivity assays. C.E.M., D.M., J.B.G., K.N.A., M.A.E., K.A.F., D.K.C., J.H.E. and T.W.G. analysed the data. K.A.F. performed histologic and immunohistochemical analysis of the data. C.E.M., D.M., D.K.C. and T.W.G. wrote the paper. All authors had access to all of the data and approved the final version of the manuscript.

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METHODS

No statistical methods were used to predetermine sample size.

Generation of N4 and N1 ZEBOV vectors. As described previously[12,19] an $rVSV_{IN}$N4CT1gag1 vector (Indian serotype) expressing HIV-1 gag was used as the backbone for generating the attenuated rVSVN4CT1 vector expressing the Zaire ebolavirus (ZEBOV) glycoprotein (GP). The corresponding $rVSV_{IN}$N4CT1gag1 genomic cDNA was modified by exchanging the gag gene expression cassette via XhoI/NotI restriction sites with an expression cassette encoding a full length ZEBOV GP [1976, Mayinga strain], generating the $rVSV_{IN}$N4CT1-ZEBOVGP1 cDNA (Fig. 1a, N4). The N1 vector was generated by first inserting ZEBOV GP into a VSV-N1ΔG backbone via XhoI/NotI restriction sites within a transcriptional cassette located at position 3 in the genome; followed by the insertion of a PCR fragment containing a portion of VSV *L*, a modified VSV *G* CT1 gene and trailer into the N1 genome at position 6 via the HindIII/RsrII sites, generating the $rVSV_{IN}$N1(G CT1)6-ZEBOVGP3 cDNA (Fig. 1a, N1).

The rVSV-ZEBOV vectors were rescued from genomic cDNA as previously described[20]. Rescued virus was plaque purified and amplified on Vero cell monolayers (ATCC, CCL-81). For animal studies, virus vectors were purified from infected BHK-21(ATCC CCL-10) cell supernatants by centrifugation through a 10% sucrose cushion. Purified virus was resuspended in PBS, pH 7.0, mixed with a sucrose phosphate (SP) stabilizer (7 mM K_2HPO_4, 4 mM KH_2PO_4, 218 mM sucrose), snap frozen in ethanol/dry ice and stored at −80 °C until ready for use.

Growth kinetics study of ΔG, N4 and N1 ZEBOV vectors. Single-step growth curves were performed by adsorbing the N4, N1 and a ΔG control virus to duplicate monolayers of Vero cells (ATCC, CCL-81) in six-well plates at a multiplicity of infection (MOI) of 10 for 15 min at room temperature with continued rocking followed by incubation at 37 °C with 5% CO_2 for 30 min without agitation. The inoculum was aspirated, the cells washed 3× with serum-free Dulbecco's minimal Eagle's medium (DMEM) and then DMEM containing 5% fetal bovine serum (FBS) was added to the plates, which were placed at 32 °C with 5% CO_2. Samples for titration were taken at 4, 8, 12, 16, 24 and 48 h post infection and replaced with the same volume of fresh media. Virus titres were determined in duplicate by plaque assay on Vero cells. Growth curves were performed in triplicate for each virus. Plaque images for each vector were taken at 48 h post infection, after staining with a 1% crystal violet solution. Statistical analysis of rVSV titres were performed using unpaired *t*-test with a 95% confidence level ($P < 0.05$) with the GraphPad Prism program.

Challenge virus. The ZEBOV Makona strain seed stock originated from serum from a fatal case early during the 2014 outbreak in Guékédou, Guinea[16] (NCBI accession number KJ660347) and was passaged twice in Vero E6 cells (ATCC, CRL-1586). The virus stock was deep sequenced as 100% 7U at the GP editing site in the viral genome (see below).

Deep sequencing. Approximately 1 ml of the ZEBOV Makona strain seed stock was removed from the seed stock vial and placed in 5 ml of TRIzol LS and vortexed three times and allowed to sit for 10 min. The 6 ml were then placed into two separate 3 ml Nunc cryo-vials for removal from the BSL-4. RNA was isolated from the TRIzol LS/sample mixture using Zymo Research Direct-zol RNA mini-prep per manufacturer's instructions. Approximately 150 ng of purified RNA were used to make cDNA using the NuGen Ovation RNA-seq 2.0 kit ultimately for the preparation of the double-stranded DNA library using Encore Ion Torrent library prep kit. Sequencing was performed by the UTMB Molecular Core on the Ion Torrent using 318-v2 deep sequencing chips. Sequence analysis was performed using DNA Star Seqman NGen software based on paired-end analysis of 100 base pairs overlaps.

Vaccination and animal challenge. Ten, healthy, filovirus-naive, adult (~3 to 9.5 kg, 7 female and 3 male), Chinese origin cynomolgus macaques (*Macaca fascicularis*) were randomized with Microsoft Excel into two experiment groups of four animals each and a control group of two animals. Animals in one experimental group were vaccinated by intramuscular injection of approximately 2×10^7 PFU of the rVSVN4CT1GP1vaccine while animals in the other experimental group were vaccinated with approximately 2×10^7 PFU of the VSV-N1CT1 ZEBOVGP vaccine. The two control animals were not vaccinated. Four weeks after the single injection vaccination all ten animals were challenged by intramuscular injection with 1,000 PFU of the ZEBOV Makona strain virus. All animals were given physical exams and blood was collected before vaccination, at day 10 after vaccination, at the time of ZEBOV challenge and on days 3, 6, 10, 14, 21 and 28 after ZEBOV challenge (Fig. 1d, arrows). Animals were monitored daily and scored for disease progression with an internal filovirus scoring protocol approved by the UTMB Institutional Animal Care and Use Committee. The scoring changes measured from baseline included posture/activity level, attitude/behaviour, food and water intake, weight, respiration, and disease manifestations such as visible rash, haemorrhage, ecchymosis, or flushed skin. A score of ≥ 9 indicated that an animal met criteria for euthanasia. This study was not blinded.

Anti-ZEBOV GP IgG ELISA. Serum collected at indicated time points was tested for immunoglobulin G (IgG) antibodies against ZEBOV. Enzyme-linked immunosorbent assay (ELISA) using recombinant ZEBOV GPdTM purified protein (Integrated BioTherapeutics, Inc.) was used to detect cross-reactive IgG. ZEBOV GPdTM was diluted to an optimal working concentration of 100 ng per well in 0.1 ml carbonate/bicarbonate buffer (carbonate/bicarbonate buffer with azide tablets from Sigma catalogue number 08058-50TAB-F) and used to coat Immulon 2HB flat bottom ELISA plates (Thermo Labsystem catalogue number 3455) for 18 h at 4 °C. Coated plates were blocked (10% FBS + 1× PBS) for at least 2 h. The serum samples were assayed at twofold dilutions starting at a 1:100 dilution in ELISA diluent (1% heat inactivated fetal bovine serum (HI-FBS), 1× PBS, and 0.2% Tween-20). Samples were incubated for 1 h at room temperature, removed, and plates were washed. Wells were then incubated at room temperature for 1 h with anti-monkey IgG conjugated to horseradish peroxidase (Fitzgerald Industries International) at a 1:2,500 dilution. These wells were washed and then incubated with 2,2'-azine-di(3ethylbenzthiazoline-6-sulfonate) peroxidase substrate system (KPL) at room temperature for approximately 10 min. Reaction was stopped with 1% SDS and read for dilution endpoints at 405 nm on a microplate reader (Molecular Devices Emax system). Absorbance values were normalized by subtraction of background $A_{405\,nm}$ from uncoated wells for each serum dilution. Antigen-specific serum IgG end-point titres were defined as the reciprocal of the last normalized serum dilution giving an $A_{405\,nm}$ greater than 0.1.

ZEBOV neutralization assay. Neutralization assays were performed by measuring plaque reduction in a constant virus:serum dilution format as previously described[9]. Briefly, a standard amount of ZEBOV (~100 PFU) was incubated with serial twofold dilutions of the serum sample for 60 min. The mixture was used to inoculate Vero E6 cells (ATCC, CRL-1586) for 60 min. Cells were overlaid with an agar medium, incubated for 7 days, and plaques were counted 48 h after neutral red staining. Endpoint titres were determined by the dilution of serum, which neutralized 50% of the plaques ($PRNT_{50}$).

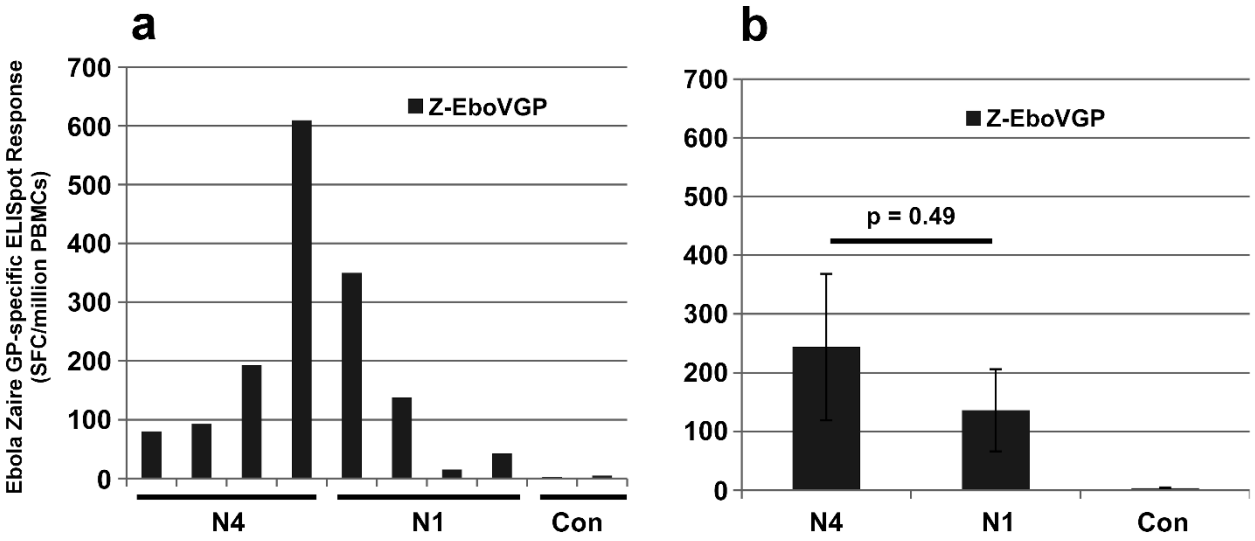
IFN-γ ELISpot assay. Ninety-six-well flat-bottomed ELISpot plates (Millipore) were coated overnight with a mouse anti-human IFN-γ monoclonal antibody (clone 27; BD-Pharmingen) at a concentration of 10 μg ml^{-1}, after which the plates were washed three times with 1× PBS and then blocked for 2 h with PBS containing 5% heat-inactivated FBS. Heparinized whole blood was collected 10 days after immunization of macaques, and peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by Ficoll-Hypaque density gradient centrifugation, and resuspended in complete R05 culture medium. The isolated macaque PBMCs were washed once with complete R05 culture medium and resuspended in complete R05 culture medium containing either 5 μg ml^{-1} phytohaemagglutinin mucoprotein (Sigma), peptide pools (15-mers overlapping by 11 amino acids; final peptide concentration, 1 μM each) spanning the ZEBOV Mayinga strain GP, or medium alone. The input cell number was 2×10^5 PBMCs per well (2×10^6 PBMCs per ml), and cells were assayed in duplicate wells. Cells were incubated for 18 to 24 h at 37 °C and then removed from the ELISpot plate by first being washed with deionized water and then being washed six times with 1× PBS containing 0.25% Tween 20. Thereafter, plates were treated with a rabbit polyclonal anti-human IFN-γ biotinylated detection antibody (0.65 μg per well; Life Technologies) diluted with 1× PBS containing 1% bovine serum albumin (BSA) and were incubated at 37 °C for 2 h. ELISpot plates were then washed 6 times with 1× PBS containing 0.25% Tween 20, treated with 100 μl per well of streptavidin–horseradish peroxidase conjugate (BD Biosciences) diluted 1:250 with 1× PBS containing 10% FBS and 0.005% Tween 20, and incubated for an additional 1 h at room temperature. Unbound conjugate was removed by rinsing the plate six times with 1× PBS containing 0.25% Tween 20 and three times with 1× PBS. A chromogenic substrate (100 μl per well) (one-step nitroblue tetrazolium/5-bromo-4-chloro-3-indolylphosphate (NBT/BCIP); Pierce) was then added for 3 to 5 min before being rinsed away with water, after which the plates were air dried and the resulting spots counted using an ImmunoSpot reader (CTL Inc.). Peptide-specific IFN-γ ELISpot responses were considered positive if the responses (minus the medium background) were threefold above the medium response and 50 spot-forming cells (SFC) per 10^6 PBMCs. Unpaired *t*-test analysis of IFN-γ ELISpot data was performed on GraphPad Prism version 5.02 software. Two-tailed *P* values less than 0.05 indicated that the tests were statistically significant.

Detection of viraemia. Virus titration of the rVSV vaccine vectors and ZEBOV was performed by plaque assay with Vero E6 cells (ATCC, CRL-1586) from cell culture or serum samples as previously described[9]. Briefly, increasing tenfold dilutions of the samples were adsorbed to Vero E6 monolayers in duplicate wells (200 μl); the limit of detection was 25 PFU per ml.

Haematology and serum biochemistry. Total white blood cell counts, white blood cell differentials, red blood cell counts, platelet counts, haematocrit values, total haemoglobin concentrations, mean cell volumes, mean corpuscular volumes, and mean corpuscular haemoglobin concentrations were analysed from blood collected in tubes containing EDTA using a laser based haematologic analyser (Beckman Coulter). Serum samples were tested for concentrations of albumin, amylase, alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, gamma-glutamyltransferase, glucose, cholesterol, total protein, total bilirubin, blood urea nitrogen, creatine, and C-reactive protein by using a Piccolo point-of-care analyser and Biochemistry Panel Plus analyser discs (Abaxis).

Histopathology and immunohistochemistry. Necropsy was performed on all subjects. Tissue samples of all major organs were collected for histopathological and immunohistochemical examination, immersion-fixed in 10% neutral buffered formalin, and processed for histopathology as previously described[21]. For immunohistochemistry, specific anti-ZEBOV immunoreactivity was detected using an anti-ZEBOV VP40 protein rabbit primary antibody (Integrated BioTherapeutics, Inc.) at a 1:4,000 dilution. In brief, tissue sections were processed for immunohistochemistry using the Dako Autostainer (Dako). Secondary antibody used was biotinylated goat anti-rabbit IgG (Vector Laboratories) at 1:200 followed by Dako LSAB2 streptavidin–horseradish peroxidase (Dako). Slides were developed with Dako DAB chromagen (Dako) and counterstained with haematoxylin. Non-immune rabbit IgG was used as a negative control.

19. Cooper, D. *et al.* Attenuation of recombinant vesicular stomatitis virus HIV-1 vaccine vectors by gene translocations and G gene truncation reduces neurovirulence and enhances immunogenicity in mice. *J. Virol.* **82,** 207–219 (2008). Medline CrossRef.
20. Witko, S. E. *et al.* An efficient helper-virus-free method for rescue of recombinant paramyxoviruses and rhadoviruses from a cell line suitable for vaccine development. *J. Virol. Methods* **135,** 91–101 (2006). Medline CrossRef.
21. Thi, E. P. *et al.* Marburg virus infection in nonhuman primates: Therapeutic treatment by lipid-encapsulated siRNA. *Sci. Transl. Med.* **6,** 250ra116 (2014).



Extended Data Figure 1 | Relative immunogenicity of rVSV/ZEBOV vectors in cynomolgus macaques. At study day −28, cynomolgus macaques were immunized intramuscularly with 2×10^7 PFU of either N4 or N1 vectors. Ten days after a single immunization, PBMCs were prepared and ZEBOV GP-specific T-cell responses were quantified by IFN-γ ELISpot assay. **a**, ZEBOV GP-specific IFN-γ ELISpot responses in individual macaques. **b**, Average ZEBOV GP-specific IFN-γ ELISpot responses with s.e.m. indicated.

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To: (b) (6) .; Broder, Christopher; (b) (6)
Subject: rVSV constructs - gen3?
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Some food for thought - would appreciate some feedback.

I really like the idea of focusing on the N4CT1 constructs to attenuate the rVSV constructs, particularly for the virus families we're dealing with where neruovirulence of the rVSVs has been shown or could be a limitation.

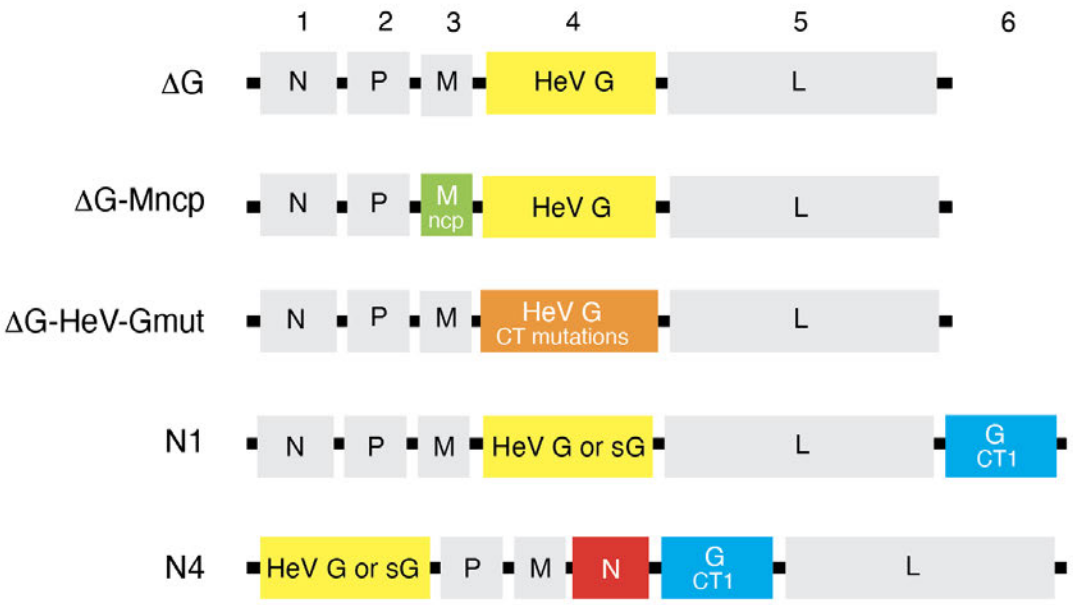
I coming to think that we should also propose testing other attenuating mutations as in Clarke et al. [attached]

I also like inserting sG constructs.

Additionally, I think it's possible that we could both increase expression of our viral glycoproteins AND attenuate them by truncating and mutating the cytoplasmic tails. IOWS mutate not only the VSV G cytoplasmic tail but also or mutate the cytoplasmic tail of the inserted viral glycoprotein.

This makes a lot of sense [in my mind] for all our prototype pathogens but especially for JUNV - check out the Nunberg paper attached. By truncating the CT of JUNC GPC you eliminate the need for the SSP but more importantly drive the GP to the cell surface.

This draft figure is derived from Tom's Makona paper and shows what I think might be a good grantsmanship approach of not putting all the eggs in one rVSV basket for any one virus.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viruses

MDPI

Article

A Recombinant Chimeric Cedar Virus-Based Surrogate Neutralization Assay Platform for Pathogenic Henipaviruses

Moushimi Amaya [1,*], Randy Yin [1,2], Lianying Yan [1,2], Viktoriya Borisevich [3,4], Bishwo N. Adhikari [5,6], Andrew Bennett [6,7], Francisco Malagon [6,7], Regina Z. Cer [5], Kimberly A. Bishop-Lilly [5], Antony S. Dimitrov [1,2], Robert W. Cross [3,4], Thomas W. Geisbert [3,4] and Christopher C. Broder [1,*]

1 Department of Microbiology and Immunology, Uniformed Services University, Bethesda, MD 20814, USA
2 Henry M. Jackson Foundation for the Advancement of Military Medicine Inc., Bethesda, MD 20814, USA
3 Department of Microbiology and Immunology, University of Texas Medical Branch, Galveston, TX 77555, USA
4 Galveston National Laboratory, University of Texas Medical Branch, Galveston, TX 77555, USA
5 Genomics and Bioinformatics Department, Biological Defense Research Directorate, Naval Medical Research Command–Frederick, Fort Detrick, Frederick, MD 21702, USA
6 Defense Threat Reduction Agency, Fort Belvoir, VA 22060, USA
7 Leidos, Inc., Reston, VA 20190, USA
* Correspondence: moushimi.amaya@usuhs.edu (M.A.); christopher.broder@usuhs.edu (C.C.B.)

Abstract: The henipaviruses, Nipah virus (NiV), and Hendra virus (HeV) can cause fatal diseases in humans and animals, whereas Cedar virus is a nonpathogenic henipavirus. Here, using a recombinant Cedar virus (rCedV) reverse genetics platform, the fusion (F) and attachment (G) glycoprotein genes of rCedV were replaced with those of NiV-Bangladesh (NiV-B) or HeV, generating replication-competent chimeric viruses (rCedV-NiV-B and rCedV-HeV), both with and without green fluorescent protein (GFP) or luciferase protein genes. The rCedV chimeras induced a Type I interferon response and utilized only ephrin-B2 and ephrin-B3 as entry receptors compared to rCedV. The neutralizing potencies of well-characterized cross-reactive NiV/HeV F and G specific monoclonal antibodies against rCedV-NiV-B-GFP and rCedV-HeV-GFP highly correlated with measurements obtained using authentic NiV-B and HeV when tested in parallel by plaque reduction neutralization tests (PRNT). A rapid, high-throughput, and quantitative fluorescence reduction neutralization test (FRNT) using the GFP-encoding chimeras was established, and monoclonal antibody neutralization data derived by FRNT highly correlated with data derived by PRNT. The FRNT assay could also measure serum neutralization titers from henipavirus G glycoprotein immunized animals. These rCedV chimeras are an authentic henipavirus-based surrogate neutralization assay that is rapid, cost-effective, and can be utilized outside high containment.

Keywords: Hendra virus; Nipah virus; Cedar virus; henipavirus; chimera; reverse genetics; virus neutralization; vaccine; virus-host cell interaction; antibody; serum

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1. Introduction

The bat-borne highly pathogenic Hendra virus (HeV) and Nipah virus (NiV) are the prototype members of the genus *Henipavirus* within the family *Paramyxoviridae* [1]. HeV and NiV are classified as Biosafety Level-4 (BSL-4) pathogens because of their high lethality and lack of approved vaccines or antivirals and are transboundary agents of significant disease threats to livestock and people in Australia and South and Southeast Asia, respectively. The genus now includes nine other reported henipaviruses; the four viral isolates of Cedar virus (CedV), Gamak virus, Daeryong virus, and Langya virus (LayV), [2–4] and five additional species known only from nucleic acid sequence information; Ghana bat virus (GhV), Mòjiāng virus, Melian virus, Denwin virus, and Angavokely virus (AngV) [5–8]. The recognized or apparent natural reservoir of all isolates of NiV, HeV, and CedV, along with the genomic data of GhV and AngV, are old-world fruit bats of the family *Pteropodidae*.

Whereas the six other reported henipaviruses are, or are likely, of rodent origins, including the isolate LayV. Only HeV and NiV are known to be associated with severe and often fatal henipaviral disease in humans and a number of animal species (reviewed in: [9,10]), while LayV was associated with nonfatal febrile illnesses in humans [4]. In contrast, CedV is the only henipavirus isolate demonstrated to be nonpathogenic in well-established animal models of NiV and HeV infection and disease, including guinea pigs, ferrets, hamsters [2,11], and African green monkeys (Geisbert, T.W. and Broder, C.C., unpublished). An important distinction between CedV and other henipaviruses lies within the *P* gene, which encodes the phosphoprotein (P), and the *P* gene transcripts of NiV and HeV undergo RNA editing to produce the V and W nonstructural proteins that are key interferon (IFN) antagonists (reviewed in [12,13]). The CedV *P* gene lacks both RNA editing and does not encode V or W [2,14]. Several studies with recombinant NiV variants have demonstrated the differential importance of the V and W proteins in the pathogenesis brought about by NiV infection in both the hamster and ferret models, and a lack of the V protein resulted in nonlethal infections [15–18]. All other recognized or proposed henipaviruses have the potential to express V and W proteins based on current genetic data. The absence of these proteins in CedV is hypothesized to be the key factor underlying its nonpathogenic nature in established NiV and HeV animal models. These data permitted the rescue and characterization of recombinant CedV (rCedV) by reverse genetics at BSL-2 containment, and CedV is now recognized as a BSL-2 restricted agent [19–21].

The development of effective countermeasures against NiV and HeV has been a research priority since their discovery [22]. NiV and henipaviral diseases are also included on the WHO's Blueprint List of Priority Pathogens [23], and NiV is among the Coalition for Epidemic Preparedness Innovations (CEPI) list of Priority Diseases needing urgent research and countermeasure development [24,25]. There are currently no licensed NiV/HeV vaccines or antivirals approved for human use, although a licensed vaccine to prevent HeV infection in horses, based on a soluble form of the attachment (G) glycoprotein (sG) from HeV (HeV-sG), was launched in Australia (Equivac® HeV) by Zoetis, Inc., in November 2012 [26]. A NiV vaccine formulated for human use with the HeV-sG immunogen is currently in Phase 1 human clinical trials [27]. As a therapeutic approach development for henipavirus human infection, the human monoclonal antibody (mAb) m102.4, specific to the NiV and HeV G glycoprotein receptor binding site, has completed a Phase I clinical trial in Australia [28]. To date, 18 individuals exposed to either HeV in Australia (n = 17) or NiV in the United States (n = 1) have received high-dose, post-exposure, m102.4 therapy (15–20 mg/kg) by emergency use protocols and no evidence of virus infection has been reported.

The G glycoprotein (also referred to as the receptor-binding protein (RBP)), together with the fusion (F) glycoprotein on the surface of the henipavirus virion, are the mediators of virus attachment and infection [29]. The RBP determines the cellular tropism of infection. The NiV and HeV G glycoproteins specifically bind to cells expressing the ephrin-B class ligands, ephrin-B2, and ephrin-B3 [30–33]. In contrast, CedV has a uniquely broad ephrin protein tropism and can utilize both B-class and A-class ephrins for cell entry and infection [21,34]. The binding of NiV or HeV G to their ephrin entry receptors on cells triggers a well-characterized activation and refolding of F from a pre- to post-fusion conformation that facilitates the merger of the virion and host cell membranes and subsequent delivery of the viral nucleocapsid into the cell cytoplasm (reviewed in [35]). Importantly, the F and G glycoproteins are also the major viral structural protein targets of neutralizing antibodies and the relevant antigens employed in all henipavirus vaccine strategies [22]. However, for pathogenic henipavirus vaccines or antibody-based countermeasure strategies, the assessment and quantification of neutralizing antibody responses or their potencies against authentic NiV and HeV, which requires BSL-4 containment, can be a major challenge.

Using a rCedV reverse genetics platform, the CedV F and G glycoprotein genes were replaced with those of NiV-B or HeV, and replication-competent chimeric henipaviruses (rCedV-NiV-B and rCedV-HeV) were rescued. Both non-reporter and two reporter gene

versions, encoding a green fluorescent protein (GFP) or luciferase protein (Luc), of the rCedV chimeras were also produced. Characterization of the chimeric viruses revealed no significant differences in their replication kinetics or ability to induce a type I IFN response compared to rCedV and possessed the same ephrin B-class entry receptor tropisms as NiV-B and HeV. The neutralization potencies of several well-characterized cross-reactive NiV and HeV F and G specific mAbs against rCedV-NiV-B-GFP and rCedV-HeV-GFP were highly correlated with those measured using authentic NiV-B and HeV when tested in parallel by a plaque reduction neutralization test (PRNT). A rapid high-throughput and quantitative fluorescence reduction neutralization test (FRNT) using the GFP-encoding chimeras was established that also yielded highly correlated mAb neutralization potencies with those derived by PRNT. The FRNT assay was also suitable for measuring serum neutralization titers from animals immunized with recombinant HeV or NiV soluble G glycoproteins. Taken together, the rCedV chimera platform is an authentic henipavirus-based surrogate neutralization assay for pathogenic henipaviruses that is rapid, cost-effective and can be utilized outside BSL-4 containment.

2. Materials and Methods

2.1. Construction of pOLTV5-rCedV Chimeric Antigenomes

A full description of the synthesis of the rCedV antigenome clone (pOLTV5-rCedV) has previously been described [20]. Here, an optimized version of the pOLTV5-rCedV antigenome clone was designed, and the 3′ end of the T7 minimal promoter ($T7_{min}$) sequence (TAATACGACTCACTATA) was modified by the addition of nucleotides GGGAGA to generate a T7 optimal promoter ($T7_{opt}$) [36]. The $T7_{opt}$ sequence was then followed by the insertion of a self-cleaving autocatalytic hammerhead ribozyme A (HHRbzA) sequence (GGGAGATTGGTCTGATGAGTCCGTGAGGACGAAACGGAGTCTAGACTCCGTC) [36]. This synthesized gene fragment ($T7_{opt}$-HHRbzA) (Genscript; NJ, USA) was enzymatically inserted to precede the CedV 3′ Leader (3′ Le) sequence in the pOLTV5-rCedV plasmid to yield pOLTV5$_{opt}$-rCedV (Figure 1A).

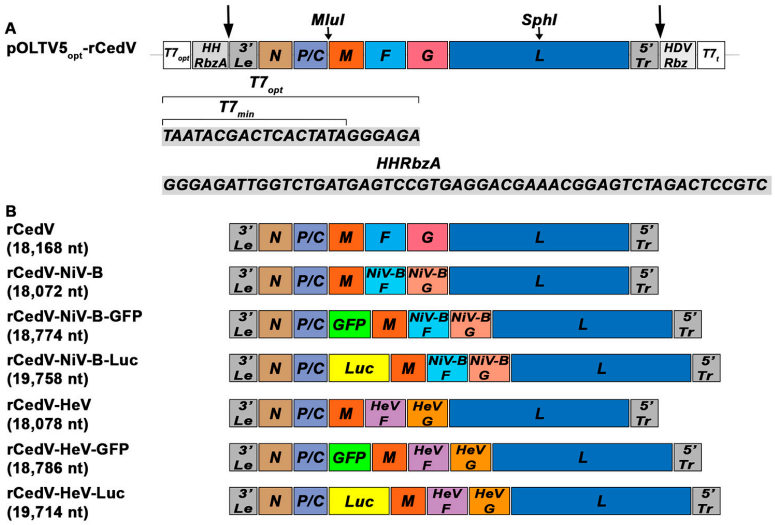

Figure 1. Schematic representation of the optimized rCedV plasmid and the genomes of the generated rCedV chimeric viruses. (**A**) The pOLTV5$_{opt}$-rCedV plasmid illustrates the location and sequences of the T7 optimal promoter ($T7_{opt}$) and the Hammerhead Ribozyme A (HHRbzA). The long arrows indicate regions of self-cleavage. Unique restriction sites MluI and SphI used to construct the rCedV chimeric plasmids are shown. (**B**) The genomes and the lengths of the generated chimeras are schematically diagrammed as rCedV-NiV-B, rCedV-NiV-B-GFP, rCedV-NiV-B-Luc, rCedV-HeV, rCedV-HeV-GFP, and rCedV-HeV-Luc. pOLTV5$_{opt}$-rCedV, optimized pOLTV5-rCedV plasmid; $T7_{min}$, T7 minimal promoter; $T7_{opt}$, T7 optimal promoter; HHRbzA, Hammerhead Ribozyme A; 3′Le, 3′ Leader; 5′Tr, 5′ Trailer; HDVRbz, hepatitis delta virus ribozyme; $T7_t$, T7 terminator.

Large gene cassettes comprising CedV F and G untranslated intergenic regions flanking the respective NiV-B or HeV F and G coding sequences were synthesized (Genscript). The NiV-B F and G coding sequences were based on the NiV-B 2010 Faridpur isolate (GenBank: JN808864.1). The HeV F and G protein sequences used here, HeV genome (GenBank: MN062017.1), are identical to that of the HeV 2008 Redlands isolate (GenBank: JN255805.1). The isolates and GenBank accession numbers for each F and G protein are listed in Table 1. Unique restriction enzyme sites facilitated the insertion of the NiV-B or HeV F and G gene cassettes into pOLTV5$_{opt}$-rCedV (Figure 1A) to ultimately generate non-reporter versions, pOLTV5$_{opt}$-rCedV-NiV-B or pOLTV5$_{opt}$-rCedV-HeV. The "rule-of-six" was maintained by removing the last three nucleotides (ACG; amino acid Threonine) from the NiV-B F coding sequence and adding a stop codon (TAA) to the end of the HeV F coding region. The insertion of a modified turbo Green Fluorescent Protein (*GFP*) gene (Lonza Inc., Allendale, NJ, USA) or firefly (*Photinus pyralis*) luciferase (*Luc*) gene (a kind gift from Dr. B Schaefer, USU) between CedV *P* and *M* genes of the newly constructed pOLTV5$_{opt}$-rCedV chimeric plasmids using standard molecular techniques was as previously described [20,37] and yielded pOLTV5$_{opt}$-rCedV chimeric reporter gene encoding versions of the rCedV antigenome clones. All cloning was performed with *Escherichia coli* Stbl2 cells (Invitrogen; Carlsbad, CA, USA). The insertions were sequenced to obtain at least 2-fold coverage.

Table 1. GenBank accession numbers of NiV-B and HeV envelope glycoproteins.

Henipavirus	Isolate	Protein	GenBank Accession Number
NiV-B	2010 Faridpur	F	AEZ01396.1
		G	AEZ01397.1
HeV	2008 Redlands	F	AEQ38070.1
		G	AEQ38071.1

2.2. Cells, Monoclonal Antibodies, Rhesus Macaque, and Rabbit Immune Sera

BSR-T7/5 cells, a BHK-derived cell line stably expressing T7 RNA polymerase [38], HeLa-USU, HeLa (ATCC CCL-2), Vero E6 (ATCC CCL-81), and Vero 76 (ATCC CRL-1587) cell lines were maintained at 37 °C, 5% CO_2 in Dulbecco's modified eagle media (DMEM) (Quality Biological; Gaithersburg, MD, USA) supplemented with 10% cosmic calf serum (CCS) and 1% L-glutamine (Quality Biological) (DMEM-10). HeLa-USU-ephrin-B2 and HeLa-USU-ephrin-B3 stable cell lines were maintained in DMEM-10% CCS, 1% L-glutamine supplemented with 0.4 mg/mL Hygromycin B (Invitrogen).

The neutralizing HeV and NiV cross-reactive human mAb, m102.4, is a G glycoprotein-specific IgG1 subclass antibody [28,39–43]. The humanized 5B3.1 (h5B3.1) mAb [44,45] and the murine mAbs 12B2 and 1F5 [46] are IgG1 mAbs cross-reactive to HeV and NiV F glycoprotein. Anti-NiV G glycoprotein-specific sera were from four rhesus macaques immunized at the University of Texas Medical Branch at Galveston, TX, on days 0, 28, and 56 with an equal mixture of 0.1 mg NiV-B and 0.1 mg NiV-M recombinant soluble G (sG) glycoproteins adjuvanted with aluminum hydroxide suspension (Auro Vaccines, LLC, Pearl River, NY, USA) [47]. Sera were collected on days 42 and 84 post-immunization and stored at −80 °C. Anti-HeV G glycoprotein-specific sera were prepared using the HeV/NiV recombinant soluble HeV G glycoprotein vaccine candidate (HeV-sG) [26,27,48–54] or HeV-sG$_{tet}$ [55], a tetrameric version of the HeV sG glycoprotein which was constructed similarly to other henipavirus sG glycoproteins as described in Cheliout Da Silva et al. [56]. Sera from rabbits immunized on days 0 and 28 with 0.1 mg HeV-sG or HeV-sG$_{tet}$ formulated with complete Freund's adjuvant (initial injection) and boosted with immunogen formulated with incomplete Freund's adjuvant (booster injection) were prepared by Noble Life Sciences; Woodbine, MD. Sera were collected on day 45 post-immunization and stored at −80 °C.

2.3. Rescue of Recombinant CedV Chimeras

BSR-T7/5 cells in a 12-well plate (2.5×10^5 cells/well) were co-transfected with pCMV-CedV helper plasmids pCMV-CedV-N (1.25 µg), pCMV-CedV-P (0.8 µg) and pCMV-CedV-L (0.4 µg) together with one of the pOLTV5$_{opt}$-rCedV chimera antigenome constructs (3.5 µg) using TransIT-LT1 transfection reagent (Mirus Bio; Madison, WI, USA) according to the manufacturer's recommendations. After 4–5 days, transfected cells were observed for syncytia formation and/or GFP expression. Supernatants from successful rescue wells were collected and passaged onto naïve Vero E6 cells in a T-75 flask to prepare a master stock of each of the rCedV chimeras. When maximal syncytia and/or GFP expression was observed (~2–3 days), viral supernatants were collected and clarified by centrifugation at 948× *g* (2400 rpm) for 10 min to pellet cell debris. The supernatant was transferred to screw-cap tubes as single-use aliquots and stored at −80 °C. All rCedV chimeras were deep sequenced using Illumina short reads, and variants were analyzed. Briefly, sequencing libraries were prepared from total RNA using the TruSeq Stranded Total RNA Sample Prep kit (Illumina, San Diego, CA, USA) and subjected to multiplexed sequencing on either the Illumina MiSeq platform using 600 cycles, V3 chemistry, or the Illumina NextSeq platform using 300 cycles, V2 chemistry. The resulting sequencing reads were analyzed using EDGE Bioinformatics tools [57] and an in-house metagenomics pipeline called MetaDetector (unpublished). EDGE Bioinformatics suite was used for read processing, and the host read subtraction, de novo assembly, taxonomic classification, and variant detection. Sequencing reads were also processed in parallel using MetaDetector, which checked for quality using FASTQC [58], trimmed for quality using BBDuk (Q20) [59], and removed incidental matching human genome reads using BBMAP [59]. The remaining reads were assembled using metaSPAdes and SPAdes [60]; the resulting contigs, along with all the cleaned singleton reads, were BLAST searched using Diamond [61] for taxonomic classification. The final assemblies were examined and constructs were identified to be annotated using BLASTn and BLASTx implemented in CLC Genomics Workbench (QIAGEN Bioinformatics; Redwood City, CA). Variant analysis was performed by mapping the reads to a publicly available reference Cedar virus genome from isolate CG1a (GenBank accession JQ001776) using EDGE Bioinformatics tools and iVar [62].

2.4. Viral Plaque Assay

Viral stocks were titrated by plaque assay as previously described [20,37,63]. Briefly, a ten-fold serial dilution of the virus stock was prepared in DMEM-10, 200 µL of which was applied to pre-seeded Vero E6 cells in duplicate (5×10^5 cells/well) in a 12-well plate and incubated for 1 h at 37 °C, 5% CO_2. A 2 mL overlay of a 1:1 mix of DMEM containing 5% CCS and 1% L-glutamine (DMEM-5) with 2% carboxymethylcellulose sodium salt (medium viscosity) (Sigma-Aldrich; St. Louis, MO, USA) was applied to all wells and incubated for 4 days at 37 °C, 5% CO_2. Cells were fixed with 4% Formaldehyde in 1× PBS for 1 h at room temperature and stained with 0.5% crystal violet in 80% methanol for 15 min at room temperature. The stain was removed and washed with diH_2O, and plaque-forming units (PFU) were counted and expressed as PFU/mL.

2.5. Virus Biosafety Procedures and Regulations

Laboratory manipulation guidelines and standard operating procedures for rCedV-NiV-B, rCedV-NiV-B-GFP, rCedV-NiV-B-Luc, rCedV-HeV, rCedV-HeV-GFP, rCedV-HeV-Luc, rCedV, rCedV-GFP and rCedV-Luc under BSL-2 conditions have been established, reviewed, and approved by the Uniformed Services University (USU), Institutional Biosafety Committee in accordance with NIH guidelines. The rCedV-HeV-GFP and/or rCedV-NiV-B-GFP chimeras have been previously used in mAb neutralization and mAb synergy neutralization studies [47,64,65]. A P2 stock of NiV-B was used in these studies. There were four mutations of sufficient frequency in comparison to the reference sequence GenBank Accession number AY988601.1. Of these, one was non-coding, and the other three led to single amino acid changes: one in the M protein and two in the F protein [66]. The HeV

isolate used in these studies (GenBank Accession number NC_001906) was obtained from a patient from the 1994 outbreak in Australia and was provided by Dr. Thomas Ksiazek [45]. All studies with authentic NiV-B and HeV were performed within the BSL-4 facilities of the Galveston National Laboratory, The University of Texas Medical Branch at Galveston, TX, USA.

2.6. Western Blot Analysis

Vero E6 cells in a 6-well plate were infected at a density of 1×10^6 cells/well with rCedV-NiV-B, rCedV-NiV-B-GFP, rCedV-NiV-B-Luc, rCedV-HeV, rCedV-HeV-GFP, rCedV-HeV-Luc, rCedV, rCedV-GFP or rCedV-Luc at a multiplicity of infection (MOI) of 0.01. Simultaneously, the cells were co-transfected with a total of 2 µg of the plasmid to express both F and G glycoproteins from either NiV or HeV. A promoter-modified pcDNA3.1 vector with a hygromycin selection marker [67] encoding either NiV-F (pcDNA3.1-NiV-F) or NiV-G (pcDNA3.1-NiV-G) was used for the NiV-F and NiV-G expression. A promoter-modified pcDNA3.1 encoding either HeV-F (pcDNA3.1-HeV-F) or HeV-G (pcDNA3.1-HeV-G) was used for the HeV-F and HeV-G expression. At 24 h and 48 h post-infection, cells were collected and lysed with 1× RIPA (radioimmunoprecipitation assay) Lysis and Extraction Buffer (ThermoFisher Scientific; Waltham, MA, USA) containing a protein inhibitor cocktail (ThermoFisher Scientific). Total protein (~30 µg) in reducing sample buffer (2× lithium dodecyl sulfate (LDS) NuPage® sample buffer (Invitrogen), 5% β-mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA) was boiled for 10 min at 100 °C. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 4–12% Bis-Tris gel (ThermoFisher Scientific) and then transferred on nitrocellulose membranes (ThermoFisher Scientific). The membranes were blocked in 5% milk in 1× PBS with 0.1% Tween-20 at room temperature. Cross-reactive murine mAbs specific to NiV and HeV F (mAb 5G7) or to NiV and HeV G (mAb 48D3) glycoproteins, polyclonal rabbit sera to CedV-N (CSIRO, Victoria, Australia), and β-actin (ThermoFisher Scientific) were used as primary antibodies and subsequently probed with a corresponding secondary HRP-coupled antibody.

2.7. Virus Replication Kinetics

Vero E6 cells were seeded at a density of 2×10^4 cells/well in a 96-well cell culture plate. The next day, cells were infected at an MOI of 0.01 at 37 °C, 5% CO_2. After 1 h, the viral inoculum was removed, and fresh DMEM-10 was added to all wells. Supernatants were collected at 0, 8, 24, 48, and 72 h post-infection and stored at −80 °C until ready to analyze. Viral titers were determined by plaque assay as described in Section 2.4 and were expressed as plaque-forming units (PFU) per mL (PFU/mL). To determine intracellular luciferase activity, cells infected with either the non-reporter or Luc expressing viral chimeras were lysed with the Steady-Glo® Luciferase Assay System (Promega, Madison, WI, USA) in a 1:1 mixture with DMEM-10. After a 10 min incubation at room temperature, the homogenate was transferred to a white opaque 96-well cell culture plate, Nunc™ F96 MicroWell™ White Polystyrene Plate (ThermoFisher Scientific), and luminescence read using the GloMax®—Multi Detection System (Promega, Madison, WI, USA). Relative light units (RLU) were measured and normalized by subtracting the luminescence values of rCedV-NiV-B or rCedV-HeV infected cells from the luminescence values of rCedV-NiV-B-Luc or rCedV-HeV-Luc infected cells, respectively. Virus titers and luciferase activity levels at 0 h post-infection indicate the lower limit of detection for the plaque assay and the luminometer, respectively.

2.8. Ephrin Entry Receptor Tropism

HeLa-USU, HeLa-USU-ephrin-B2, and HeLa-USU-ephrin-B3 cell lines were seeded at a density of 2.5×10^5 cells/well in a 12-well cell culture plate. When confluent, the cell culture medium was removed, and cells were left uninfected (Mock) or infected at an MOI of 0.5 with either rCedV-NiV-B-GFP, rCedV-HeV-GFP, or rCedV-GFP individually diluted in DMEM-10. At 24 h post-infection, rCedV-NiV-B-GFP, rCedV-HeV-GFP, and rCedV-GFP

infected cell cultures were monitored for GFP fluorescence and syncytia. Images were captured with a Zeiss Axio Observer A1 inverted microscope using the 5× objective.

2.9. Reverse Transcriptase Quantitative PCR (RT-qPCR) and Type I IFN Response

HeLa-CCL-2 cells were seeded at a density of 1.25×10^5 cells/well in a 24-well plate and incubated overnight. Cells were left intact (Mock), transfected with polyinosinic:polycytidylic acid (Poly I:C) (InvivoGen; San Diego, CA, USA) (10 μg/mL) using Lipofectamine LTX (ThermoFisher Scientific), or infected with rCedV-NiV-B, rCedV-HeV or rCedV at either an MOI of 0.5 or 1.0. At 24 h post-infection, total RNA was extracted using the RNeasy Mini Kit (Qiagen Sciences Inc., Germantown; MD, USA). An amount of 500 ng of DNase I digested RNA was converted to cDNA using the Superscript III First-Strand Synthesis System (ThermoFisher Scientific) with oligo(dT) primers. Quantitative PCR (qPCR) was then performed with the synthesized cDNA using the Power SYBR Green PCR Master Mix (ThermoFisher Scientific) and the Applied Biosystems 7500 Real-Time PCR System. PCR cycling conditions were: 95 °C, 10 min; 40× cycles of 95 °C, 15 s; 60 °C, 1 min; followed by a melt curve analysis at the completion of each experiment. Each sample was analyzed for IFN-α, IFN-β and 18S ribosomal RNA in triplicate, and fold changes were calculated relative to 18S ribosomal RNA and normalized to mock samples using the $2^{(-\Delta\Delta Ct)}$ method. IFN-α forward primer, 5′ TTTCTCCTGCCTGAAGGACAG 3′, IFN-α reverse primer, 5′ ACAGTCTCGTCTTTAGTACTCG 3′ [68]. IFN-β forward primer, 5′ GTCAGAGTGGAAATCCTAAG 3′, IFN-β reverse primer, 5′ ACAGCATCTGCTGGTTGAAG 3′ [69]. An 18S ribosomal RNA forward primer 5′ GGGCATTCGTATTTCATAGTCAGAG 3′, 18S ribosomal RNA reverse primer 5′ CGGTTCTTGATTAATGAAAACATCCT 3′ [70].

2.10. Plaque Reduction Neutralization Test (PRNT)

Vero 76 cells were seeded at a density of 6×10^5 cells/well in a 6-well plate and incubated overnight at 37 °C, 5% CO_2. The mAbs were serially diluted 3-fold in DMEM-10 such that an initial concentration of 10 μg/mL was used for the 9-point dose-response curve. The diluted mAbs were incubated with an equal volume of either rCedV-NiV-B-GFP, rCedV-HeV-GFP, NiV-B, or HeV at an MOI of 0.0001 for 1 h at 37 °C, 5% CO_2. MOI was calculated for a tentative 10^6 cells/well and 0.4 mL virus and antibody mixture per well. Each virus-mAb mixture (400 μL/well) was added to duplicate wells. Following a 1 h incubation at 37 °C, 5% CO_2, the wells were overlaid with a 1:1 mix of 0.8% agarose/DMEM-10 and incubated for 4 days. A neutral red solution was added to each well and incubated for 24 h, at which time plaques were counted. Neutralization percent (%) was calculated by subtracting the PFU_{mAb} for each virus from the respective PFU without the antibody, i.e., $Neutralization(\%) = 100 \times \frac{(PFU_0 - PFU_{mAb})}{PFU_0}$, where the PFU_{mAb} is the PFU at the respective mAb concentration, and PFU_0 is the PFU without the antibody. The 50% inhibitory concentration (IC_{50}) was determined as the antibody concentration at which there was a 50% reduction in plaque counts versus untreated control wells. The IC_{50} values were calculated by non-linear regression curve fitting with a variable slope using GraphPad Prism 9 (GraphPad Software Inc., San Diego, CA, USA). The limit of detection for this assay was 50 PFU.

2.11. Fluorescent Reduction Neutralization Test (FRNT)

Vero 76 cells were seeded at a density of 2×10^4 cells/well in black-walled clear bottom 96-well plates (Corning Life Sciences; Corning, NY, USA) and incubated for 24 h at 37 °C, 5% CO_2. m102.4, h5B3.1, 12B2, and 1F5 mAbs were serially diluted 3-fold such that an initial concentration of 1.1 μg/mL was used for the 7-point dose-response curve. Immunized sera were 3-fold serially diluted in DMEM-10 such that NiV-B and NiV-M sG immunized rhesus macaque sera were at a starting dilution of 1:200, and the HeV-sG and HeV-sG_{tet} rabbit sera were at a starting dilution of 1:400. An equal volume of DMEM-10 containing either rCedV-NiV-B-GFP or rCedV-HeV-GFP was added to each dilution for a final MOI of 0.05 and incubated for 2 h at 37 °C, 5% CO_2. Each of the virus-mAb or

virus-sera mixtures (90 µL/well) was added to the pre-seeded Vero 76 cells in triplicate and incubated for an additional 24 h at 37 °C, 5% CO_2. The virus-mAb supernatants were removed, and the plates were fixed with 4% Formaldehyde in 1× PBS for 20 min at room temperature. The plates were then washed 3 times by hand with a slow stream of diH_2O, and the last wash was discarded before the plates were imaged using a CTL S6 analyzer (Cellular Technology Limited; Shaker Heights, OH, USA). Fluorescent foci were counted using the CTL Basic Count software. The 50% inhibitory concentration (IC_{50}) was determined as the antibody concentration or serum dilution at which there was a 50% reduction in fluorescent foci versus untreated control wells. The IC_{50} values were calculated by non-linear regression curve fitting with a variable slope using GraphPad Prism 9 (GraphPad Software Inc., San Diego, CA, USA). The limit of detection for this assay was 50 fluorescent foci.

2.12. Statistical Analysis

Data were analyzed and graphed using GraphPad Prism 9 (GraphPad Software Inc.). Unless otherwise stated, graphs and images are the average of three independent experiments and are expressed as the arithmetic mean. Standard deviations were calculated and represented accordingly. Statistical analyses for viral replication kinetics were performed with two-way ANOVA followed by the Tukey post hoc test ($\alpha = 0.05$). Statistical analyses for qPCR experiments were performed with the unpaired, two-tailed Student *t*-test using GraphPad Prism 9. Correlation analyses were performed using Pearson correlation coefficient analyses.

3. Results

3.1. Construction and Rescue of Recombinant Cedar Virus-Based Chimeras

To generate rCedV chimeric viruses encoding the NiV-B or HeV envelope glycoprotein genes, we first optimized the virus rescue efficiency of the previously described rCedV reverse genetics system [20]. A pOLTV5$_{opt}$-rCedV antigenome plasmid was constructed by inserting a DNA fragment containing sequences for a T7 optimal promoter (T7$_{opt}$) and an autocatalytic Hammerhead Ribozyme A (HHRbzA) sequence upstream of the rCedV 3′ leader antigenome sequence (see Materials and Methods) (Figure 1A). Next, a large fragment of the pOLTV5$_{opt}$-rCedV plasmid flanked by unique restriction enzyme sites MluI and SphI was replaced with synthesized DNA fragments containing the open reading frames of either the NiV-B or HeV F and G glycoproteins in place of the CedV F and G glycoprotein encoding region. Reporter genes containing antigenome plasmids encoding either *GFP* or *Luc* genes for each chimera were also generated (see Materials and Methods). The reverse genetics method was then used to rescue a panel of replication-competent rCedV chimeras as non-reporter gene versions (rCedV-NiV-B and rCedV-HeV) and reporter gene encoding versions expressing GFP (rCedV-NiV-B-GFP and rCedV-HeV-GFP) or Luc (rCedV-NiV-B-Luc and rCedV-HeV-Luc) proteins. A schematic representation of the genomes and genome lengths of all rescued viruses is illustrated in Figure 1B. Successful rescue of the viruses was confirmed by the detection of syncytia formation (cytopathic effect (CPE)) when supernatants from BSR-T7/5 cells transfected with the rCedV chimeric antigenome and CedV helper plasmids were then passaged onto Vero E6 cells. Stock virus preparations were subsequently prepared, and virus genomes were sequenced. When compared to the predicted genome sequences, the following mutations were detected within coding sequences in the chimera genomes (e.g., excluding intergenic regions) (Table S1). There were two mutations in the rCedV-HeV genome: one synonymous single nucleotide variation (SNV) and one SNV that resulted in a single amino acid change in the F protein. Three mutations were detected in the rCedV-HeV-GFP genome: one synonymous SNV and two that resulted in single amino acid changes: one in the N protein and the other in the M protein. There were four mutations in the rCedV-HeV-Luc genome, and all four were synonymous mutations. The rCedV-NiV-B genome contained four mutations, all resulting in single amino acid changes: one in the N protein, one in the M

protein, and two in the L protein. There were eight mutations in the rCedV-NiV-B-GFP genome: six were synonymous SNVs, and two resulted in single amino acid changes in the N protein. There were nine mutations detected in the rCedV-NiV-B-Luc genome: five were synonymous SNVs, and four resulted in single amino acid changes: one in the N protein, one in the Luc protein, one in the M protein, and one in the L protein. No apparent loss of rCedV chimera reproductive capacity or reporter gene loss or integrity has been observed to date, probably owing to the requirement of these paramyxoviruses to the 'rule-of-six.'

3.2. Characterization of Recombinant Cedar Virus-Based Chimeric Viruses

We assessed the ability of the rCedV-NiV-B chimeras and the rCedV-HeV chimeras to facilitate membrane fusion and syncytia formation when used to infect cells (Figure 2). Vero E6 cells were either uninfected (Mock) or infected with either rCedV-NiV-B, rCedV-NiV-B-GFP, rCedV-NiV-B-Luc, rCedV-HeV, rCedV-HeV-GFP, or rCedV-HeV-Luc and comparisons then made to Vero E6 cells infected with rCedV, rCedV-GFP or rCedV-Luc. At 24 h post-infection, cells infected with the GFP-expressing viruses were imaged for fluorescence and syncytia (Figure 2A), while cells infected with the non-reporter or Luc expressing rCedV chimeras were imaged following fixation and crystal violet staining (Figure 2B). Fluorescence and/or syncytia (yellow arrows) were observed in all infected Vero E6 cells. The syncytia observed in cells infected with either the rCedV-NiV-B chimeras or the rCedV-HeV chimeras were noticeably larger and contained more nuclei than those syncytia observed in rCedV-infected cells (Figure 2). These data confirmed the functionality of the NiV-B and HeV F and G glycoproteins expressed in the context of rCedV.

We next evaluated the relative expression levels of NiV-B and HeV F and G envelope glycoproteins from cells infected with the rCedV chimeras in comparison to rCedV (comparative control). Vero E6 cells were uninfected (Mock) or infected with either rCedV-NiV-B, rCedV-NiV-B-GFP, rCedV-NiV-B-Luc, rCedV-HeV, rCedV-HeV-GFP, rCedV-HeV-Luc, rCedV, rCedV-GFP or rCedV-Luc. For additional comparative purposes, separate populations of Vero E6 cells were co-transfected with plasmids, pcDNA3.1-NiV-F and pcDNA3.1-NiV-G (pcDNA3.1-NiV F + G), or pcDNA3.1-HeV-F and pcDNA3.1-HeV-G (pcDNA3.1-HeV F + G). Representative western blot images for NiV-B and HeV F and G glycoproteins probed with cross-reactive NiV/HeV F or G specific mAbs are shown in Figure 3. We observed the precursor protein F_0 and the processed F_1 subunit in the lysates of cells infected with the rCedV-NiV-B chimeras (Figure 3A) or the rCedV-HeV chimeras (Figure 3B,C). A distinct band representing the G glycoprotein was detected in all rCedV-NiV-B (Figure 3A) and rCedV-HeV (Figure 3B,C) infected lysates. Furthermore, the NiV-B and HeV F and G glycoprotein SDS-PAGE gel migration profiles were similar to those observed in the pcDNA3.1-NiV F + G (Figure 3A) and pcDNA3.1-HeV F + G (Figure 3B,C) transfected cell lysates, respectively. Whereas, HeV/NiV-B F_0, F_1, or G bands were not observed in any of the rCedV infected lysates (Figure 3). The lower levels of F_0, F_1, and G observed in the rCedV-HeV-Luc infected lysates in comparison to the other rCedV-HeV chimera infected lysates at 24 h post-infection (Figure 3B) could be attributed to slower virus replication kinetics of rCedV-HeV-Luc (see Figure 4B). To address this, we analyzed lysates of all rCedV-HeV chimeras at 48 h post-infection by western blot. As shown in Figure 3C, the levels of HeV F_0, F_1, and G glycoproteins in all rCedV-HeV chimeras were comparable at this later time point. The presence of CedV N protein served as an expressed viral protein control and was observed in all infected cell lysates, while β-actin served as a lysate loading control. These data confirm the expression of NiV-B and HeV F and G glycoproteins in infected cells and indicate functional compatibility between rCedV and NiV-B and HeV envelope glycoproteins in a relevant viral context.

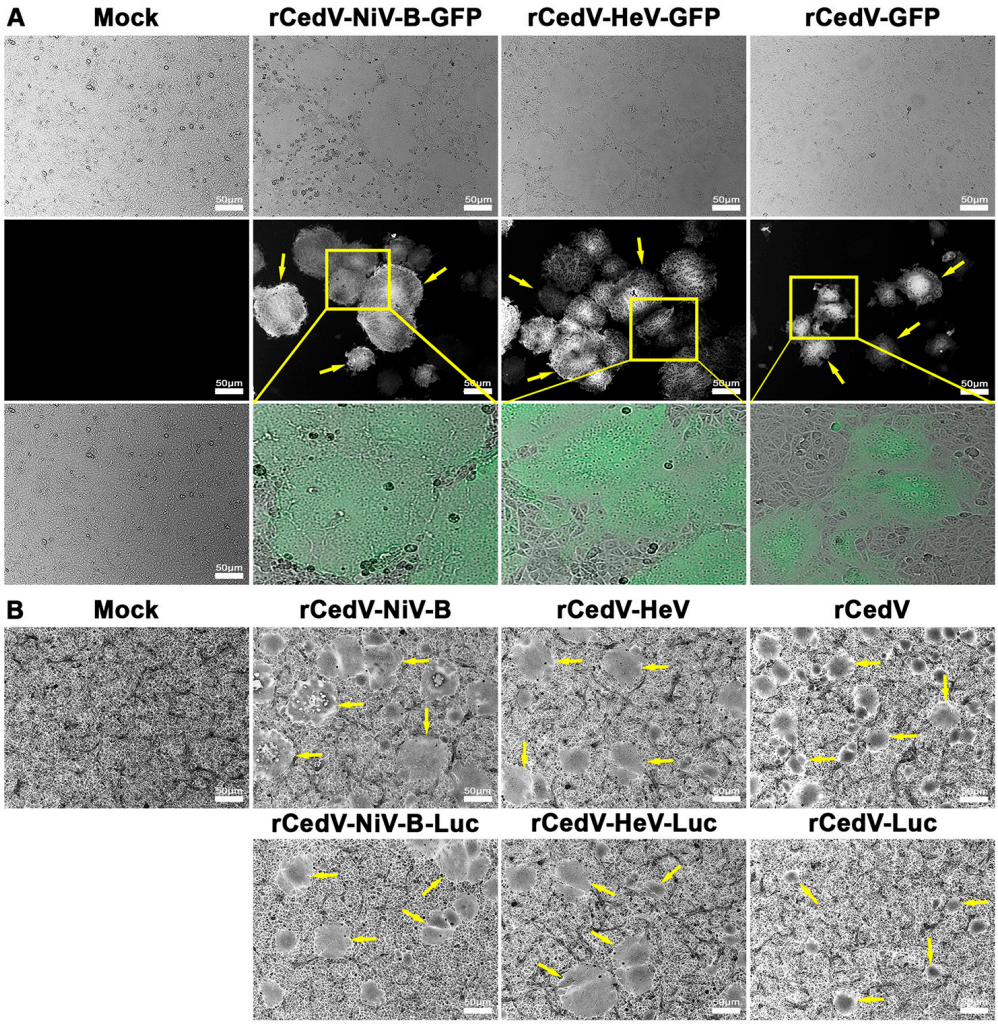

Figure 2. Syncytia induced by rCedV expressing NiV-B or HeV envelope glycoproteins. Vero E6 cells were uninfected (Mock) or infected with either rCedV-NiV-B, rCedV-NiV-B-GFP, rCedV-NiV-B-Luc, rCedV-HeV, rCedV-HeV-GFP, rCedV-HeV-Luc, rCedV, rCedV-GFP or rCedV-Luc at a MOI of 0.01. All images were taken 24 h post-infection. (**A**) Cells infected with GFP-expressing viruses. Transmitted light (**top** row), fluorescence (**middle** row), and merged (**bottom** row) images are shown. The respective zoomed-in fluorescence images (3rd row) are regions from the yellow boxes. (**B**) Cells infected with non-reporter or Luc expressing rCedV chimeras were fixed, stained, and then imaged for syncytia. The images taken with transmitted light are shown. Images were captured with a Zeiss Axio Observer A1 inverted microscope using a 5× objective. Arrows indicate giant multinucleated cells (syncytia). Representative images from three independent experiments are shown. Scale bar, 50 μm.

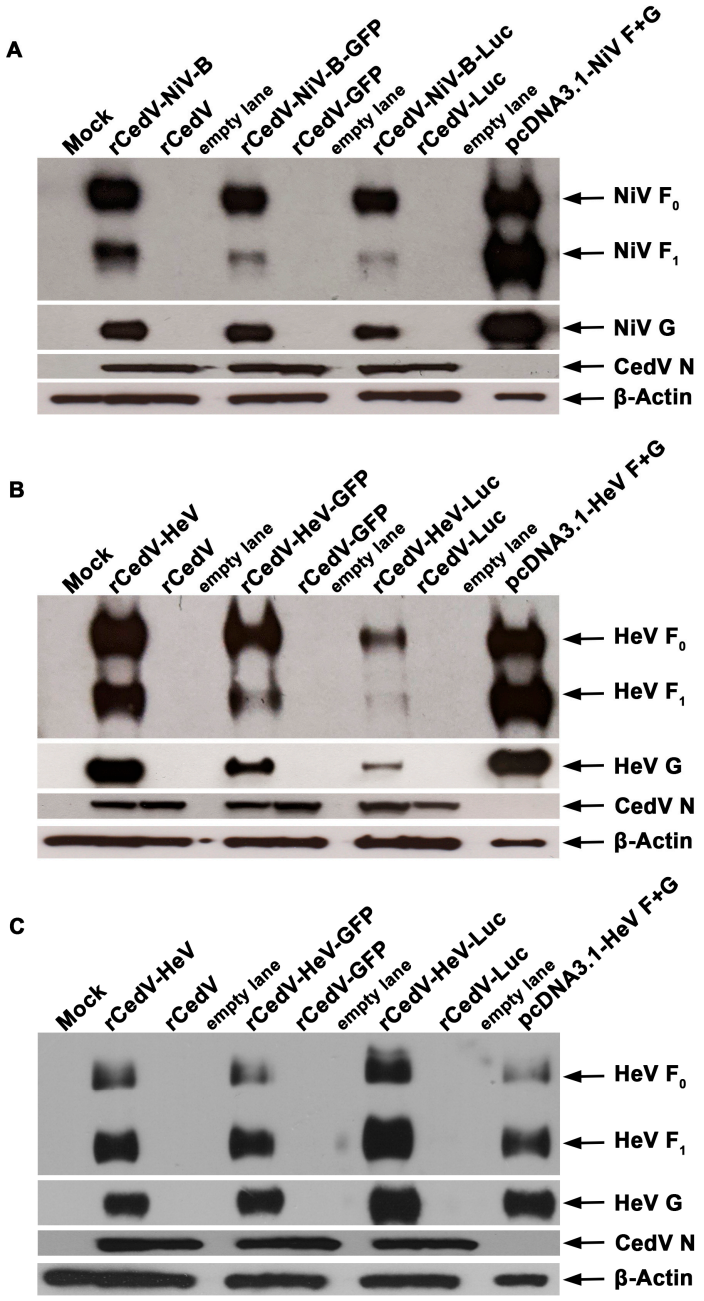

Figure 3. Expression of NiV-B and HeV envelope glycoproteins in infected cells. Vero E6 cells were uninfected (Mock) or infected at a MOI of 0.01 with either rCedV-NiV-B, rCedV-NiV-B-GFP, rCedV-NiV-B-Luc (**A**), rCedV-HeV, rCedV-HeV-GFP, rCedV-HeV-Luc (**B**,**C**), rCedV, rCedV-GFP or rCedV-Luc. As a reference, cells were co-transfected with a total of 2 µg of pcDNA3.1-NiV-F and pcDNA3.1-NiV-G (pcDNA3.1-NiV F + G), or pcDNA3.1-HeV-F and pcDNA3.1-HeV-G (pcDNA3.1-HeV F + G). Cells were harvested at 24 h post-infection (**A**,**B**) (rCedV-NiV-B and rCedV-HeV chimeras) or 48 h post-infection (**C**) (rCedV-HeV chimeras only), lysates were prepared and total protein (~30 µg) resolved by SDS-PAGE followed by western blot assay. The subsequent membrane was probed with HeV and NiV cross-reactive monoclonal antibodies (mAbs) against F glycoprotein (mAb 5G7) and G glycoprotein (mAb 48D3), polyclonal rabbit serum to CedV-N and β-actin. Representative images from two independent experiments are shown.

The replication kinetics of rCedV-NiV-B, rCedV-NiV-B-GFP, and rCedV-NiV-B-Luc (Figure 4A) and rCedV-HeV, rCedV-HeV-GFP, and rCedV-HeV-Luc (Figure 4B) were also compared to rCedV. Plaque assays were performed on harvested viral supernatants, and

infectious virus titers were determined. We observed a gradual increase in infectious virus titers of all rCedV chimeric viruses that peaked 48 h post-infection (Figure 4). Specifically, the rCedV-NiV-B chimeras reached maximum virus titers of ~4–7 × 10^5 PFU/mL, while the rCedV-HeV chimeras peaked at ~2–9 × 10^5 PFU/mL. No statistically significant differences in replicated virus titers were observed between any of the rCedV-NiV-B chimeras or the rCedV-HeV chimeras or when compared to rCedV. In parallel, luciferase activity in rCedV-NiV-B-Luc and rCedV-HeV-Luc infected cells was measured. Figure 4 (right *y*-axes, black dashed lines) shows an increase in luminescence signal for both rCedV-NiV-B-Luc (Figure 4A) and rCedV-HeV-Luc (Figure 4B), which corresponded to the increase in infectious virus titers of their respective chimeras. Maximum luminescence signal was measured at 3.5 × 10^7 RLU at 48 h post-infection for rCedV-HeV-Luc and at ~8 × 10^6 RLU at 24 h post-infection for rCedV-NiV-B-Luc. The latter is likely due to extensive syncytia, and CPE observed in rCedV-NiV-B infected cells. These data illustrate that the rCedV chimeric viruses replicated efficiently and were comparable to rCedV and that luciferase activity is an indicator of viral genome expression.

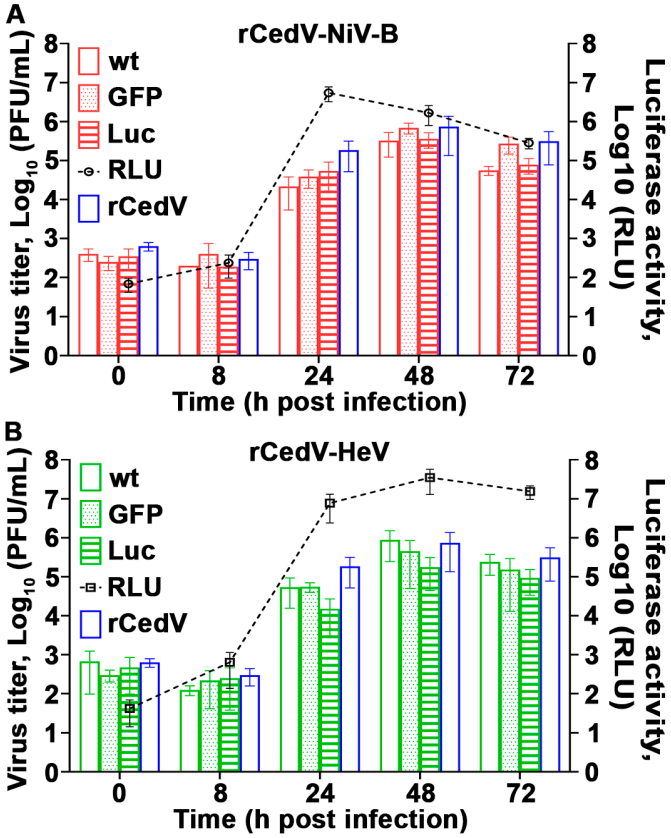

Figure 4. Replication kinetics of rCedV chimeras. Infectious virus titers (PFU/mL) determined from supernatants harvested at the indicated time points from Vero E6 cells infected at a MOI of 0.01 with rCedV-NiV-B (clear red bar), rCedV-NiV-B-GFP (dotted red bar), rCedV-NiV-B-Luc (striped red bar) (**A**), rCedV-HeV (clear green bar), rCedV-HeV-GFP (dotted green bar) or rCedV-HeV-Luc (striped green bar) (**B**). As a reference, separate populations of Vero E6 cells were also infected with rCedV (blue bar) (**A**,**B**). Normalized relative light units (RLU) for CedV-NiV-B-Luc (**A**) and rCedV-HeV-Luc (**B**) infected cells are represented on the right *y*-axes as black dashed lines. These data represent mean ± standard deviation from three independent experiments. Virus titers and luciferase activity levels at 0 h post-infection indicate the lower limit of detection for the plaque assay and the luminometer, respectively. Statistical analysis was performed in GraphPad Prism 9 by two-way ANOVA followed by Tukey's post hoc test ($\alpha = 0.05$).

3.3. Ephrin Entry Receptor Tropism of Recombinant Cedar Virus Chimeras

To define the receptor tropism of the newly generated rCedV chimeras, we used the NiV and HeV non-permissive cell line, HeLa-USU [31] and HeLa-USU cells stably expressing either ephrin-B2 (HeLa-USU-ephrin-B2) or ephrin-B3 (HeLa-USU-ephrin-B3) [20]. Here, all cells were either uninfected (Mock) or infected with rCedV-NiV-B-GFP, rCedV-HeV-GFP, or rCedV-GFP and at 24 h post-infection imaged for GFP expression. We observed fluorescence and/or syncytia in rCedV-NiV-B-GFP, and rCedV-HeV-GFP infected HeLa-USU-ephrin-B2 (Figure 5A) and HeLa-USU-ephrin-B3 (Figure 5B) cells, but not in the HeLa-USU infected cells (Figure 5C). In addition, GFP expression was detected in all rCedV-GFP infected cells, although syncytia were only observed in HeLa-USU-ephrin-B2 infected cells and were consistent with prior observations [20].

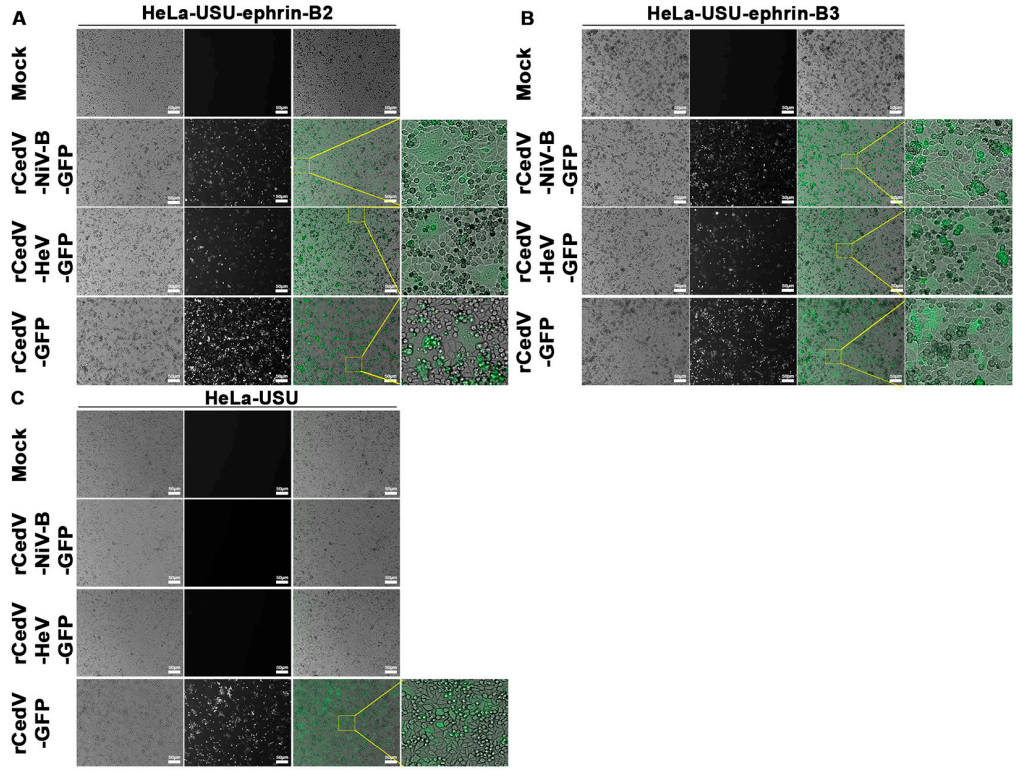

Figure 5. Ephrin-B2 and ephrin-B3 receptors facilitate rCedV-NiV-B-GFP and rCedV-HeV-GFP infection. Confluent HeLa-USU-ephrin-B2 (**A**), HeLa-USU-ephrin-B3 (**B**), and HeLa-USU (**C**) cells were uninfected (Mock) or infected with rCedV-NiV-B-GFP, rCedV-HeV-GFP, or rCedV-GFP at a MOI of 0.5. Infected cells were imaged for fluorescence and syncytia at 24 h post-infection. In each panel, transmitted light (1st column), fluorescence (2nd column), and merged (3rd column) images are shown. Zoomed-in regions are from the yellow boxes. Images were captured with a Zeiss Axio Observer A1 inverted microscope using a 5× objective. Representative images from two independent experiments are shown. Scale bar, 50 µm.

3.4. Recombinant Cedar Virus Chimeras Induce an Interferon Response

We next evaluated the induction of a type I IFN response in cells infected with the rCedV chimeras. HeLa-CCL-2 cells were uninfected (Mock) or infected with rCedV-NiV-B, rCedV-HeV, or rCedV. Additional HeLa-CCL-2 cells were transfected with Poly I:C to verify the induction of IFN-β (positive control). At 24 h post-infection, total RNA was extracted from all samples, and IFN-α and IFN-β mRNA levels were quantified by qPCR. As shown in Figure 6, in contrast to the mock samples, we observed a significant dose-dependent increase in IFN-β mRNA expression levels following rCedV-NiV-B, rCedV-HeV, and rCedV infection. A significant increase in the expression levels of IFN-α mRNA was not observed in any of the infected samples. These data demonstrate that rCedV chimeric

viruses induced a robust and dose-dependent IFN-β response similar to rCedV [20] and also CedV [2].

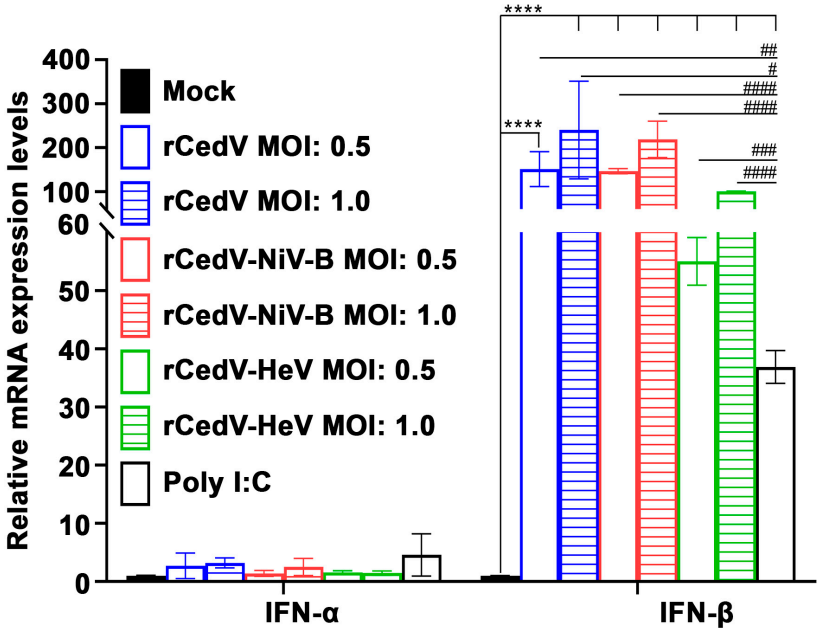

Figure 6. rCedV chimeras induce an IFN-β response. HeLa-CCL-2 cells were uninfected (Mock) or infected with rCedV-NiV-B, rCedV-HeV, or rCedV at a MOI of either 0.5 or 1.0 or transfected with Poly I:C (10 μg/mL) for 24 h. IFN-α and IFN-β mRNA expression were determined by qPCR. Fold changes were calculated relative to 18S ribosomal RNA and normalized to mock samples using the $2^{(-\Delta\Delta Ct)}$ method. These data represent mean ± standard deviation from two independent experiments, each performed in triplicate. Statistical analysis was performed with all samples in GraphPad Prism 9 by performing *t*-tests of each virus against Mock (asterisk *) or each virus against Poly I:C (hash #). **** $p < 0.0001$, # $p = 0.011$, ## $p = 0.012$, ### $p = 0.0001$ and #### $p < 0.0001$.

3.5. Plaque Reduction Neutralization Test (PRNT) of Chimeric Recombinant Cedar Viruses by Cross-Reactive NiV and HeV Specific Monoclonal Antibodies

To determine whether the rCedV-NiV-B-GFP and rCedV-HeV-GFP chimeras could serve as suitable surrogate viruses for authentic NiV-B and HeV, respectively, the ability of the GFP expressing rCedV chimeras to be neutralized by a panel of well-characterized NiV/HeV cross-reactive neutralizing mAbs was conducted by PRNT. The antibody panel included the human mAb m102.4 specific to the G glycoprotein and the humanized mAb h5B3.1 and murine mAbs 12B2 and 1F5 specific to the F glycoprotein [44–46,71]. Figure 7 illustrates the dose-response neutralization profiles for each mAb against rCedV-NiV-B-GFP and rCedV-HeV-GFP performed at BSL-2 (Figure 7A). In addition, a set of parallel PRNTs using authentic NiV-B and HeV and both rCedV chimeras were also performed simultaneously in BSL-4 containment (Figure 7B,C). Each mAb tested neutralized the infectivity of rCedV-NiV-B-GFP and rCedV-HeV-GFP and NiV-B and HeV (Figure 7) with highly similar dose-response virus neutralization profiles. The mean 50% inhibitory concentrations (IC_{50}) for each of the mAbs against rCedV-NiV-B-GFP, rCedV-HeV-GFP, NiV-B, and HeV are summarized in Table 2. The most potent mAb was m102.4 with average IC_{50} values of ~20 ng/mL against rCedV-NiV-B-GFP and NiV-B, ~101 ng/mL against rCedV-HeV-GFP and ~50 ng/mL against HeV. The IC_{50} values for the mAbs tested here are within comparable ranges when compared to previous in vitro PRNT studies conducted with authentic NiV and HeV with the same set of cross-reactive neutralizing mAbs [43,44,46,66].

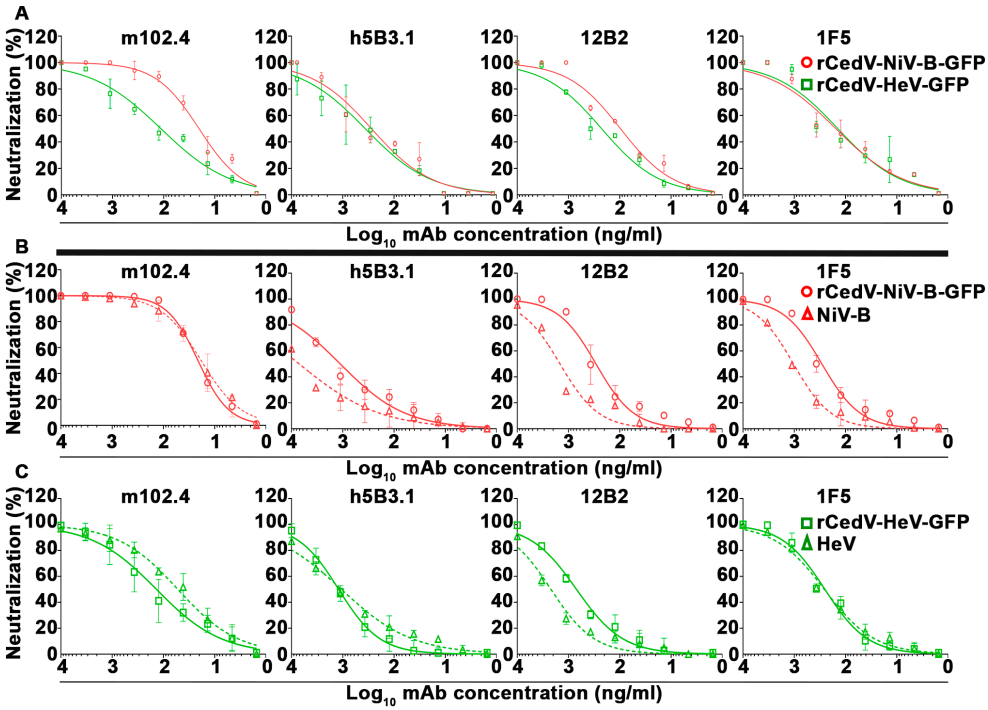

Figure 7. Neutralization of rCedV-NiV-B-GFP and rCedV-HeV-GFP by plaque reduction neutralization test (PRNT). Nine-point dose-response neutralization profiles for mAbs m102.4, h5B3.1, 12B2, and 1F5 against rCedV-NiV-B-GFP and rCedV-HeV-GFP at BSL-2 (**A**), at BSL-4 (**B**,**C**) and authentic NiV-B and HeV at BSL-4 (**B**,**C**). The diluted mAbs were incubated with an equal volume of either rCedV-NiV-B-GFP, rCedV-HeV-GFP, NiV-B, or HeV at an MOI of 0.0001 for 1 h at 37 °C, 5% CO_2. MOI was calculated for a tentative 10^6 cells/well and 0.4 mL virus and antibody mixture per well. Neutralization percent (%) was calculated based on PFU for each virus without mAb. These data represent mean ± standard deviation and are plotted as a non-linear regression curve fit with variable slope. BSL-2 studies are representative of two independent experiments, each performed in duplicate, and BSL-4 studies are from a single experiment performed in duplicate. The limit of detection for this assay was 50 PFU. Red circles and lines represent rCedV-NiV-B-GFP, red triangles and dashed lines represent NiV-B, green squares and lines represent rCedV-HeV-GFP, and green triangles and dashed lines represent HeV. The thick black line divides the BSL-2 PRNT from the BSL-4 PRNT.

Table 2. IC_{50} values of NiV and HeV cross-reactive monoclonal antibodies against henipavirus infection by PRNT.

Monoclonal Antibody (mAb)	IC_{50} (95% CI) (ng/mL)					
	BSL-2	BSL-4		BSL-2	BSL-4	
	rCedV-NiV-B-GFP	rCedV-NiV-B-GFP	NiV-B	rCedV-HeV-GFP	rCedV-HeV-GFP	HeV
m102.4	20.30 (16.58–24.99)	21.20 (18.80–23.89)	18.36 (15.21–22.17)	112.9 (82.82–154.1)	137.0 (89.09–208.8)	52.41 (39.32–70.07)
h5B3.1	274.8 (185.9–403.7)	1122 (813.6–1548)	7101 (4323–15,087)	363.5 (241.6–546.3)	1202 (975.2–1481)	1064 (827.4–1372)
12B2	130.0 (97.10–174.0)	291.9 (219.9–381.5)	1467 (1098–1925)	502.3 (377.4–658.3)	700.1 (570.0–857.0)	2202 (1692–2846)
1F5	153.8 (107.0–219.4)	289.4 (229.9–360.9)	1036 (812.3–1298)	140.6 (83.29–232.0)	253.2 (200.5–318.8)	259.8 (213.2–315.8)

Note: All IC_{50} values are calculated by a nonlinear fit model and are shown with 95% confidence intervals (95% CI). BSL-2 studies are representative of two independent experiments, each performed in duplicate, and BSL-4 studies are from a single experiment performed in duplicate.

3.6. Correlation Analysis of Plaque Reduction Neutralization Tests (PRNT) Using GFP Expressing Recombinant Cedar Virus Chimeras and Authentic NiV-B and HeV

To further evaluate if the rCedV-NiV-B-GFP and rCedV-HeV-GFP chimeras are suitable surrogate virus platforms for authentic NiV-B and HeV antibody neutralization, a correlation analysis was performed. A Pearson correlation coefficient 'r' for each mAb was calculated by comparing the neutralization values derived from the rCedV chimeras BSL-2 PRNT with those of the NiV-B or HeV BSL-4 PRNT. The analysis indicated strong and statistically significant positive correlations between the two PRNTs (r values ranging from 0.86 to 0.99, *p* values from 0.005 to 0.0001) (Figure 8 and Table 3).

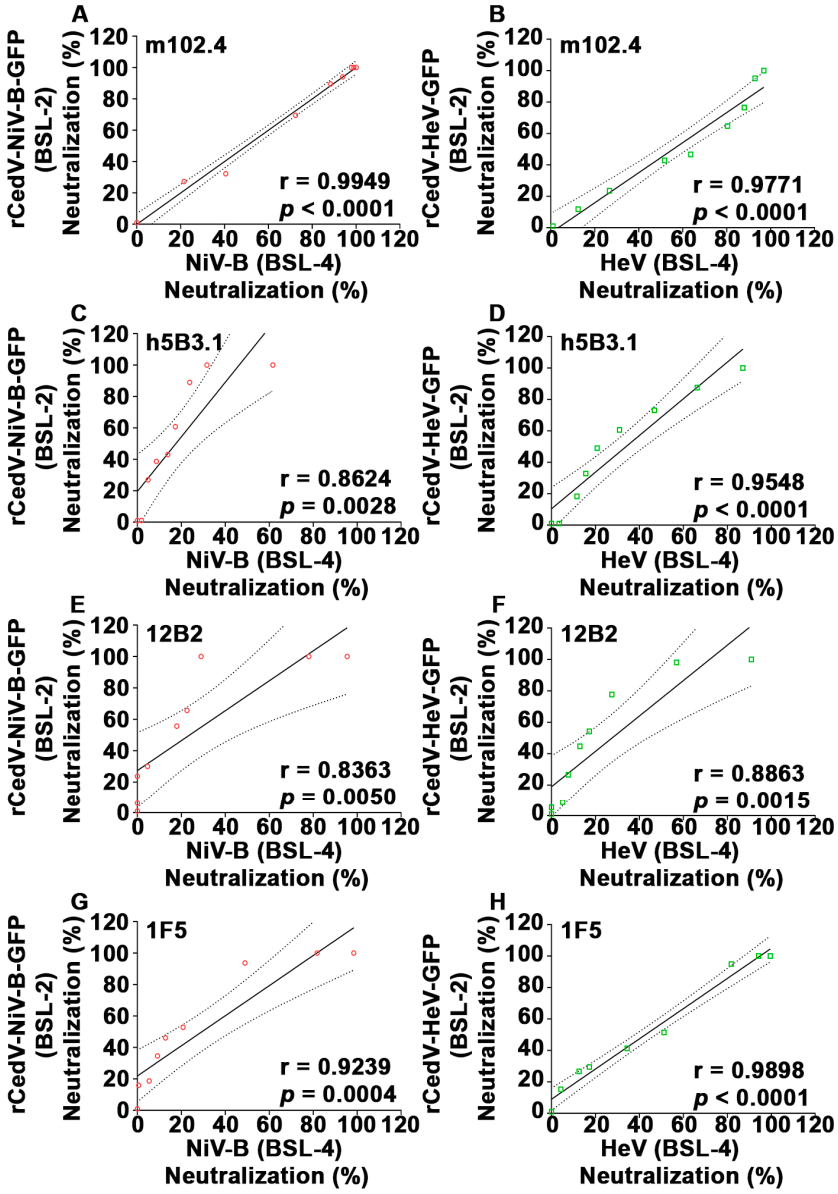

Figure 8. Correlation analysis of plaque reduction neutralization test (PRNT) neutralization values. Pearson correlation analysis of PRNT neutralization (%) values of rCedV-NiV-B-GFP versus NiV-B (**A**,**C**,**E**,**G**) and rCedV-HeV-GFP versus HeV (**B**,**D**,**F**,**H**) with mAbs m102.4, h5B3.1, 12B2 or 1F5. The Pearson correlation coefficient 'r,' *p*-value (two-tailed), linear regression line (solid lines), and 95% confidence intervals (dashed lines) are represented. Pearson's r $\geq$ 0.8 and *p*-value < 0.05 indicate a strong significant positive correlation.

Table 3. Correlation analysis of rCedV chimeric viruses in BSL-2 PRNT versus NiV-B and HeV BSL-4 PRNT assays.

Virus	Monoclonal Antibody (mAb)	Pearson's Correlation Coefficient (r)	Coefficient of Determination (R^2)	Significance (*p*)	95% Confidence Interval (CI)
rCedV-NiV-B-GFP vs. NiV-B	m102.4	0.9949	0.9898	<0.0001	0.9750–0.990
	h5B3.1	0.8624	0.7437	0.0028	0.4640–0.9706
	12B2	0.8363	0.6994	0.0050	0.3873–0.9647
	1F5	0.9239	0.8536	0.0004	0.6722–0.9842
rCedV-HeV-GFP vs. HeV	m102.4	0.9771	0.9547	<0.0001	0.8914–0.9953
	h5B3.1	0.9548	0.9117	<0.0001	0.7945–0.9907
	12B2	0.8863	0.7855	0.0015	0.5400–0.9760
	1F5	0.9898	0.9796	<0.0001	0.9503–0.9979

Note: Correlation analysis was performed with the neutralization values from Figure 7.

3.7. Establishment of a Fluorescence Reduction Neutralization Test (FRNT)

To further develop the utility of rCedV-NiV-B-GFP and rCedV-HeV-GFP chimeras as a surrogate platform for authentic NiV-B and HeV neutralization testing, we developed a high-throughput and quantitative assay based on the reduction in GFP fluorescent virus infection foci. Here, the virus neutralization efficacies of the same panel of mAbs used in the PRNT assays (Figure 7) were analyzed against rCedV-NiV-B-GFP and rCedV-HeV-GFP in a FRNT assay. As shown in Figure 9, the dose-response neutralization data were similar to those obtained by a PRNT (Figures 7A and 9). The IC_{50} values for each mAb against rCedV-NiV-B-GFP and rCedV-HeV-GFP are summarized in Table 4. The mAb m102.4 potently neutralized rCedV-NiV-B-GFP at an IC_{50} of 16.91 ng/mL, while rCedV-HeV-GFP was neutralized by m102.4 and 1F5 with similar potencies at IC_{50} values of 58.12 ng/mL and 50.16 ng/mL, respectively. These data reveal that rCedV-NiV-B-GFP and rCedV-HeV-GFP mAb neutralization values in a FRNT are comparable to those obtained in a PRNT (comparisons of Tables 2 and 4).

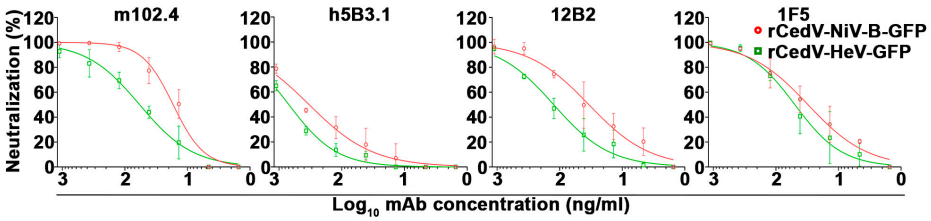

Figure 9. Neutralization profiles of NiV and HeV cross-reactive monoclonal antibodies by fluorescence reduction neutralization test (FRNT). Seven-point dose-response neutralization profiles for mAbs m102.4, h5B3.1, 12B2, and 1F5 against rCedV-NiV-B-GFP and rCedV-HeV-GFP. Neutralization percent (%) was calculated based on fluorescent foci for each virus without mAb. These data represent mean ± standard deviation from three independent experiments, each performed in triplicate. Data are plotted as non-linear regression curve fit with variable slope. The limit of detection for this assay was 50 fluorescent foci. Red circles and lines represent rCedV-NiV-B-GFP, and green squares and lines represent rCedV-HeV-GFP.

Table 4. IC_{50} values of NiV and HeV cross-reactive specific monoclonal antibodies against rCedV-NiV-B-GFP and rCedV-HeV-GFP infection by FRNT.

Monoclonal Antibody (mAb)	IC_{50} (95% CI) (ng/mL)	
	rCedV-NiV-B-GFP	rCedV-HeV-GFP
m102.4	16.91 (14.72–19.45)	58.12 (49.27–68.70)
h5B3.1	333.0 (255.5–439.9)	700.2 (620.0–798.8)
12B2	34.07 (24.88–46.48)	124.5 (98.17–157.2)
1F5	28.97 (22.86–36.65)	50.16 (40.95–61.07)

Note: All IC_{50} values are calculated by a nonlinear fit model from three independent experiments, each performed in triplicate, and are shown with 95% confidence intervals (95% CI).

3.8. Correlation Analysis of the Conventional PRNT and the FRNT Neutralization Assays

To further evaluate the FRNT assay as a suitable alternative virus neutralization assay to the standard PRNT, a correlation analysis was performed. A Pearson correlation analysis using the neutralization values obtained with each mAb against the rCedV-GFP chimeras by PRNT and FRNT assays was conducted (Figure 10 and Table 5), and a strong and significant positive correlation between the neutralization values obtained by PRNT versus the corresponding FRNT assay derived values was observed ($r \geq 0.9$ and $p \leq 0.001$). Taken together, these data demonstrate that rCedV-NiV-B-GFP and rCedV-HeV-GFP chimeric viruses are an ideal set of suitable surrogate viruses for authentic NiV-B and HeV for conducting a rapid FRNT-based assay for assessing NiV and HeV antibody neutralization.

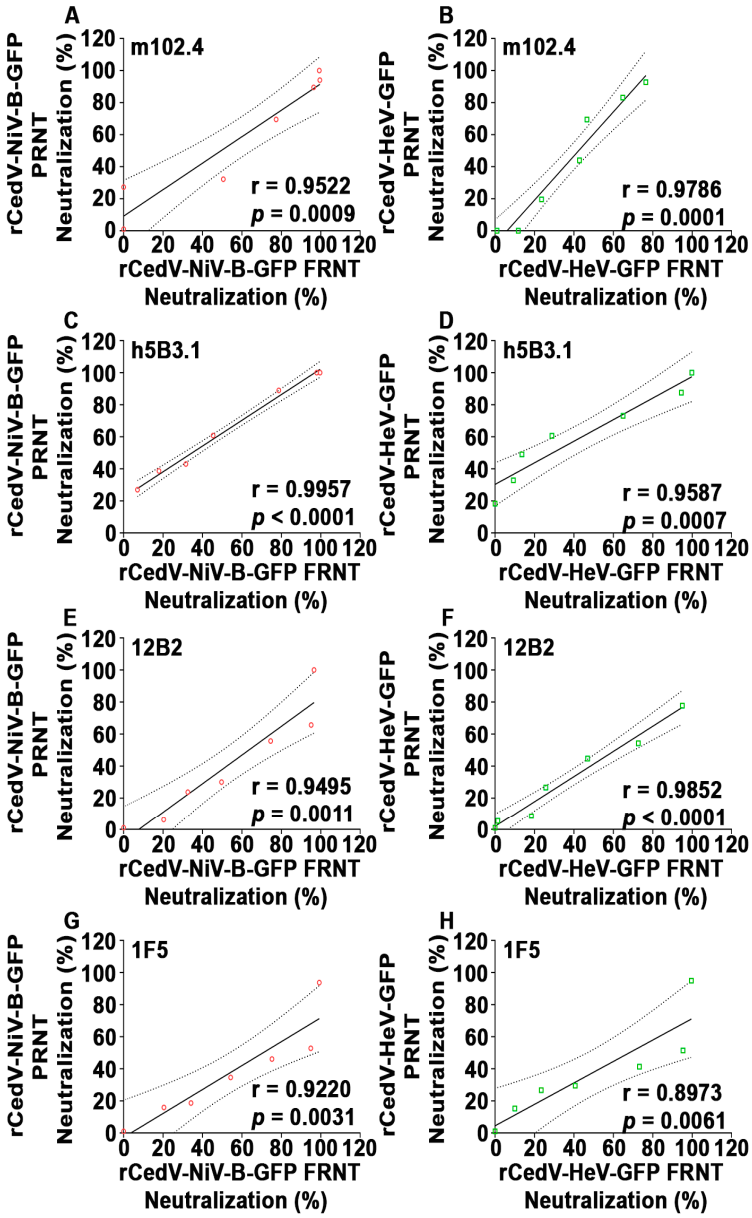

Figure 10. Correlation analysis of neutralization assays using the GFP expressing rCedV chimeric viruses. Pearson correlation analysis of neutralization (%) values from plaque reduction neutralization tests (PRNTs) (*y*-axes) and fluorescence reduction neutralization tests (FRNTs) (*x*-axes) performed with rCedV-NiV-B-GFP (**A**,**C**,**E**,**G**) and with rCedV-HeV-GFP (**B**,**D**,**F**,**H**) with mAbs m102.4, h5B3.1, 12B2 or 1F5. The Pearson correlation coefficient 'r,' *p*-value (two-tailed), linear regression line (solid lines), and 95% confidence intervals (dashed lines) are represented. Pearson's $r \geq 0.8$ and *p*-value < 0.05 indicate a strong significant positive correlation.

Table 5. Correlation analysis of rCedV chimeric viruses in the PRNT versus FRNT assays.

Virus	Monoclonal Antibody (mAb)	Pearson's Correlation Coefficient (r)	Coefficient of Determination (R^2)	Significance (*p*)	95% Confidence Interval
rCedV-NiV-B-GFP	m102.4	0.9522	0.9067	0.0009	0.7038–0.9931
	h5B3.1	0.9957	0.9914	<0.0001	0.9698–0.9994
	12B2	0.9495	0.9016	0.0011	0.6894–0.9927
	1F5	0.9220	0.8501	0.0031	0.5527–0.9886
rCedV-HeV-GFP	m102.4	0.9786	0.9576	0.0001	0.8573–0.9970
	h5B3.1	0.9587	0.9191	0.0007	0.7397–0.9941
	12B2	0.9852	0.9707	<0.0001	0.8997–0.9979
	1F5	0.8973	0.8051	0.0061	0.4448–0.9849

Note: Correlation analysis was performed with the neutralization values from Figures 7A and 9.

3.9. Neutralization of rCedV-NiV-B-GFP and rCedV-HeV-GFP Using Henipavirus sG Immune Antisera

The utility of the rCedV-NiV-B-GFP and rCedV-HeV-GFP viruses in the FRNT assay for measuring immune serum neutralization was examined by testing sera from NiV-sG or HeV-sG immunized nonhuman primates (NHP) (rhesus macaques) and rabbits, respectively. NHP subjects were immunized with an equal mixture of recombinant NiV-M and NiV-B sG glycoproteins (see Section 2) [47]. Figure 11 shows the dose-response neutralization profiles for each NHP sera against the rCedV-NiV-B-GFP and rCedV-HeV-GFP chimeras. The IC_{50} titers for each of the sera against rCedV-NiV-B-GFP and rCedV-HeV-GFP are summarized in Table 6. Sera collected on day 42 from subject 171269 had the highest neutralizing titer against rCedV-NiV-B-GFP (1:32,147) and rCedV-HeV-GFP (1:4157). In addition, we were also able to test sera collected on day 84 from 2 subjects, 171269 and 180227, and the IC_{50} titers are summarized in Table 6. Although still potently neutralizing, the 50% serum neutralization titers for animal 171269 declined 2-fold against rCedV-NiV-B-GFP to 1:16,101 and 4.8-fold against rCedV-HeV-GFP to 1:873.6. As expected, although the NHP sera were cross-neutralizing against rCedV-HeV-GFP the IC_{50} titers were much greater against the homologous immunized subjects (NiV).

Table 6. IC_{50} Anti-NiV G glycoprotein immunized rhesus macaque serum titers against rCedV-NiV-B-GFP and rCedV-HeV-GFP infection.

Animal ID	IC_{50} (95% CI) (Serum Titer)	
	rCedV-NiV-B-GFP	rCedV-HeV-GFP
171269 Day 42	1:32,147 (1:29,414–1:35,182)	1:4157 (1:3711–1:4658)
171269 Day 84	1:16,101 (1:12,288–1:21,024)	1:873.6 (1:809.4–1:941.3)
180274 Day 42	1:14,860 (1:14,018–1:15,761)	1:2704 (1:2375–1:3082)
180606 Day 42	1:19,480 (1:18,181–1:20,948)	1:3739 (1:3094–1:4542)
180227 Day 42	1:19,408 (1:17,817–1:21,158)	1:2048 (1:1668–1:2539)
180227 Day 84	1:7283 (1:6079–1:8739)	1:689.0 (1:499.2–1:904.4)

Note: All IC_{50} values are calculated by a nonlinear fit model from two independent experiments, each performed in triplicate, and are shown with 95% confidence intervals (95% CI).

We also analyzed sera from rabbits immunized with either HeV-sG or HeV-sG tetramer ($HeV\text{-}sG_{tet}$) (see Materials and Methods). The sG immune rabbit sera neutralized the infectivity of both rCedV-NiV-B-GFP and rCedV-HeV-GFP, as shown by the dose-response neutralization profiles in Figure 12. The IC_{50} titers for each of the rabbit sera against rCedV-NiV-B-GFP and rCedV-HeV-GFP are summarized in Table 7. The HeV-sG and $HeV\text{-}sG_{tet}$ sera had similar and very high neutralizing titers of 1:65,820 and 1:59,457, respectively, against rCedV-HeV-GFP. Here, the HeV-sG-specific rabbit sera had higher cross-neutralizing heterologous titers against rCedV-NiV-B-GFP (HeV-sG: 1:6881 and $HeV\text{-}sG_{tet}$: 1:7367, respectively) with IC_{50} values again greater against the homologous immunized

subjects (HeV), consistent with prior authentic NiV and HeV neutralization data derived from NiV-sG versus HeV-sG immunized cats [49].

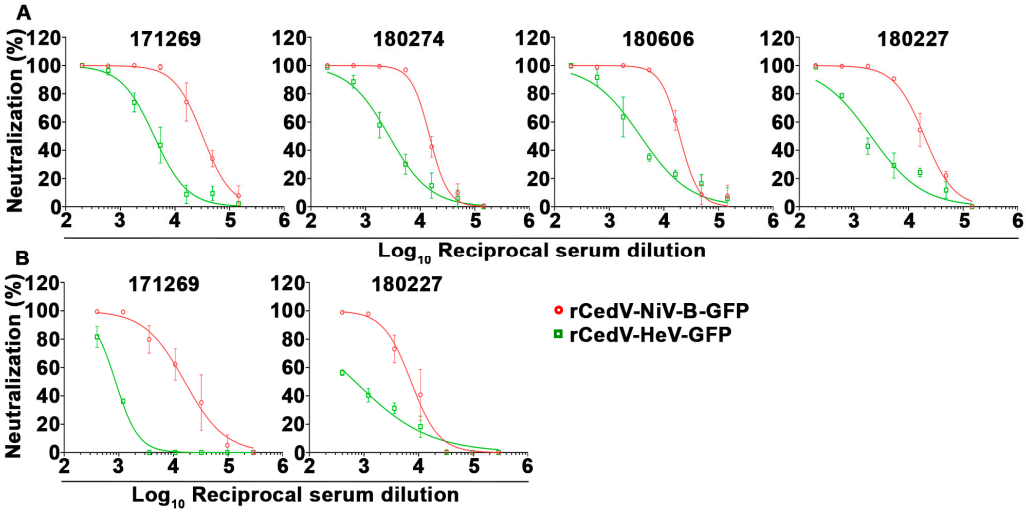

Figure 11. Neutralization profiles of NHP immunized sera against the GFP expressing rCedV chimeras by fluorescence reduction neutralization test (FRNT). Seven-point dose-response neutralization profiles of rhesus macaque sera collected on day 42 (**A**) and day 84 (**B**) post-immunization against rCedV-NiV-B-GFP and rCedV-HeV-GFP are shown. Neutralization percent (%) was calculated based on fluorescent foci for each virus without serum. These data represent mean ± standard deviation from two independent experiments, each performed in triplicate. Data are plotted as non-linear regression curve fit with variable slope. The limit of detection for this assay was 50 fluorescent foci. Animal ID numbers are 171269, 180274, 180606, and 180227. Red circles and lines represent rCedV-NiV-B-GFP, and green squares and lines represent rCedV-HeV-GFP.

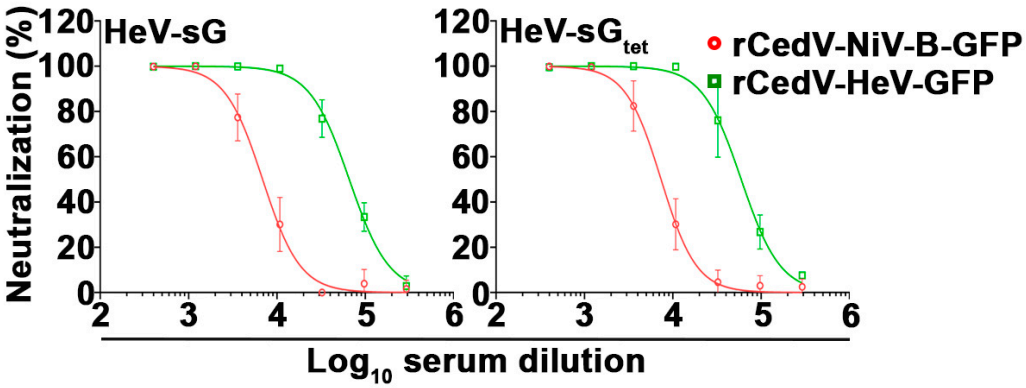

Figure 12. Neutralization profiles of rabbit immunized sera against rCedV chimeras expressing GFP. Seven-point dose-response neutralization profiles of HeV-sG (**left**) and HeV-sG$_{tet}$ (**right**) immunized sera against rCedV-NiV-B-GFP and rCedV-HeV-GFP are shown. Neutralization percent (%) was calculated based on fluorescent foci for each virus without serum. These data represent mean ± standard deviation from two independent experiments, each performed in triplicate. Data are plotted as non-linear regression curve fit with variable slope. The limit of detection for this assay was 50 fluorescent foci. Red circles and lines represent rCedV-NiV-B-GFP, and green squares and lines represent rCedV-HeV-GFP.

Table 7. IC_{50} rabbit serum titers against rCedV-NiV-B-GFP and rCedV-HeV-GFP infection.

Immunogen	IC_{50} (95% CI) (Serum Titer)	
	rCedV-NiV-B-GFP	rCedV-HeV-GFP
HeV-sG	1:6881 (1:6211–1:7616)	1:65,820 (1:61,407–1:70,409)
HeV-sG_{tet}	1:7367 (1:6642–1:8158)	1:59,457 (1:54,002–1:65,333)

Note: All IC_{50} values are calculated by a nonlinear fit model from two independent experiments, each performed in triplicate, and are shown with 95% confidence intervals (95% CI).

4. Discussion

There has been increased concern regarding respiratory pathogens such as NiV as a consequence of the COVID-19 pandemic [72]. The most recent significant outbreak of NiV-B, which occurred in Kerala, India, in 2018, had a case-fatality rate of 91% and revealed a high incidence of acute respiratory distress syndrome among those infected, correlating with nosocomial respiratory droplet-mediated human-to-human transmission by exposure to patient's coughing [73]. Experimentally, both NiV-M and NiV-B have been shown to cause lethal infection in NHPs when delivered as an aerosol [74,75], also the likely route of infection from deliberate release. Indeed, in 2020 the US Department of Health and Human Services (HHS) and Centers for Disease Control and Prevention (CDC) recommended that NiV be added to the list of Tier 1 Select Agents [76]. NiV-B has several characteristics enhancing its pandemic potential, including its respiratory tissue tropism; human susceptibility to infection; person-to-person transmission capability; and its potential to mutate, and the emergence of a human-adapted strain in South Asia could lead to the rapid spread of infection [77]. NiV and HeV have been important targets for vaccine development for more than 20 years, and these efforts have recently intensified [22,25].

In the present study, the rCedV reverse genetics platform [20,21] was modified by employing an optimized T7 promoter ($T7_{opt}$) and the self-cleaving HHRbzA in the pOLTV5-rCedV antigenome plasmid, which improved the rescue efficiency of rCedVs. A similar strategy was used to rescue a number of other single-stranded, negative-sense RNA viruses, including NiV [36,78–82]. We then expanded the utility of the rCedV platform by replacing the coding sequences of the CedV F and G glycoproteins with their NiV-B or HeV counterparts to generate a panel of non-reporter gene and reporter gene encoding versions of rCedV-NiV-B and rCedV-HeV chimeric viruses. Interestingly, all chimeras appeared more fusogenic than those observed in rCedV-infected cells, and a similar phenotype was observed by Yeo et al., where cell fusion levels of CedV were consistently lower than NiV in transfected HEK293T cells [83]. All rCedV chimeras expressed the heterologous envelope glycoproteins in infected cells, replicated similarly in comparison to rCedV, and infection tropism was specific for ephrin-B2 and ephrin-B3 as entry receptors.

Previous in vitro cell-based assays demonstrated that CedV and rCedV induced a robust IFN-β response [2,20], and CedV infection stimulated the expression of interferon response genes, such as *IFNA7*, *CCL5*, *STAT1*, and *STAT2* in primary hamster endothelial cells [11]. Here, the rCedV chimeric viruses also induced the expression of IFN-β mRNA in an infection dose-dependent manner to comparable levels observed with rCedV infection and Poly I:C treatment.

Several surrogate NiV neutralization assays using recombinant Vesicular Stomatitis Virus (VSV) as a replication-incompetent pseudovirus with a deletion of the VSV G glycoprotein gene have been developed as a tool to measure NiV neutralization at BSL-2 containment [84–86]. The VSV-based pseudotype virus particle system has also been utilized with the HeV and GhV envelope glycoproteins for measuring neutralization [87]. Preparation of VSV pseudoviruses involves the budding progeny virions from cells that are transiently expressing henipavirus F and G glycoproteins which can sometimes be technically challenging to produce large quantities of pseudovirus stocks with reproducible characteristics. We previously found similar challenges in developing a retrovirus-based pseudotyped virus assay system that also required significant optimization [88]. These replication-incompetent pseudovirus assays are sensitive and have a high correlation when

samples are scored as either positive or negative for henipavirus neutralizing antibody. However, specific mAb neutralization potencies or the virus-neutralizing titers of sera against NiV using VSV pseudotypes, as examples, are often quite different in comparison to sera titers obtained using authentic NiV [84–86].

Here, we sought to develop an improved surrogate neutralization assay system for NiV and HeV using rCedV as replication-competent chimeric viruses. The rCedV chimeric viruses developed and characterized here can be readily produced and stored in large quantities and are an authentic replication-competent henipavirus platform that can be used to study NiV and HeV F and G glycoprotein-mediated infection and also as surrogate viruses for authentic NiV and HeV in neutralization assays without the requirement for BSL-4 containment. Indeed, similar dose-response neutralization data and comparable IC_{50} concentrations of well-characterized NiV and HeV cross-reactive mAbs were observed between PRNTs using rCedV-NiV-B-GFP and NiV-B or rCedV-HeV-GFP and HeV. A strong and significant correlation between the overall neutralization values of the BSL-2 and BSL-4 PRNTs (Figure 8 and Table 3) validated the utility of the rCedV chimeric platform as suitable surrogate viruses for authentic NiV and HeV by PRNT.

We also expanded the utility of these novel reporter genes encoding rCedV chimeric viruses by developing a rapid and high-throughput fluorescence-based neutralization assay, FRNT. In contrast to the PRNT, which is the current gold standard for determining the presence of neutralizing antibodies and measuring the neutralizing titer in henipavirus-specific antisera, the FRNT (i) is high-throughput and allows for more samples to be assayed with more replicates in a 96-well plate format, (ii) requires smaller sample volumes, (iii) is less time consuming taking less than 36 h from infection to assay completion, and (iv) reduces the requirement for other reagents such as luciferase substrate. The rCedV-NiV-B-GFP and rCedV-HeV-GFP chimeric viruses were used to assess the neutralization potencies of mAbs by FRNT assay, and the neutralization values at each mAb concentration obtained by FRNT were found to be highly correlated with those values obtained by PRNT (Table 5). We further evaluated the utility of the rCedV-NiV-B-GFP and rCedV-HeV-GFP FRNT by measuring the neutralization activities of henipavirus sG immunized NHP sera and rabbit sera. The NiV-sG immunized NHP sera, and the HeV-sG immunized rabbit sera were both cross-neutralizing to rCedV-NiV-B-GFP and rCedV-HeV-GFP, with higher homotypic serum neutralization titers as expected. The rabbit HeV-sG immune sera exhibited greater heterologous neutralization titers in comparison to the NHP NiV-sG immune sera, which was also consistent with neutralization data derived from NiV-sG versus HeV-sG immunized cats against authentic NiV-M and HeV [49].

In summary, a surrogate henipavirus-based system for NiV and HeV using the rCedV platform suitable for use at BSL-2 containment has been developed and well-characterized. These rCedV chimeras can serve as useful tools to study NiV and HeV entry, membrane fusion mechanisms, and F and G glycoprotein interactions and aid in the discovery and development of henipavirus countermeasures. More importantly, the specificity and utility of the rCedV-NiV-B-GFP and rCedV-HeV-GFP viruses as a surrogate neutralization assay for authentic NiV and HeV to evaluate the neutralization potential of mAbs and NiV/HeV specific antisera has also been demonstrated. The rCedV chimeras will reduce the cost and technical challenges of the high-containment environment, particularly when large numbers of serum samples derived from NiV or HeV vaccine development programs will require testing and quantitation.

5. Patents

C.C.B. and M.A. are United States federal employees and co-inventors on US and foreign patents pertaining to Recombinant Cedar Virus Chimeras, whose assignees are the United States as represented by the Henry M Jackson Foundation for the Advancement of Military Medicine, Inc. (Bethesda, MD, USA).

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/v15051077/s1, Table S1: Mutations identified by genome sequencing of recombinant Cedar virus chimeras.

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Conflicts of Interest: C.C.B is a United States federal employee and co-inventor on United States and foreign patents pertaining to soluble forms of Nipah virus and Hendra virus G glycoproteins, and Cedar Virus and Methods of Use, whose assignees are the United States as represented by the Henry M. Jackson Foundation for the Advancement of Military Medicine, Inc. (Bethesda, MD, USA). All other authors declare no conflict of interest.

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ANNUAL REVIEWS

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Vaccines to Emerging Viruses: Nipah and Hendra

Moushimi Amaya and Christopher C. Broder

Department of Microbiology and Immunology, Uniformed Services University of the Health Sciences, Bethesda, Maryland 20814, USA; email: christopher.broder@usuhs.edu

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Abstract

Hendra virus (HeV) and Nipah virus (NiV) are bat-borne zoonotic paramyxoviruses identified in the mid- to late 1990s in outbreaks of severe disease in livestock and people in Australia and Malaysia, respectively. HeV repeatedly re-emerges in Australia while NiV continues to cause outbreaks in South Asia (Bangladesh and India), and these viruses have remained transboundary threats. In people and several mammalian species, HeV and NiV infections present as a severe systemic and often fatal neurologic and/or respiratory disease. NiV stands out as a potential pandemic threat because of its associated high case-fatality rates and capacity for human-to-human transmission. The development of effective vaccines, suitable for people and livestock, against HeV and NiV has been a research focus. Here, we review the progress made in NiV and HeV vaccine development, with an emphasis on those approaches that have been tested in established animal challenge models of NiV and HeV infection and disease.

INTRODUCTION

Nipah virus (NiV) and Hendra virus (HeV) are bat-borne viral zoonoses that were discovered in the mid- to late 1990s in outbreaks of severe disease in livestock and people in Australia (HeV) and Malaysia [NiV-Malaysia (NiV-M)] (1). They are the prototype members of the genus *Henipavirus* in the family *Paramyxoviridae* (2). NiV outbreaks have also been recorded in Bangladesh and India by a closely related strain, NiV-Bangladesh (NiV-B) (3). Three other henipaviruses are also recognized: Cedar virus (CedV) as an isolate and Ghana virus (GhV) and Mojiang virus (MojV) known only from sequence data (4–7). Both NiV and HeV are highly pathogenic in a broad range of mammalian hosts that are capable of infecting and causing severe disease in humans, monkeys, pigs, horses, cats, dogs, ferrets, hamsters, and guinea pigs and that span six mammalian orders including bats, although bats do not exhibit disease when infected (8–21). In contrast, CedV is nonpathogenic in well-characterized models of HeV and NiV disease including ferrets and hamsters (4, 22). The pathogenic potential of GhV and MojV is unknown.

Several species of *Pteropus* fruit bats are the natural reservoir hosts of NiV, HeV, and CedV (4, 23–27). NiV- or HeV-mediated disease has not been reported in wild or experimentally infected bats (13, 28–30). NiV and HeV infections in people and many animals manifest as severe systemic and often fatal neurologic and/or respiratory diseases (31–33). Both NiV and HeV are regarded as transboundary biological threats to both human and animal health and are classified as biosafety level 4 (BSL-4) select agents (34, 35). NiV and henipaviral diseases are included in the World Health Organization (WHO) R&D Blueprint list of priority pathogens with epidemic potential that need research attention (36). This review summarizes the important characteristics of the NiV and HeV pathogens, the modes of virus transmission, and the immunization strategies being developed against them.

Emergence and Outbreaks of Hendra and Nipah Viruses

In 1994 in the Brisbane suburb of Hendra, Australia, an outbreak of severe respiratory disease resulted in the deaths of 14 horses and their trainer, along with the nonfatal infection of 7 other horses and 1 other person. This led to the discovery of a novel paramyxovirus initially termed equine morbillivirus, now known as HeV (37–39). The first known cases of HeV in horses and a human actually occurred a few months prior, where one person became ill after assisting in the necropsies of two horses later shown to have died from HeV (40, 41). This individual experienced a relapsed fatal encephalitis caused by HeV 13 months later (42). HeV has since re-emerged in Australia 62 times with a total of 104 horse deaths (fatal or euthanized), along with 4 human fatalities of 7 cases (43). Every recorded occurrence of HeV in Australia has involved horses, all resulting in a severe or fatal disease, and all cases of human infection were acquired from virus-shedding horses (31, 44).

In 1998, an outbreak of encephalitis among pig farmers in Peninsular Malaysia occurred and a virus was isolated from samples of cerebrospinal fluid (CSF) of two patients who had died; cells infected with this virus cross-reacted with antibodies against HeV (45). Genetic studies revealed a new paramyxovirus that was closely related to HeV, and it was named Nipah after the village in Malaysia where one of the patients had lived (45). There were 265 cases of human infection with 105 fatalities in Malaysia and 11 cases and 1 fatality among abattoir workers in Singapore (46, 47). This outbreak was controlled through the culling of more than 1 million pigs, resulting in significant economic impacts to the region (48, 49).

A genetically similar but distinct strain of NiV was identified as the causative agent of fatal encephalitis in people in Bangladesh (NiV-B) (3, 50). Since 2001, nearly annual occurrences of human NiV-B infections have occurred in Bangladesh, and there have been three outbreaks in

India (51–54). The recent 2018 NiV outbreak in Kerala, India, was significant, having occurred in a new geographic region far from locations in Bangladesh and India where all prior outbreaks had occurred and with a case fatality rate of 91% (51). In 2014, an outbreak of NiV-M encephalitis occurred in the Philippines with 9 fatalities of 11 human cases of acute encephalitis and influenza-like illness or meningitis in another 6 individuals (55). Altogether, there have been over 650 cases of human NiV infection (combined ~60% fatality rate) in South Asia and Southeast Asia in five countries (54, 56).

Transmission of Hendra and Nipah Viruses

The routes of transmission of virus infection to humans from animals are different for HeV and NiV, with horses the only spillover host of HeV in Australia, while for NiV it was pigs in Malaysia and horses in the Philippines (**Figure 1**). However, human NiV infections in Bangladesh, India, and the Philippines also include bat-to-human and human-to-human transmission (57–60). Transmission routes of HeV and NiV to animals are likely urine from infected bats contaminating pastures or pigsties and/or virus-contaminated fruit spat from bats that is ingested (61, 62) (**Figure 1**). Recoverable virus is shed in the urine of experimentally infected bats and can also be detected in throat and rectal swabs (13, 28–30). Pooled urine samples from flying foxes are also routinely used to detect and isolate henipaviruses (4, 13, 23, 27, 63–65).

It was previously suggested that infected horses could transmit HeV to people during the feeding of ill animals (38). Also, the majority of all HeV-infected horse cases have involved a single animal, suggesting that HeV is not readily transmitted between horses, and multiple horse outbreaks are likely via contamination of fomites (43, 66). The transmission risk of HeV from infected horses to humans appears to be virus-contaminated fluids or tissues during examination procedures and/or the necropsy of horses (31, 67) (**Figure 1**). Indeed, all cases of human HeV infection have been associated with postmortem examination of horses or close contact with ill horses (31, 38, 42, 68). In Malaysia, it was contact with infected pigs or fresh infected pig products that was required for transmission of virus to humans (45, 69, 70) (**Figure 1**). NiV shedding in respiratory fluids of infected pigs suggested that it probably spread among farmed animals by aerosol droplets or direct contact (16, 71, 72). In Bangladesh, the transmission of NiV from bats to people has been linked to the consumption of virus-contaminated fresh date palm sap, and bats will consume sap during its collection (57, 73, 74). Domestic animals have also been linked to NiV infection in people in Bangladesh from unwell animals (cows and goats) and pigs (50, 59). Human-to-human transmission of NiV has been well documented in Bangladesh and India (52, 58–60, 75) (**Figure 1**). A study of human NiV-B cases in Bangladesh spanning 14 years reported that of 248 cases studied, one-third were caused by human-to-human transmission (56). Human-to-human transmission of NiV-M was not apparent in Malaysia (76, 77), whereas in the Philippines' NiV-M outbreak, human cases were linked to horse slaughtering and horse meat consumption or exposure to other human patients, indicating both horse-to-human and human-to-human transmission (55) (**Figure 1**). The NiV-B outbreak in Kerala had a very high rate of human-to-human transmission (22 of 23 cases) at three different hospital locations (51).

Naturally acquired NiV infections were also recorded in cats, dogs, and horses in the initial Malaysian outbreak (**Figure 1**), and serological evidence of natural NiV infection in dogs was linked to outbreak farms (11, 61, 78). In the Philippines, both dogs and cats were linked to NiV-M infection, with cats dying after eating horse meat and dogs having NiV-neutralizing antibodies (55) (**Figure 1**). In Australia, a dog was found to be seropositive for HeV and later euthanized but showed no signs of disease, and a second HeV-positive dog was identified in 2013 following exposure to blood from an infected horse (79) (**Figure 1**). Dogs are susceptible to experimental HeV infection and shed virus but show little evidence of clinical illness (80).

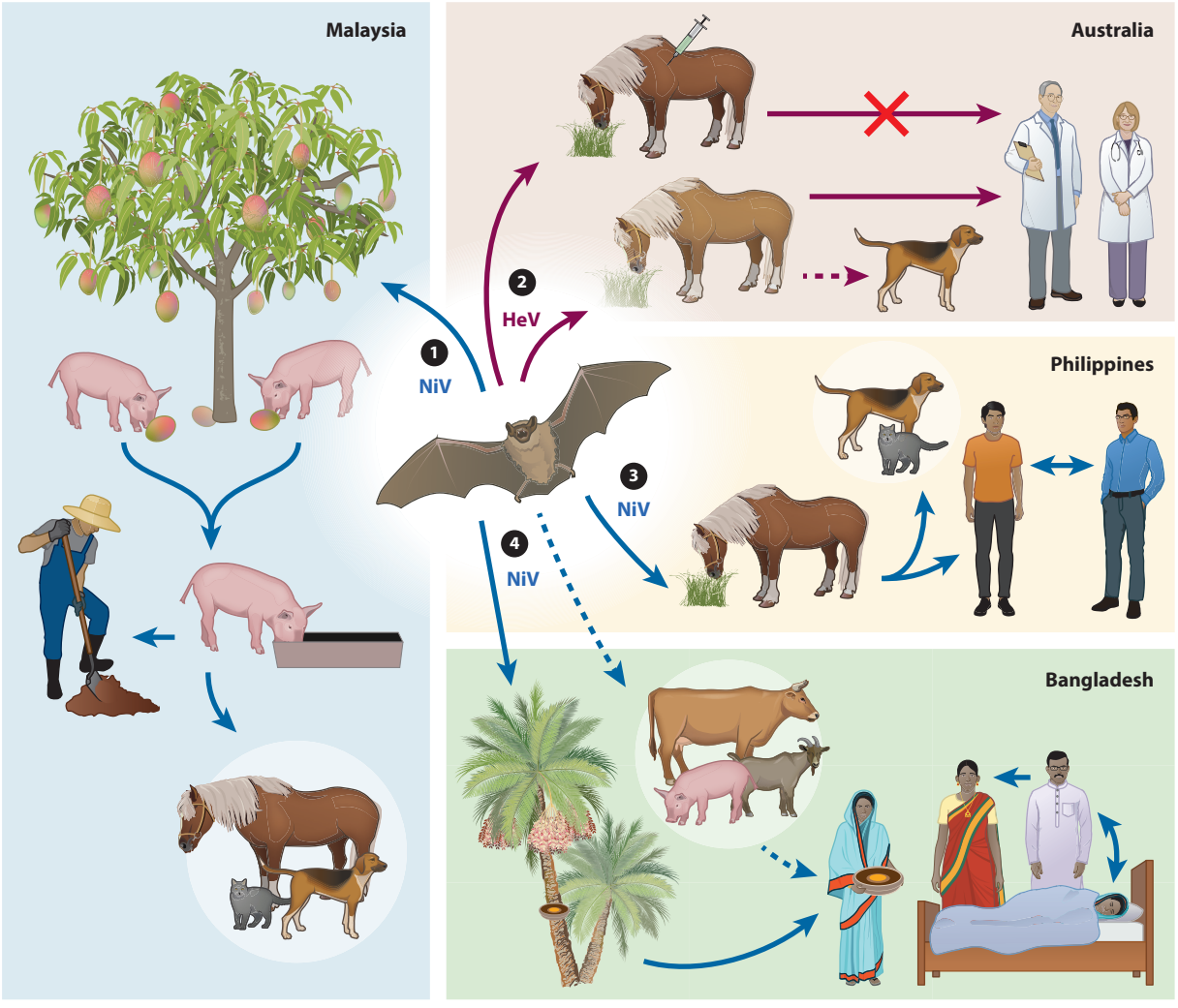

Figure 1

Nipah virus (NiV) and Hendra virus (HeV) modes of transmission in different countries. The transmission routes of NiV in Malaysia (*left*), Philippines (*middle right*), Bangladesh (*bottom right*), and HeV (*top right*) are depicted. Solid lines represent transmission that has been observed and documented, and dashed lines represent suspected transmission in natural conditions. Fruit bats are the natural reservoirs of NiV and HeV. (①) Pigs are infected by consuming partially eaten or contaminated fruit from infected bats (urine, saliva) and transmit NiV to other pigs, pig farmers, or other animals (dogs, cats, and horses) through close or direct contact. (②) Horses can be infected from grazing in contaminated pastures and transmit HeV to humans and on occasion domestic dogs through close contact. A One Health vaccine approach was developed for vaccination of horses in Australia with the dual purpose of saving horses from lethal HeV infection and preventing HeV transmission from horses to humans. (③) NiV is transmitted to humans through close contact with infected horses. NiV transmission to humans, cats, and dogs appears to have occurred following close contact with or consumption of infected horse meat. Human-to-human NiV transmission can occur through close contact. (④) Bat-to-human NiV transmission occurs through consumption of contaminated date palm sap. Human-to-human transmission can occur through close contact with infected patients. Humans may also become infected through contact with infected animals. Figure adapted with permission from Reference 171.

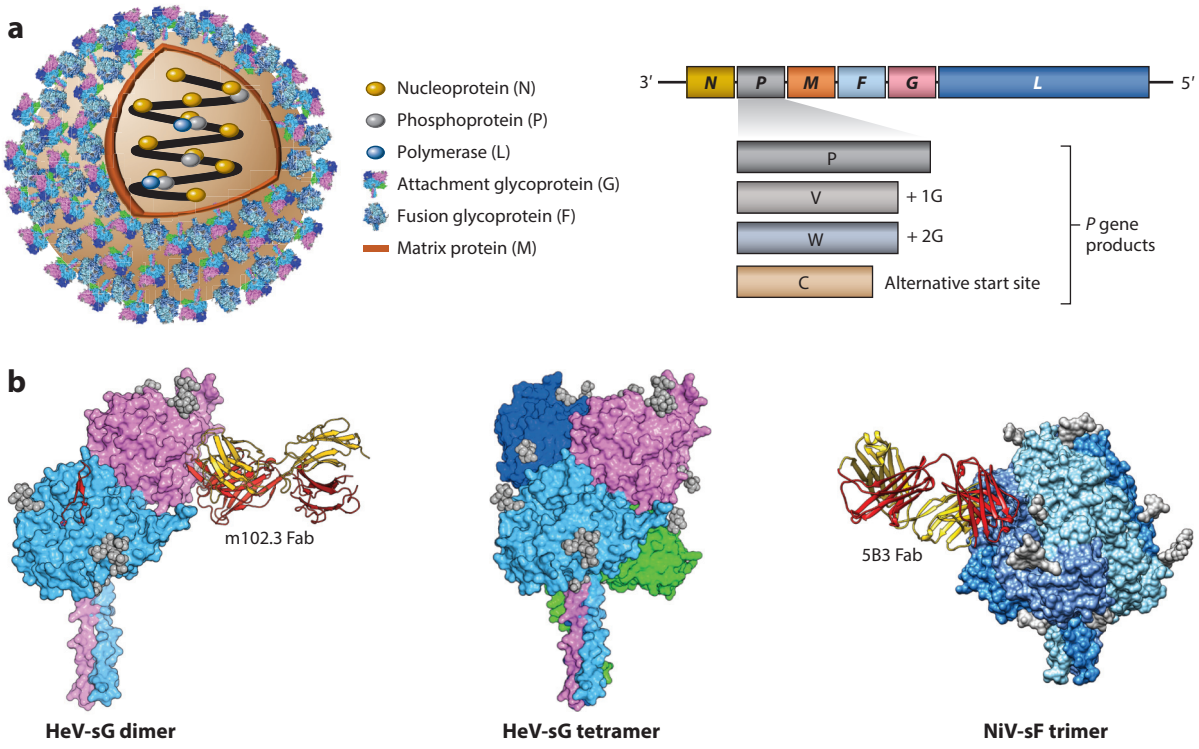

Figure 2

Henipavirus structure and genome organization and models of the G and F glycoprotein soluble ectodomains, Hendra virus (HeV-sG) and Nipah virus (NiV-sF), respectively, and their complexes with respective NiV and HeV cross-reactive neutralizing monoclonal antibodies m102.3 (anti-G) and 5B3 (anti-F). (*a*) Schematic representation of a henipavirus particle with the structural proteins depicted in different colors (*left*) and the henipavirus genome (*right*). HeV and NiV *P* genes encode 3 nonstructural proteins: The C protein is expressed from an alternative start site, and the V and W proteins are expressed following the addition of one or two G residues at the messenger RNA editing site, respectively (*right*). (*b*, *left*) HeV-sG shown as a dimer solvent-accessible surface view with one monomer (*cyan*) overlaid with the monoclonal antibody m102.3 CDR-H3 loop (*red*) at the receptor binding site, and the other monomer (*magenta*) in complex with m102.3 Fab, which has an identical heavy chain and a similar light chain, that was used in place of the m102.4 monoclonal antibody (mAb) in the structural solution of the complex (109). The HeV-sG consists of amino acids 76–604, and the structures of the two globular head domains of HeV-sG are derived from the crystal structure (103, 172). The stalk regions of each G monomer (residues 77–136) are modeled (173). The light chain of m102.3 Fab is colored in yellow, and the heavy chain is colored in red. (*b*, *middle*) The HeV-sG tetramer surface view is modeled with one dimer (*cyan* and *magenta*) in front and the other dimer (*blue* and *green*) in back. *N*-linked glycans are gray spheres. (*b*, *right*) Structural model of the NiV-sF trimer in complex with the 5B3 Fab derived from the cryo–electron microscopy structure (110). The NiV-sF consists of amino acid residues 1–494 with a FLAG tag (DYKDDDK) introduced between residues L104-V105 and a C-terminal GCN4 motif. Each monomer of NiV-sF is in a different shade of blue, 5B3 heavy chain is in red, and light chain is in gold. *N*-linked glycans are illustrated in gray.

Entry and Tropism of Nipah and Hendra Viruses

NiV and HeV are enveloped viruses containing an unsegmented, single-stranded, negative-sense RNA genome (2). **Figure 2*a*** is an illustration of the viral particle and the associated viral proteins. The genomes of HeV and NiV, and also CedV, GhV, and MojV, are considerably longer than the genomes of other paramyxoviruses, at greater than 18 kb. Henipavirus genomes encode 6 structural proteins: nucleoprotein (N), phosphoprotein (P), matrix protein (M), fusion glycoprotein (F), attachment glycoprotein (G), and the polymerase protein (L) (**Figure 2*a***). The N, P, and L proteins comprise the replication complex. The *P* gene undergoes RNA editing

to produce 2 additional nonstructural proteins, V and W, that are interferon (IFN) antagonists (81–84). The C protein is transcribed from a second open reading frame in the *P* gene (**Figure 2*a***). NiV has been central to understanding the V, W, P, and C protein roles in antagonizing the innate immune responses via a diverse set of mechanisms (85, 86). Recent in vivo studies with recombinant NiV variants have further defined the varying importance of these nonstructural proteins in pathogenesis, but only a lack of the V protein results in a nonlethal infection (87–89).

The henipavirus virion bears surface projections composed of the F and G glycoproteins that are anchored in the viral membrane and together mediate infection of host cells, and they are the major antigens of vaccine strategies (1) (**Figure 2*a***). The F glycoprotein facilitates membrane fusion between the virus and host cell. The G glycoprotein consists of a characteristic stalk with a globular head that engages entry receptors on host cells, leading to the fusion activation of F and virus infection. The native structure of G is a tetramer while F is a trimer, and together they are the key determinants of infection and tropism (90–92). Models of the soluble ectodomain of the HeV G (HeV-sG) as a dimer and tetramer and the soluble ectodomain of the NiV F (NiV-sF) as a trimer are shown in **Figure 2*b***. NiV and HeV utilize the host cell proteins ephrin-B2 and ephrin-B3 for entry (93–96). Ephrin-B2 and ephrin-B3 are members of a large family of ligands that bind to Eph receptors and are highly sequence conserved among mammals (97, 98). Ephrin-B2 expression is prominent in the vasculature of multiple organs, whereas ephrin-B3 is found predominantly in the nervous system (99–101). The ability of HeV and NiV to use these ephrins as receptors provided explanations of their broad host and tissue tropism (32, 33, 102). The NiV and HeV G head domain structures alone and in complex with ephrin-B2 and ephrin-B3 receptors have been determined (103–106). The structures of both the NiV and HeV F in their prefusion conformation have also been determined (107, 108). These studies have provided insights into understanding the virus entry receptors and host tropism features of the viruses on the molecular level and also facilitated further structural studies of henipavirus G and F glycoproteins in complexes with specific virus-neutralizing antibodies, providing valuable information that has aided vaccine design and choice (109, 110).

Nipah Virus and Hendra Virus Infection in Humans and Animals

Human NiV and HeV infections are generally accepted to occur via the oronasal route, and the incubation periods for both have been estimated to be 1 to 2 weeks (31, 51, 111). Acute infection in people is a systemic infection likely via hematogenous spread of the virus from the respiratory system (112). In general, HeV and NiV disease onset is characterized by fever, myalgia, shortness of breath, and cough (38, 111). Human HeV infections have resulted in both fatal respiratory or encephalitic disease and also recovery from infection (31, 38, 42, 68). The predominant clinical feature in the NiV-M outbreak in Malaysia was encephalitis, but respiratory symptoms were also common with fever, cough, and headache (47, 111, 112). The clinical presentation of NiV-B infections in Bangladesh also includes severe respiratory disease. In the 2018 NiV-B outbreak in Kerala, 83% of cases presented with acute respiratory distress syndrome (ARDS) (51, 113). Central findings of human NiV and HeV infection are a widespread endothelial cell tropism and systemic vasculitis, with prominent parenchymal cell infection in most major organs with the brain and lung significantly affected (45, 112, 114). Human NiV and HeV infections can also take a protracted course following apparent recovery, and some patients can experience late-onset encephalitis or relapsed encephalitis can occur in patients who previously recovered (42, 115). Relapsed encephalitis caused by NiV appears to result from a recrudescence of virus replication in the central nervous system (CNS), with cases presenting from a few months to as long as 11 years later (116–118). Recrudescence of virus has important implications for vaccine development.

The development of animal models of NiV and HeV infection and pathogenesis has been a major focus since the late 1990s and an essential component of vaccine development and testing. Also, the approval process of countermeasures for NiV and HeV would fall under the animal rule requirement set forth by the US Food and Drug Administration (FDA) in 2002 as an alternative licensing pathway for countermeasures against highly pathogenic agents when human efficacy studies are not feasible or ethical (119, 120). Several animal models of NiV and HeV infection have emerged that well reflect the pathogenesis seen in infected people, which includes a systemic vasculitis with both respiratory and neurological diseases. Detailed reviews of NiV and HeV infections of a variety of mammalian species have recently been published (33, 121–123). It is generally accepted that the pathogenic processes of NiV and HeV infection in the hamster, ferret, and African green monkey (AGM) best reflect the pathogenesis observed in humans, whereas the most appropriate models for livestock are the horse and pig themselves.

VACCINATION

The attachment and fusion glycoproteins of paramyxoviruses such as measles, mumps, and parainfluenza viruses are the viral antigens to which virtually all neutralizing antibodies are directed (124–126). Likewise, immunization strategies for NiV and HeV have largely targeted their G and F glycoproteins.

Passive Immunization Strategies

Early passive immunization studies in the hamster model demonstrated that polyclonal antiserums or mouse monoclonal antibodies (mAbs) to NiV F or G could provide complete protection against NiV-M or HeV when administered before and immediately after virus infection (10, 127, 128). These studies demonstrated a major role of a viral glycoprotein-specific antibody in protection.

Recombinant human antibody technology was used to generate a potent cross-neutralizing mAb against NiV and HeV (m102.4) (129, 130). The m102.4 mAb epitope maps to the ephrin receptor binding site of G and blocks virus infection (see the left side of **Figure 2*b***), and it can neutralize NiV-M, NiV-B, and HeV (8, 109). The m102.4 mAb provided complete protection from NiV-M-mediated disease in ferrets as a single 50 mg dose administered 10 h post-challenge (8). In the AGM model, m102.4 administered as two 20 mg/kg doses, intravenously, at 10 h and again on day 3, on days 1 and 3 (days 1/3), or on days 3/5, after HeV challenge [4×10^5 50% tissue culture infectious dose ($TCID_{50}$)] by intratracheal (i.t.) administration, protected 100% of treated subjects (131). All treated subjects seroconverted against HeV F glycoprotein with a rise in antibody titer over time, indicating all animals had become infected with HeV and recovered, whereas untreated control subjects succumbed to HeV disease and failed to mount a protective immune response. No clinical signs were evident at any time in the early treatment groups; although neurological symptoms were observed in subjects in the late treatment group (days 3/5), all later recovered from infection. There was no HeV antigen or virus-specific histopathology detected in the lung or brain at the conclusion of the study in any treated subject, and infectious virus could not be recovered from any tissue. A similar study evaluated m102.4 against NiV-M disease in the AGM model at several time points following virus challenge (5×10^5 PFU), including a late cohort where treatment was initiated at the onset of clinical illness (day 5) (132). All subjects became infected after challenge, and all subjects that received m102.4 survived infection and all controls succumbed to disease. Subjects in the late day 5/7 treatment group exhibited disease, but all recovered. A comparative study in AGMs using NiV-M and NiV-B [5×10^5 PFU divided by i.t. and intranasal (i.n.) administration] revealed that NiV-B caused a more aggressive disease, with a

shortened time to death and higher virus loads in tissues and fluids (133). When m102.4 was tested in this model, all subjects in the days 1/3 and days 3/5 post-infection treatment groups survived NiV-B challenge, but subjects in the days 5/7 treatment group succumbed, indicating a shorter therapeutic window in treating NiV-B infection (133). Another well-characterized, humanized mouse mAb, 5B3 (h5B3.1), that is cross-reactive to the F glycoprotein of NiV and HeV and binds a prefusion conformation epitope on F, preventing membrane fusion, was recently tested (110, 134) (**Figure 2*b***). The h5B3.1 mAb was given to ferrets in 20 mg/kg doses by intraperitoneal (i.p.) injection, at 1 to several days post-challenge, with either NiV or HeV ($\sim 5 \times 10^3$ PFU) delivered i.n. (135). All subjects that received h5B3.1 after infection were protected from disease and had increasing neutralizing antibody titers, whereas all controls died. No pathology was observed, and no infectious virus could be isolated at the study endpoint. Altogether, these studies demonstrate that passive immunization with mAbs can provide therapeutic benefit and allow the infected host an extended period to mount a protective immune response. The findings from these experiments were also important because they suggest that vaccine approaches designed to induce adequate neutralizing antibody responses to NiV and HeV should be effective.

The m102.4 mAb producing cell line was provided to the Queensland Government, Australia, to produce the mAb for compassionate use in future cases of high-risk human HeV infection. To date, 14 individuals exposed to either HeV in Australia ($n = 13$) or NiV in the United States ($n = 1$) have been given high-dose m102.4 therapy (15–20 mg/kg) by emergency use protocols, and all have remained well. In Australia, m102.4 was used in a randomized, controlled phase I study in healthy adults (136). The study included four single and one repeat dosing groups, and the m102.4 mAb was found to be safe and well tolerated, with a half-life ranging between ~16.5 and 27 days, and no observed immunogenicity was reported. Two doses of 20 mg/kg (days 1/3) were as well tolerated as a single dose. This study's findings will aid in the design of future dosing regimens of mAbs for evaluating their ability to prevent and/or treat HeV and NiV human infections.

Active Immunization Strategies

A variety of immunization strategies have been developed to prevent NiV and HeV infection including several live-recombinant virus vectors, protein subunit, and virus-like particle (VLP) approaches, and all target the virus attachment and entry steps of infection by employing the G and/or F glycoprotein antigens. Here we summarize these various vaccination countermeasure approaches to NiV and HeV infection (**Tables 1** and **2**).

Poxvirus vectored. Poxviruses have a long history as a platform for the expression of heterologous genes to study protein function and serve as vaccine candidates as a live-attenuated viral vaccine platform capable of inducing both cell-mediated and humoral immune responses (137). The F and G glycoproteins of NiV and HeV were functionally characterized using recombinant vaccinia viruses in the early 2000s (138, 139). The first NiV vaccine tested used a highly attenuated vaccinia virus strain (NYVAC) encoding either the F or G glycoproteins from NiV-M (127). Hamsters were vaccinated by subcutaneous (s.c.) injection in a prime-boost strategy with NYVAC-NiV-F or NYVAC-NiV-G, individually and in combination, and then 3 months later challenged i.p. with NiV-M. Vaccination yielded complete protection from NiV-M with no detection of viral RNA, and control subjects succumbed 7–10 days after challenge (127) (**Table 1**). Another poxvirus-based approach was examined as a vaccine for pigs using canarypox (ALVAC) vaccine vectors encoding either NiV-M F or G glycoprotein (140). A prime-boost strategy with ALVAC-NiV-F or ALVAC-NiV-G vectors was tested alone or in combination in pigs. The animals were then challenged 28 days later with NiV-M via i.n. administration. All vaccinated animals survived NiV-M challenge

Table 1 Virus vectored vaccine strategies for NiV and HeV

Approach	Name(s)	Animal model	Vaccination dose, route, schedule	Adjuvant	Challenge	Challenge dose, route, schedule	Survival	Correlate(s) of immunity/ protection	Reference
Poxvirus	NYVAC-NiV-F and/or -G	Hamster	2 doses at 1×10^7 PFU, s.c., 1 month apart	None	NiV-M	1×10^3 PFU, i.p., 3 months later	100%	NAb response, viral RNA	127
	ALVAC-NiV-F and/or -G	Pigs	2 doses at 1×10^8 PFU, i.m., 2 weeks apart	None	NiV-M	2.5×10^5 PFU, i.n., 28 days later	100%	NAb response, viral RNA, infectious virus, viral shedding, cytokine production	140
	ALVAC-HeV-F and/or -G	Hamster	2 doses at 7.4 or 5.4 log10 $CCID_{50}$, s.c., 3 weeks apart	None	HeV	1×10^3 LD_{50}, i.p., 21 days later	89% and 63%	NAb response, viral RNA, viral antigen, viral shedding	141
		Ponies	2 doses at 6 log10 $CCID_{50}$, i.m., 3 weeks apart	None	NT	NA	NA	High NAb titers	
	MVA-NiV-sG and/or MVA-NiV-G	IFNAR−/− mice	1 or 2 doses at 1×10^8 PFU, i.m., 3 weeks apart	None	NT	NA	NA	High serum IgG titers, NiV-G-specific CD8 and CD4 T cells	142

(*Continued*)

Table 1 (*Continued*)

Approach	Name(s)	Animal model	Vaccination dose, route, schedule	Adjuvant	Challenge	Challenge dose, route, schedule	Survival	Correlate(s) of immunity/ protection	Reference
VSV	VSV-NiV-F and/or -G	Mice	5×10^3 PFU, i.n. or i.m.	None	NT	NA	NA	High NAb titers	144
		Hamster	1×10^6 infectious particles, i.m.		NiV-M	1×10^5 $TCID_{50}$, i.p., 32 days later	100%	NAb response, viral RNA, viral antigen	145
	VSV-NiV-B F and/or G	Ferret	1×10^7 PFU, i.m.	None	NiV-M	5×10^3 PFU, i.n., 28 days later	100%	Serum IgG response, viral RNA, viral antigen	146
		AGM			NiV-B	5×10^5 PFU, i.t. and i.n., 28 days later		NAb response, viral RNA, viral antigen	147
	VSV-ZEBOV-GP-NiV F, G, or N	Hamster	1×10^5 PFU, i.p.	None	NiV-M	1×10^3 LD_{50}, i.p., 28 days later	100%	NAb response, viral RNA, infectious virus	148
		AGM	1×10^7 PFU, i.m.			1×10^5 $TCID_{50}$, i.t., 29 days later		NAb response, viral RNA, infectious virus, viral shedding	149
	VSV-HeV-G	Mice	1×10^5 PFU, i.m.	None	NT	NA	NA	Serum IgG, NAb response	150
AAV	AAV8 NiV.G	Mice	2×10^{10} genome particles, i.m. or 1×10^{10} genome particles, i.d.	None	NT	NA	NA	Serum IgG, NAb response	151
		Hamster	6×10^{11} genome particles, i.m.		NiV-M	1×10^4 PFU, i.p., 5 weeks later	100%	Serum IgG, NAb response, viral RNA, viral antigen	
					HeV		50%		

(*Continued*)

Table 1 (*Continued*)

Approach	Name(s)	Animal model	Vaccination dose, route, schedule	Adjuvant	Challenge	Challenge dose, route, schedule	Survival	Correlate(s) of immunity/ protection	Reference
Adenovirus	ChAdOx1 NiV-B G	Hamster	2 doses at 1×10^8 IU, i.m., 28 days apart	None	NiV-B	5.3×10^5 $TCID_{50}$, i.p., 28 days later	100%	NAb response, viral RNA, infectious virus, virus shedding	153
			1×10^8 IU, i.m.		NiV-M	6.8×10^4 $TCID_{50}$, i.p., 28 days later	100%		
					HeV	6×10^3 $TCID_{50}$, i.p., 28 days later	33%		
Measles virus	rMV-Ed-G or rMV-HL-G	Hamster	2 doses at 2×10^4 $TCID_{50}$, i.p., 3 weeks apart	None	NiV-M	1×10^3 $TCID_{50}$, i.p., 1 week later	100%	Serum IgG response	NA
	rMV-Ed-G	AGM	2 doses at 1×10^5 $TCID_{50}$, s.c., 4 weeks apart			1×10^5 $TCID_{50}$, i.p., 1 week later		Serum IgG response, viral RNA	154
Inactivated RABV	RABV-HeV-G	Mice	3 doses at 10 μg, i.m., 2 weeks apart	None	NT	NA	NA	High NAb titers, serum IgG response	150
	RABV-NiV-B G		2 doses at 10 μg, i.m., 4 weeks apart						155
RABV	RABV-NiV-F and/or -G	Mice	$1 \times 10^{6.5}$ FFU, oral	None	NT	NA	NA	Serum IgG, NAb response	156

All NiV glycoprotein vaccines employ the NiV-M strain unless otherwise indicated.

Abbreviations: AAV, adeno-associated virus; AGM, African green monkey; $CCID_{50}$, 50% cell culture infectious dose; ChAdOx1, chimpanzee adenovirus Oxford 1; F, fusion glycoprotein; FFU, focus forming units; G, attachment glycoprotein; HeV, Hendra virus; i.d., intradermal; i.m., intramuscular; i.n., intranasal; i.p., intraperitoneal; i.t., intratracheal; IFNAR, interferon receptor; IgG, immunoglobulin G; IU, infectious unit; LD_{50}, 50% lethal dose; MVA, modified vaccinia virus Ankara; NA, not applicable; NAb, neutralizing antibody; NiV, Nipah virus; NiV-B, Nipah virus Bangladesh; NiV-M, Nipah virus Malaysia; NT, not tested; PFU, plaque forming unit; RABV, rabies virus; rMV-Ed, recombinant measles virus Edmonston; rMV-HL, recombinant measles virus HL; s.c., subcutaneous; sF, F glycoprotein soluble ectodomain; sG, G glycoprotein soluble ectodomain; $TCID_{50}$, 50% tissue culture infectious dose; VSV, vesicular stomatitis virus; ZEBOV-GP, *Zaire ebolavirus* glycoprotein.

Table 2 VLP, subunit, and nucleic acid–based vaccine strategies for NiV and HeV

Approach	Name	Animal model	Immunization dose, route, schedule	Adjuvant	Challenge	Challenge dose, route, schedule	Survival	Immune correlate(s) of survival	Reference
VLPs	VLPs-NiV M/F/G	Mice	2 doses at 1.75, 3.5, 7, or 14 μg and 6 μg, s.c., 2 weeks apart	None	NT	NA	NA	High NAb titers	159
		Hamster	1 dose or 3 doses at 30 μg, i.m., 3 weeks apart	Alhydrogel/MPLA or Alhydrogel/CpG	NiV-M	1.6×10^4 PFU (3-dose trial) or 3.3×10^4 PFU (1-dose trial), i.p., 28 days later	100%	NAb response, viral RNA	160
Subunit vaccines	NiV-sG	Cat	3 doses at 100 μg, s.c., 2 weeks apart	CSIRO triple adjuvant	NiV-M	5×10^2 $TCID_{50}$, s.c., 2 months later	100%	NAb response, viral antigen, viral genome	14
	HeV-sG	Cat	3 doses at 100 μg, s.c., 2 weeks apart	CSIRO triple adjuvant	NiV-M	5×10^2 $TCID_{50}$, s.c., 2 months later	100%	NAb response, viral antigen, viral genome	14
			2 doses at 50, 25, or 5 μg, i.m., 3 weeks apart	CpG/Alhydrogel		5×10^4 $TCID_{50}$, o.n., 2 weeks later	100%	Serum IgG, NAb response, viral RNA, viral shedding, infectious virus	162
		Ferret	2 doses at 100, 20, or 4 μg, i.m., 20 days apart	CpG	HeV	5×10^3 $TCID_{50}$, o.n., 3 weeks later	100%	NAb response, viral RNA, infectious virus	163
				CpG/Alhydrogel	NiV-B	5×10^4 $TCID_{50}$, 20 days later or 12 months later	100%	viral RNA, viral antigen, infectious virus	164

(*Continued*)

Table 2 (*Continued*)

Approach	Name	Animal model	Immunization dose, route, schedule	Adjuvant	Challenge	Challenge dose, route, schedule	Survival	Immune correlate(s) of survival	Reference
		AGM	2 doses at 100, 50, or 10 μg, i.m., 3 weeks apart	CpG/Alhydrogel	NiV-M	1×10^5 $TCID_{50}$, i.t., 3 weeks later	100%	Serum IgG, NAb response, viral RNA, viral antigen, infectious virus	165
			2 doses at 100 μg, i.m., 3 weeks apart	Alhydrogel or CpG/Alhydrogel	HeV	5×10^5 PFU, i.t., 21 days later	100%	NAb response, viral RNA	166
		Horse	2 doses at 100 or 50 μg, i.m., 3 weeks apart	Zoetis	HeV	2×10^6 $TCID_{50}$, o.n., 28 or 194 days later	100%	NAb response, viral RNA, viral antigen, infectious virus	167
		Pig	2 doses of 2 mL preformulation, i.m., 3 weeks apart	Zoetis	HeV	5×10^5 PFU, i.n., 35 days later	Partial	NAb response, viral RNA, infectious virus, viral shedding	168
					NiV-M		0%		
Nucleic acid–based vaccine	HeV-sG mRNA LNP	Hamster	10 or 30 μg, i.m.	None	NiV-M	1×10^5 $TCID_{50}$, i.p., 30 days later	30% or 70%	Serum IgG, NAb response	169

All NiV glycoprotein vaccines employ the NiV-M strain.

Abbreviations: AGM, African green monkey; CSIRO, Commonwealth Scientific and Industrial Research Organisation; F, fusion glycoprotein; G, attachment glycoprotein; HeV, Hendra virus; i.m., intramuscular; i.n., intranasal; i.p., intraperitoneal; i.t., intratracheal; IgG, immunoglobulin G; LNP, lipid nanoparticle; M, matrix protein; MPLA, monophosphoryl lipid A; mRNA, messenger RNA; NA, not applicable; NAb, neutralizing antibody; NiV, Nipah virus; NiV-B, Nipah virus Bangladesh; NiV-M, Nipah virus Malaysia; NT, not tested; o.n., oronasal; PFU, plaque forming unit; s.c., subcutaneous; sG, G glycoprotein soluble ectodomain; $TCID_{50}$, 50% tissue culture infectious dose; VLP, virus-like particle.

as determined by the lack of NiV RNA and infectious virus from nasal washes, pharyngeal swabs, and a variety of sampled organs (140).

ALVAC-vectored vaccines encoding HeV F or G glycoprotein for potential use in horses were also examined (141). ALVAC-HeV-F or ALVAC-HeV-G vectors were combined and first used to vaccinate hamsters at a high or low dose of each vector, by s.c. injection, and then challenged with HeV by i.p. administration. Vaccination did not result in complete protection, with 8 out of 9 subjects in the high-dose group and 5 out of 8 subjects in the low-dose group surviving challenge. No signs of disease were noted, and viral antigen or viral RNA could not be detected in survivors. Nine ponies vaccinated using the same prime-boost regimen were able to develop high cross-neutralizing antibody titers to HeV and NiV-M at day 28 after vaccination. Although ponies were not challenged, most animals yielded titers of at least 1:32 and were considered likely protective (141).

More recently, a modified vaccinia virus Ankara (MVA) vector encoding NiV-M G glycoprotein and a soluble version of G (NiV-sG) were examined in interferon receptor α and β (IFNAR−/−) knockout mice (142) (**Table 1**). IFNAR−/− mice were immunized once with MVA-NiV-G or MVA-NiV-sG or prime-boosted. IFNAR−/− mice developed high serum immunoglobulin G (IgG) titers to NiV-G and also generated NiV-G-specific CD8 and CD4 T cells following vaccination. MVA-NiV-sG vaccination induced rapid and significantly higher amounts of NiV-G epitope-specific CD8 T cells compared with the MVA-NiV-G candidate vaccine, suggesting superior immunogenicity. Together, these immunization studies with poxvirus vectors highlight that both T cell and B cell responses play a role in an adaptive immune response to NiV and HeV. However, detailed studies on the adaptive immune responses in animal experiments with henipaviruses have been limited. Future studies evaluating the role of NiV-specific T cells will be important because two human survivors of NiV-B infection in the 2018 outbreak in Kerala showed marked elevation of activated $CD8^+$ T cells, which coincided with virus clearance (143).

Vesicular stomatitis virus vectored. Recombinant vesicular stomatitis virus (rVSV) vectors as a vaccine platform suitable for single immunization strategies to potentially meet emergency use requirements have been tested by several groups (**Table 1**). A method of using two defective VSVΔG vectors each expressing only the NiV G or F glycoprotein was devised using VSV G glycoprotein complementation that can generate replication-defective VSV vectors that could elicit NiV-neutralizing antibodies (144). Using this technique, researchers tested rVSV vaccines expressing either NiV-M F or G glycoproteins (VSV-ΔG-NiVG, VSV-ΔG-NiVF) in hamsters by intramuscular (i.m.) vaccination (145). Hamsters were then challenged 32 days later with NiV-M by i.p. administration. All vaccinated animals survived lethal infection with no clinical signs of disease. No viral RNA or viral antigen could be detected in the sampled tissues when compared with controls, and there was a lack of an anamnestic immune response in vaccinated subjects following challenge, suggesting the induction of sterilizing immunity.

Another study used rVSV-ΔG vectors expressing NiV-B F or G glycoprotein and also tested them as single-injection vaccinations in NiV-M-challenged ferrets (146). Ferrets were vaccinated i.m. with rVSV-NiV-B F or rVSV-NiV-B G complemented with VSV G or a mix of both vectors, rVSV-NiV-B F/G, that was generated as a complementing pair in the absence of VSV G and then challenged at 28 days with NiV-M by i.n. administration. All vaccinated ferrets were completely protected against NiV-M challenge. Although viral RNA was detected in blood at day 6 post-challenge in 2 of 5 animals in each group, those levels were 100 times lower than in the unvaccinated controls, and by day 21 no viral RNA was detected (146). In a second study, rVSV-NiV-B F and rVSV-NiV-B G were assessed separately and in combination in AGMs (147). Cohorts were

vaccinated with the rVSVs by i.m. injection and challenged 28 days later with NiV-B divided between the i.t. and i.n. routes (147). Complete protection was recorded from NiV-B disease with no gross pathology and no detectable NiV antigen in lung or spleen tissues. Viral RNA was detected in nasal and oral swabs of the vaccinated groups, but no viral RNA could be detected in blood samples.

Replication-competent rVSV-NiV-M F or G vectors, generated by the retention of the envelope glycoprotein from *Zaire ebolavirus* (ZEBOV-GP), which allowed virus stocks to be propagated (rVSV-ZEBOV-GP-NiVF, rVSV-ZEBOV-GP-NiVG, and rVSV-ZEBOV-GP-NiVN), have also been tested (148). These rVSVs were used to immunize hamsters by i.p. administration and were challenged 28 days later with NiV-M. All subjects vaccinated with either the NiV F or G glycoprotein encoding rVSV vectors were completely protected with no clinical disease or pathology, whereas those vaccinated with the NiV N protein were only partially protected (2 of 6 animals) with no clinical signs of disease and the other subjects succumbed to infection. The protective efficacy of the rVSV-ZEBOV-GP-NiVG was also tested in AGMs, where vaccinated subjects were challenged with NiV-M by i.t. administration 29 days later (149). All vaccinated subjects were protected from lethal challenge and showed no signs of clinical disease, no viral RNA was detected in the blood or oral and nasal swabs, and no infectious virus could be recovered. Another study using a rVSV vector expressing a codon-optimized HeV *G* gene together with an inactivated counterpart was evaluated in mice for humoral immune responses only as a comparator to a recombinant rabies virus vaccine encoding HeV G as a HeV vaccine candidate (150). Here, the live rVSV vectors induced greater levels of HeV G-specific antibodies and higher levels of HeV-neutralizing antibodies than did the recombinant rabies virus vectors (see the section titled Rabies Virus Vectored).

Adeno-associated virus and adenovirus vectored. Adeno-associated virus (AAV) vectors as a vaccine platform against infectious diseases, particularly viral pathogens, have been explored. AAV is a small, single-stranded DNA virus in the family *Parvoviridae*. Immunization of hamsters with an AAV vector expressing NiV-M G glycoprotein (AAV8 NiV.G) by i.m. injection demonstrated complete protection from a challenge of NiV-M by i.p. administration, and no signs of clinical disease were recorded (151) (**Table 1**). Neutralizing antibodies to NiV were induced, no viral RNA or viral antigen was detected in any of the sampled tissues, and there was only a moderate anamnestic response observed in a single subject, suggestive of potential sterilizing immunity. However, in a cross-protection study, AAV8 NiV.G protected only 50% of hamsters challenged with HeV.

Chimpanzee adenoviral (ChAd) vectors circumvent issues of the preexisting immunity observed with human adenovirus vectors (152). Adenoviruses are double-stranded DNA viruses in the family *Adenoviridae*. An engineered replication-deficient ChAd vector, Oxford 1 (ChAdOx1), was tested as a NiV/HeV vaccine (153). Here, ChAdOx1 encoding NiV-B (ChAdOx1 NiV-B) G glycoprotein was used to vaccinate hamsters by i.m. injection, either as a single dose or as a prime-boost protocol. Hamsters were challenged by i.p. administration with NiV-B 42 days following the booster or the single vaccination. Neutralizing antibodies were detectable, and all vaccinated hamsters were protected against lethal disease with no lung pathology, suggesting that a single dose of ChAdOx1 NiV-B was sufficient to completely protect against NiV-B. No viral RNA in the lung tissue and no viral shedding in oropharyngeal swabs could be detected, and no infectious virus could be isolated. A second cohort using a single dose of ChAdOx1 NiV-B to vaccinate hamsters was trialed, and these animals were challenged 28 days later with NiV-M or HeV. All vaccinated animals were protected from lethal NiV-M challenge, but 4 out of 6 hamsters succumbed to HeV disease between days 5 and 7 post-challenge. Neither virus shedding in oropharyngeal swabs nor

infectious virus was detected in the lung or brain tissues of NiV-M-challenged vaccinated hamsters. In contrast, infectious virus was detected in the lung tissues of 75% of the HeV-challenged vaccinated animals. The lower cross-protection observation using NiV G vaccination followed by HeV challenge was not unexpected, as it was previously shown that when the G glycoprotein (as a recombinant soluble subunit immunogen) of either HeV or NiV was used to vaccinate cats, both could completely protect against lethal NiV-M challenge, and that the HeV-sG elicited greater heterologous neutralizing antibody responses in comparison to NiV-sG (14).

Measles virus vectored. Recombinant measles virus vectors based on the HL (rMV-HL) and Edmonston (rMV-Ed) measles virus strains have also been explored in which they encoded the NiV-M G glycoprotein (rMV-HL-G and rMV-Ed-G) (154) (**Table 1**). Hamsters were immunized twice by i.p. administration of rMV-HL-G or rMV-Ed-G. All vaccinated animals produced NiV G-specific antibody titers after the booster immunization. Animals were challenged 1 week after the second immunization with NiV-M by i.p. administration. All immunized hamsters exhibited no clinical symptoms and survived challenge. The study was extended to non-human primates (NHPs), where 2 AGMs were immunized twice by s.c. injection with rMV-Ed-G. Subjects were challenged 2 weeks after the second immunization with NiV-M by i.p. administration. Here, immunization completely protected the AGMs with no observed clinical disease and no detectable pathological changes, and no viral RNA could be detected in sampled tissues. Although this was a small study, the safety profile and success of the live-attenuated measles virus vaccine suggests that a recombinant platform encoding the NiV G glycoprotein as a NiV vaccine candidate is promising and should induce a balanced and long-lasting immune response against NiV.

Rabies virus vectored. A rabies virus (RABV) SAD B19 vaccine strain, BNSP333, expressing HeV or NiV G glycoproteins has been evaluated (150, 155). Recombinant BNSP333 encoding either the wild-type or a codon-optimized HeV *G* gene, together with their inactivated counterparts, was used in mice (150) (**Table 1**). Mice were immunized by i.m. injection with a single dose of the RABV-based vectors or with 3 doses of their inactivated versions. The inactivated RABV-based vectors induced higher and more rapid HeV G-specific antibody responses and higher neutralizing antibody titers than their live counterparts. The inactivated RABV-coHeV-G induced cross-neutralizing antibodies against NiV. A similar study used the BNSP333 vector expressing NiV-B G glycoprotein (RABV-NiV-BG) (**Table 1**) and elicited NiV G-specific neutralizing antibodies (155).

Recently, the recombinant RABV Evelyn-Rokitnicki-Abelseth (ERA) strain ($rERAG_{333E}$) expressing either NiV-M F or G glycoproteins was evaluated in mice and pigs (156) (**Table 1**). This vector, $rERAG_{333E}$, serves as an oral vaccine in dogs. Here, mice were orally immunized with RABV-NiV-F or RABV-NiV-G either individually or in combination. Pigs were also immunized in a similar manner but with 2 doses of each vector either alone or in combination. RABV-NiV-F and/or RABV-NiV-G immunization induced NiV F- and G-specific IgG antibody responses and neutralizing antibodies in both mice and pigs with the combination vaccine inducing higher titers. Although not suitable for human use, the live-attenuated $rERAG_{333E}$ vector is of interest as a potential veterinary vaccine for NiV because it is already approved for use in some animals and could be adapted for emergency use to protect against NiV infection in livestock, particularly swine.

Many of these virus-vectored vaccines for NiV are promising candidates because of their established safety profiles and ease of genetic modification. Several of these virus-vectored vaccines also require no adjuvants, and some are clearly efficacious as a single immunization strategy, suitable features for emergency use circumstances. In addition, several of these platforms are able to induce both cell-mediated and humoral immune responses, which may also be desirable but as yet

are not fully explored in the development of vaccines for NiV and HeV. Although animals immunized with viral vectors encoding the NiV G glycoprotein and challenged with the homologous virus were completely protected, cross-protection studies with some of these vaccines against a HeV challenge were less effective. For example, only 50% of AAV8 NiV.G-immunized hamsters or 33% of ChAdOx1 NiV-B G glycoprotein–immunized hamsters were protected from a lethal HeV challenge (151, 153). In addition, the ALVAC-HeV-F and ALVAC-HeV-G vaccination studies showed that these vectors did not provide 100% protection in hamsters challenged with HeV, perhaps due to either a suboptimal immunization dose or the immunization route (141).

Virus-like particles. VLPs have been explored as a vaccine platform because of the resemblance of their surface structure, dimensions, and compositions to authentic virus yet are of high safety because of the lack of viral genetic material. Earlier studies revealed that the M protein of NiV was capable of orchestrating the formation and budding of NiV VLPs when expressed in cells that appeared structurally similar to authentic NiV virions, and these VLPs could also incorporate other viral proteins such as the F and G glycoproteins (157, 158). VLPs composed of NiV M, F, and G were used to vaccinate mice s.c. at weeks 0, 2, and 4 and demonstrated they could induce high neutralizing antibody titers by day 35 (159) (**Table 2**). NiV VLPs were later used in NiV-M challenge studies either alone or in combination with adjuvant, monophosphoryl lipid A (MPLA) and Alhydrogel^{TM} (15 μg/50 μg) or CpG and Alhydrogel (40 μg/50 μg) (160). Hamsters were vaccinated i.m. either as a single dose or as a 3-dose regimen and then challenged via i.p. administration of NiV-M at 28 days or 58 days, respectively. In all cohorts, 100% of the vaccinated animals survived with no clinical signs of disease and no detection of viral RNA in any of the sampled tissues, regardless of the presence of adjuvant. VLPs are thus an alternative means, with inherent safety, of producing an inactivated whole virus vaccine from an otherwise highly pathogenic virus.

Subunit vaccine. The HeV-sG subunit vaccine has been extensively evaluated in several studies. Here, a brief summary of earlier reports is made, but the focus is on studies in NHPs and livestock. Recombinant HeV-sG and NiV-sG can elicit a potent neutralizing antibody response and were first tested as vaccine immunogens in the feline model (14, 161) (**Table 2**). Both HeV-sG and NiV-sG vaccination of cats completely protected against lethal NiV-M challenge, and HeV-sG elicited greater heterologous neutralizing titers than did NiV-sG, demonstrating that a single subunit vaccine may be effective against both NiV and HeV (14). Other studies using lower doses of HeV-sG (**Table 2**) demonstrated that a pre-challenge neutralizing titer of 1:32 could protect against NiV-M (162). Additional studies in ferrets showed that low doses of HeV-sG could protect against HeV and NiV-B (163, 164) (**Table 2**). Also, a longevity study showed that vaccinated ferrets challenged with NiV-B at 14 months post-immunization, with pre-challenge neutralizing titers of 1:16 to 1:128, were also protected (164).

The HeV-sG vaccine has been extensively evaluated in AGMs (**Table 2**). In a cross-protection study, 100 μg, 50 μg, or 10 μg doses of HeV-sG in combination with Alhydrogel and CpG were administered i.m. as a prime-boost, on days 0 and 21. Pre-challenge 50% neutralization titers ranged from 1:28 to 1:379. All subjects were challenged with NiV-M by i.t. administration on day 42. All vaccinated subjects were completely protected, displaying no clinical signs of disease, and no viral RNA could be detected in blood and tissues and no infectious virus was isolated (165). Similarly, HeV-sG vaccination HeV challenge in AGMs has also been performed. Using a prime-boost regimen, AGMs were vaccinated twice, 3 weeks apart, by i.m. injection with 100 μg HeV-sG with Alhydrogel or HeV-sG with Alhydrogel and CpG, and then challenged 3 weeks later with HeV by i.t. administration (166). All vaccinated animals were completely protected from clinical

disease, and no HeV RNA or viral antigen could be detected in swabs, blood, or tissues, and notably HeV-sG formulated in only Alhydrogel protected (166).

The efficacy and inherent safety of the HeV-sG subunit led to its development as an equine vaccine to prevent HeV infection of horses and also reduce the risk of HeV transmission to people, as a One Health concept (**Figure 1**). HeV-sG, formulated in an approved equine adjuvant (Zoetis, Inc.), was evaluated in two efficacy studies; the first tested 50 μg and 100 μg doses of the same HeV-sG used in prior animal studies to vaccinate horses, and the second used 100 μg doses of HeV-sG produced in Chinese hamster ovary cells (Zoetis, Inc.). Two vaccinations were given by i.m. administration 3 weeks apart. All horses in these efficacy studies were challenged by oronasal inoculation with HeV (**Table 2**). Seven horses were challenged at 28 days and 3 horses were challenged at 194 days after the second immunization. All vaccinated horses remained clinically healthy following challenge; pre-challenge neutralization titers ranged from 1:128 to more than 4,096 in horses challenged 21 days after vaccination and only from 1:16 to 1:32 in horses challenged at 6 months. There was no gross or histologic evidence of infection in any of the vaccinated horses at study completion, and all tissues examined were negative for viral antigen, with no viral genome detected in any tissue. In 9 of 10 vaccinated horses, HeV nucleic acid was not detected in daily nasal, oral, or rectal swab samples or from blood, urine, or fecal samples collected before euthanasia, no recoverable virus was present, and no rise in antibody titer was detected in any vaccinated horse following challenge (167). The HeV-sG horse vaccine (Equivac® HeV) was launched by Zoetis, Inc., in November 2012 on a minor use permit by the regulatory authority, the Australian Pesticides and Veterinary Medicines Authority (APVMA), and is the first commercially developed and deployed vaccine against a BSL-4 agent. All vaccinated horses are microchipped, and a database is maintained. Equivac HeV received full registration by the APVMA in 2015. To date, more than 765,000 doses of Equivac HeV vaccine have been administered to more than 179,000 unique horses, and laboratory-confirmed HeV infections in horses have since occurred only in unvaccinated animals.

Studies showed HeV-sG as a NiV vaccine in the pig model (which is a non-lethal challenge model) was much less effective in comparison to results observed in the cat, ferret, NHP, and horse, and HeV-sG was only partially protective against HeV challenge and unprotective against NiV-M in the pig (168). These experiments also indicated that both humoral and cellular immune responses were required for protection of swine against NiV and HeV. Here, pigs were immunized with HeV-sG in a proprietary adjuvant (Zoetis, Inc.), and subjects were challenged with HeV or NiV via i.n. administration (**Table 2**). HeV-sG-vaccinated pigs developed neutralizing titers ranging from 1:160 to 1:320 to HeV, but only partial protection was achieved with reduced viral RNA in tissues and no recoverable virus, and there was no reduction of viral shedding in nasal washes. These HeV-sG-vaccinated pigs did not develop neutralizing antibodies to NiV-M that were considered protective (low), nor did they have measurable activation of cellular immune memory. Only a comparative group of pigs that were first orally infected (vaccinated) with NiV (and recovered) were subsequently protected against an i.n. rechallenge with NiV. This group of pigs developed protective antibody levels and cell-mediated immune memory responses (168).

Single-dose lipid nanoparticle mRNA, HeV-sG vaccine. More recently, messenger RNA (mRNA)-based vaccines have emerged as an attractive vaccine strategy because of safety, efficacy, and rapid implementation features. In a recent study, the efficacy of an mRNA vaccine approach was assessed in a NiV-M animal challenge model (169). mRNA transcripts encoding HeV-sG were complexed with lipid nanoparticles (LNPs) to generate HeV-sG mRNA LNP. Two groups of 10 hamsters were vaccinated with a single dose of HeV-sG mRNA LNP at either 10 μg or 30 μg by i.m. injection. Subjects were challenged with NiV-M by i.p. administration 30 days

post-vaccination (**Table 2**). The HeV-sG mRNA LNP was only partially protective, with 3 hamsters from the low-dose group and 7 hamsters from the high-dose group surviving challenge. Of the surviving animals, signs of clinical disease were observed in 2 low-dose group and 6 high-dose group hamsters; however, disease symptoms were gone by study termination. NiV *N* gene RNA levels in the blood and a variety of tissues in surviving hamsters were lower compared with nonsurvivors, but NiV RNA copy levels were not different compared with controls. No anti-NiV IgG or virus-neutralizing activity was detected in vaccinated animals prior to challenge; however, all post-challenge survivors were positive for anti-NiV IgG antibodies, and all survivors (in both groups) had similar neutralizing titers ranging from 1:160 to 1:640. Euthanized animals had little to undetectable neutralizing activity, highlighting the correlation of this immune response to protection. Although promising, the partial efficacy of HeV-sG mRNA LNP observed in this study suggests that further optimization of vaccination route, addition of an adjuvant, and/or a prime-boost regimen is needed.

SUMMARY AND FUTURE PERSPECTIVES

The frequency of henipavirus outbreaks and human infections is a significant global health concern. A promising passive immunization strategy has been developed using a human mAb, m102.4, shown effective in the NHP challenge model, which has also been administered numerous times to people by compassionate use protocol and has successfully completed a phase I safety trial in Australia. In addition, the Equivac HeV vaccine is available, targeting the protection of horses and also people by breaking the chain of HeV transmission to people, and is an example of a One Health approach to counter an infectious disease threat. Over the past 15 years, nearly a dozen NiV and HeV vaccine approaches have been trialed in animal challenge models, and many show promise as effective human-use vaccines. Recently, the formation of the Coalition for Epidemic Preparedness Innovations (CEPI), a global partnership between public and private organizations, was undertaken with the goals of developing vaccines against emerging infectious diseases and offering equitable access to those vaccines (170). Indeed, without the support of CEPI, the prospects of having a NiV or HeV vaccine suitable for use in people, at a deployable stage in the event of a significant outbreak, would have remained academic. Research teams can now capitalize on the large body of basic and preclinical vaccine development data on a half-dozen important emerging viral threats including NiV and, with the support of CEPI, can develop vaccine candidates for clinical use and future licensure. Several of the NiV human vaccine candidates described in this review are now supported by CEPI.

DISCLOSURE STATEMENT

C.C.B. is a US federal employee and co-inventor on US and foreign patents pertaining to soluble forms of HeV and NiV G and F glycoproteins and monoclonal antibodies against HeV and NiV whose assignee is the United States as represented by the Henry M. Jackson Foundation for the Advancement of Military Medicine (Bethesda, Maryland). The soluble forms of the HeV and NiV G glycoproteins are licensed to Zoetis, Inc., and Aurobindo Pharma USA Inc. M.A. declares no competing interests.

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Volume 7, 2020

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Errata

An online log of corrections to *Annual Review of Virology* articles may be found at http://www.annualreviews.org/errata/virology

From: Broder, Christopher (b) (6)
To: (b) (6)
Subject: IAVI ReVAMPP
Date: Tuesday, May 2, 2023 1:28:20 PM
Attachments: MAmaya-Published-Viruses-15-01077.pdf
AA-Amaya-Published-annurev-virology-021920-113833.pdf

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hi (b) (6)

great to meet you today.

attached is Cedar chimera paper.
also a recent Nipah/Hendra review

(b) (5)

v/r
(b) (6)

(b) (5)

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From: (b) (6)
To: (b) (6)
Cc: (b) (6) ; CHRISTOPHER BRODER; (b) (6)
Subject: RE: [EXTERNAL] Fwd: Revised Format for the Annual Progress Report for 1U19AI171413-01
Date: Monday, May 1, 2023 1:24:43 PM
Attachments: Other Support (b) (6) Dec 2022 v5(b) (6).pdf
All Personnel Report 67058.pdf
Other Support (b) (6) v5_signed.pdf
image001.png

Good Afternoon,
Please find the completed all personnel report and signed other support documents for Drs. (b) (6) and (b) (6). Please let us know if any additional information is needed.
Thank you,
(b) (6)

[redacted]

Henry M. Jackson Foundation for the
Advancement of Military Medicine
6720A Rockledge Drive, Suite 100
Bethesda, MD 20817

---------- Forwarded message ---------
From: (b) (6) [redacted]
Date: Wed, Apr 12, 2023 at 1:41 PM
Subject: RE: Fw: URGENT: Revised Format for the Annual Progress Report for 1U19AI171413-01
To: (b) (6) [redacted] CHRISTOPHER BRODER
(b) (6) [redacted]
Cc: (b) (6) [redacted]

Thanks – I talked to (b) (6) at Novartis, who is compiling input for the Project 3 report. I think that she is on track getting the input she needs for Form Page 5 by next week and we'll handle Form pages 1, and 6. We will need updated Other Support if either (b) (6) [redacted] have changes to report. And I'm checking to make sure, but I think we'll have to ask you to fill out Form page 7 – the All Personnel Report. For those two pieces, we've set the week of May 1st as a target date.
(b) (6)

From: (b) (6)
Sent: Wednesday, April 12, 2023 11:45 AM
To: (b) (6) CHRISTOPHER BRODER (b) (6)
Cc: (b) (6)
Subject: Re: Fw: URGENT: Revised Format for the Annual Progress Report for 1U19AI171413-01

Bringing (b) (6) into the loop on this.

From: (b) (6)
Sent: Wednesday, April 12, 2023 11:19 AM
To: CHRISTOPHER BRODER (b) (6)
Cc: (b) (6)
Subject: Re: Fw: URGENT: Revised Format for the Annual Progress Report for 1U19AI171413-01

Hello all,

I don't see a due date for us to have these materials to you. I inquired previously but want to make sure we are on time.

Thanks much .... (b) (6)

On Thu, Mar 2, 2023 at 10:30 AM Broder, Christopher (b) (6) wrote:

> fysa
>
> ---------- Forwarded message ---------
> From: (b) (6)
> Date: Thu, Mar 2, 2023 at 10:26 AM
> Subject: Fw: URGENT: Revised Format for the Annual Progress Report for 1U19AI171413-01
> To: CHRISTOPHER BRODER (b) (6)
>
> FYI
>
> ---
>
> **From:** (b) (6)

Sent: Thursday, March 2, 2023 9:24 AM
To: (b) (6)
Cc: (b) (6)
Subject: FW: URGENT: Revised Format for the Annual Progress Report for 1U19AI171413-01

Dear Dr. (b) (6),

Thank you for the new information about the AViDD progress report. We will prepare accordingly.

Best, (b) (6)

From: (b) (6)
Sent: Thursday, March 2, 2023 9:14 AM
To: (b) (6) >
Cc: (b) (6)
Subject: URGENT: Revised Format for the Annual Progress Report for 1U19AI171413-01
Importance: High

Dear Dr. (b) (6) and Authorized Institutional Official,

NIH has identified issues with the submission of Multi-Year Funded Research Performance Progress Reports (RPPRs) using the eRA Commons, consequently **all AViDD awardees must now use the PHS 2590 format** instead of the eRA Commons to prepare and submit the Annual Progress Report for Year 1. **Please disregard the instructions previously transmitted via the email sent in November 2022**.

Please see the attached instructions for the PHS 2590 format and the Revised AViDD Progress Report Guidelines. The additional information being requested in the Revised AViDD Progress Report Guidelines is the same as the previous version, however, for the PHS 2590 format you will now provide this information using Form page 5 instead of sections noted in the previous version.

The PHS 2590 forms can be downloaded from:
https://grants.nih.gov/grants/funding/2590/2590.htm

The Annual Progress Report will contain separate reports for each component of your AViDD. You will create a separate PHS 2590 report for the AViDD Overall progress, each Research Project, and each Core, and these will be combined to generate the Annual Progress Report.

The separate reports for each component will contain:

Form Page 1: Face Page – leave Items 8a and 8b blank

Form Page 1-continued: Additional form if Multiple PIs are involved

Project/Performance Site Format Page - use only if additional space is needed

Biographical Sketch: Complete a Biographical Sketch for all new senior/key personnel since the previous submission. Use form available from
https://grants.nih.gov/grants/forms/biosketch.htm

Other Support: Include other active support for all senior/key personnel whose support has changed during the past year and indicate what the change has occurred. Use form available from

https://grants.nih.gov/grants/forms/othersupport.htm

Form Page 5: Progress Report Summary - this form will be used to provide both the additional information required for AViDD awards as well as the standard information required for a Progress Report. Please refer to the Revised AViDD Progress Report Guidelines for the additional information required to be included in the Progress Summary for the AViDD Overall, Research Project and Core reports.

Use the Continuation page after Form page 5 is full for this section and any other sections that require additional pages after the initial form.

Form Page 6: Checklist

Form Page 7: All Personnel Report

If Human Subjects Research is being conducted - include the PHS Inclusion Enrollment Report

Please refer to the attached Revised AViDD Progress Report Guidelines and the phs2590 General Instructions found on pages 5 through 17 for guidance about the information required for each section.

Because your AViDD award is a Multi-Year Funded award you are not required to submit Detailed Budget for Next Budget Period or Budget Justification pages as part of the Annual Progress Report. In addition, carry forward requests are not required during the remaining time of the Multi-Year Funded project period. However, if the situation arises, please note, prior approval is still required for compliance with the significant rebudgeting policy as stated in the NIH Grants Policy Statement https://grants.nih.gov/policy/nihgps/index.htm:

Significant rebudgeting, whether or not the particular expenditure(s) require prior approval: Significant rebudgeting occurs when expenditures in a single direct cost budget category deviate (increase or decrease) from the categorical commitment level established for the budget period by 25 percent or more of the total direct costs awarded. For example, if the award budget for total direct costs is $200,000, any rebudgeting that would result in an increase or decrease of more than $50,000 in a budget category is considered significant rebudgeting

To submit the report, your Authorized Institutional Official will email the entire Annual Progress Report to Regina Kitsoulis, who is the NIAID Grants Management Official for this award, and me.

These reports are due by the project anniversary date (05/16/2023) for your award and can be submitted anytime between now and then. As noted above, please read through the Revised AViDD Report Guidelines and the PHS 2590 instructions. If questions remain, then please have your business official contact (b) (6) and me.

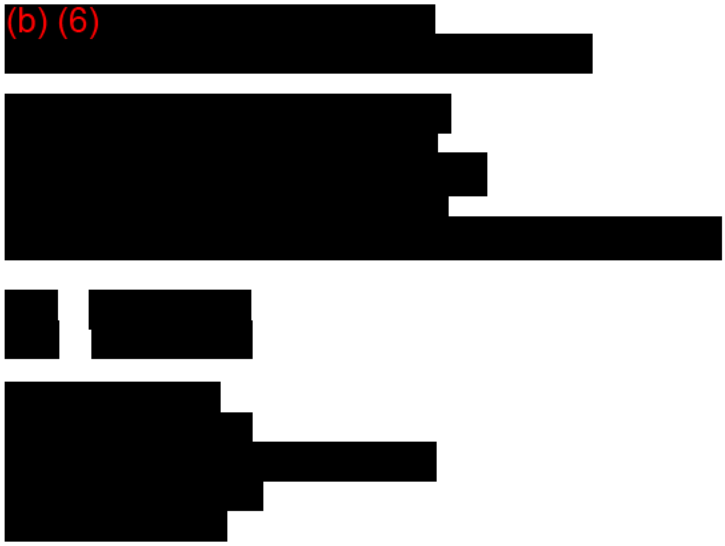
We apologize for the inconvenience.

Sincerely yours,

(b) (6)

[redacted]

(b) (6)



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(b) (6) [redacted]

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(b) (6) [redacted]

Department of Health and Human Services

Part 1. Overview Information

Participating Organization(s)

National Institutes of Health (NIH (http://www.nih.gov))

Components of Participating Organizations

National Institute of Allergy and Infectious Diseases (NIAID (https://www.niaid.nih.gov/))

Funding Opportunity Title

Research and Development of Vaccines and Monoclonal Antibodies for Pandemic Preparedness (ReVAMPP) Centers for Bunyavirales, Paramyxoviridae and Picornaviridae (U19 Clinical Trial Not Allowed)

Activity Code

U19 (//grants.nih.gov/grants/funding/ac_search_results.htm?text_curr=u19&Search.x=0&Search.y=0&sort=ac&Search_Type=Activity&text_prev=) Research Program – Cooperative Agreements

Announcement Type

New

Related Notices

NOT-OD-22-189 (https://grants.nih.gov/grants/guide/notice-files/NOT-OD-22-189.html) - Implementation Details for the NIH Data Management and Sharing Policy

NOT-OD-22-195 (https://grants.nih.gov/grants/guide/notice-files/NOT-OD-22-195.html) - New NIH "FORMS-H" Grant Application Forms and Instructions Coming for Due Dates on or after January 25, 2023

NOT-OD-22-198 (https://grants.nih.gov/grants/guide/notice-files/not-od-22-198.html) - Implementation Changes for Genomic Data Sharing Plans Included with Applications Due on or after January 25, 2023

NOT-OD-23-012 (https://grants.nih.gov/grants/guide/notice-files/NOT-OD-23-012.html) - Reminder: FORMS-H Grant Application Forms & Instructions Must be Used for Due Dates On or After January 25, 2023 - New Grant Application Instructions Now Available

Funding Opportunity Announcement (FOA) Number

RFA-AI-23-020

Companion Funding Opportunity

RFA-AI-23-019 (https://grants.nih.gov/grants/guide/rfa-files/RFA-AI-23-019.html), U19 (https://grants.nih.gov/grants/funding/ac_search_results.htm?text_curr=U19&&Search.x=0&&Search.y=0&&Search_Type=Activity) Research Program (Cooperative Agreement)
RFA-AI-23-021 (https://grants.nih.gov/grants/guide/rfa-files/RFA-AI-23-021.html), UG3 (https://grants.nih.gov/grants/funding/ac_search_results.htm?text_curr=UG3&&Search.x=0&&Search.y=0&&Search_Type=Activity)/ UH3 (https://grants.nih.gov/grants/funding/ac_search_results.htm?text_curr=UH3&&Search.x=0&&Search.y=0&&Search_Type=Activity) Phase 1 Exploratory/Developmental Cooperative Agreement/Exploratory/Developmental Cooperative Agreement Phase II

Number of Applications

See Section III. 3. Additional Information on Eligibility.

Assistance Listing Number(s)

93.855

Funding Opportunity Purpose

This Funding Opportunity Announcement (FOA) solicits applications to participate in the Research and Development of Vaccines and Monoclonal Antibodies for Pandemic Preparedness (ReVAMPP) Network. The purpose of this FOA is to establish comprehensive, cooperative basic and translational research Centers to carry out in-depth research on prototype members of select virus families that have the potential to emerge as pandemic pathogens. The goal of these Centers will be to develop vaccine and monoclonal antibody strategies for prototype pathogen(s) that can be applied to closely related family members based on shared functional and structural properties. This FOA solicits for centers proposing research on virus families from Bunyavirales, Paramyxoviridae and Picornaviridae to be part of the ReVAMPP Network.

Key Dates

Posted Date

March 16, 2023

Open Date (Earliest Submission Date)

May 08, 2023

Letter of Intent Due Date(s)

30 days prior to the application due date

Application Due Dates			Review and Award Cycles		
New	Renewal / Resubmission / Revision (as allowed)	AIDS	Scientific Merit Review	Advisory Council Review	Earliest Start Date
June 08, 2023	Not Applicable	Not Applicable	November 2023	January 2024	March 2024

All applications are due by 5:00 PM local time of applicant organization.

Applicants are encouraged to apply early to allow adequate time to make any corrections to errors found in the application during the submission process by the due date.

No late applications will be accepted for this Funding Opportunity Announcement.

Expiration Date

June 09, 2023

Due Dates for E.O. 12372

Not Applicable

Required Application Instructions

It is critical that applicants follow the Multi-Project (M) Instructions in the SF424 (R&R) Application Guide (https://grants.nih.gov/grants/guide/url_redirect.htm?id=82400), except where instructed to do otherwise (in this FOA or in a Notice from the *NIH Guide for Grants and Contracts* (//grants.nih.gov/grants/guide/url_redirect.htm?id=11164)). Conformance to all requirements (both in the Application Guide and the FOA) is required and strictly enforced. Applicants must read and follow all application instructions in the Application Guide as well as any program-specific instructions noted in Section IV. When the program-specific instructions deviate from those in the Application Guide, follow the program-specific instructions. **Applications that do not comply with these instructions may be delayed or not accepted for review.**

There are several options available to submit your application through Grants.gov to NIH and Department of Health and Human Services partners. You **must** use one of these submission options to access the application forms for this opportunity.

1. Use the NIH ASSIST system to prepare, submit and track your application online.

   Apply Online Using ASSIST

2. Use an institutional system-to-system (S2S) solution to prepare and submit your application to Grants.gov and eRA Commons (https://public.era.nih.gov/commons/) to track your application. Check with your institutional officials regarding availability.

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- Section IV. Application and Submission Information
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Part 2. Full Text of Announcement

Section I. Funding Opportunity Description

The National Institute of Allergy and Infectious Diseases (NIAID) supports complementary research programs to understand, control and prevent viral diseases and related pandemics. As part of pandemic preparedness planning, this Funding Opportunity Announcement (FOA) solicits applications to establish comprehensive, cooperative basic and translational research centers to 1) advance scientific knowledge needed to develop vaccines and monoclonal antibodies (mAbs) for prototype viral pathogens within virus families that have pandemic potential; and 2) leverage this information to develop and evaluate generalizable approaches for vaccines and mAbs for the prototype pathogens and other related family members based on shared functional and structural properties. Centers proposing research on select virus families from Bunyavirales, Paramyxoviridae and Picornaviridae are included in this FOA.

The ReVAMPP Network is comprised of ReVAMPP Centers from both this RFA (Bunyavirales, Paramyxoviridae and Picornaviridae) and its companion FOAs soliciting for ReVAMPP Centers focusing on Flaviviridae and Togaviridae (see RFA-AI-23-019 (https://grants.nih.gov/grants/guide/rfa-files/RFA-AI-23-019.html)) and the ReVAMPP Coordinating and Data Sharing Center (CDSC) (see RFA-AI-23-021 (https://grants.nih.gov/grants/guide/rfa-files/RFA-AI-23-021.html)). ReVAMPP Centers will conduct independent research project(s) and are expected to share information and collaborate within the Network under the direction of the ReVAMPP CDSC which is responsible for establishing and maintaining a collaborative ReVAMPP Network platform for data sharing and overall collaboration among the ReVAMPP Centers.

In the event of an outbreak, the ReVAMPP Centers will be poised to leverage the expertise and resources within the network to assist in a coordinated research response. This new NIAID Network will align with the goals of the American Pandemic Preparedness Plan: Transforming Our Capabilities (AP3) (https://www.whitehouse.gov/wp-content/uploads/2021/09/American-Pandemic-Preparedness-Transforming-Our-Capabilities-Final-For-Web.pdf), which was announced in September 2021, and recognizes the need for a trans-government investment and response to combat future pandemics and NIAID's Pandemic Preparedness Plan (https://www.niaid.nih.gov/sites/default/files/pandemic-preparedness-plan.pdf).

Background

The emergence and re-emergence of infectious diseases continues to threaten the health of Americans and people worldwide. Over the past two decades, the public health community has responded to emerging infectious diseases including those caused by Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV-1), the 2009 H1N1 influenza virus, Middle East Respiratory Syndrome Coronavirus (MERS-CoV), Ebola virus, Zika virus, and most recently, SARS-CoV-2. The global pandemic caused by SARS-CoV-2 further underscores the continual threat of newly emerging and re-emerging pathogens and the critical value of basic and translational research for pandemic preparedness. The unprecedented rapid development of vaccines and mAbs for SARS-CoV-2 was enabled by decades of foundational research on related coronaviruses which allowed scientists to quickly and effectively respond once SARS-CoV-2 emerged. In recent years, considerable resources have been invested in vaccine and mAb development for coronavirus and influenza viruses to prepare for the next pandemic from these families. However, viruses from other families also propose substantial risk of causing a pandemic. Thus, continuing to build a robust basic research portfolio and advancing translational science for other viral families with pandemic potential is essential for biomedical countermeasure preparedness. In addition to known threats, effective preparedness must also account for unexpected emerging disease threats. To mitigate risks associated with these yet-unknown pathogens, NIAID's intent for the ReVAMPP Network is to promote focused research needed to develop vaccines and mAbs for prototype-pathogens from viral families known to infect humans (Graham BS and Corbett KS J Clin Invest. 2020; 130(7):3348-3349 (https://www.jci.org/articles/view/139601), Cassetti MC, et al., JID. D022; jiac296). (https://academic.oup.com/jid/advance-article/doi/10.1093/infdis/jiac296/6649664?login=true) As defined in this FOA, a prototype pathogen is a representative virus from which research on the virology, pathology and immunology of the prototype will generate generalizable knowledge, and in turn vaccine and mAb strategies, which can be applied to other members of the viral family. As the prototype pathogen approach proposes, viruses are organized into families based upon shared functional and structural similarities and thus candidate vaccine strategies developed against a prototype pathogen may similarly work against other members in the same family. Through targeted basic and applied research on these prototype pathogens, a solid foundation of knowledge will enable a rapid response when a previously unknown (or known but understudied) pathogen emerges or spreads from any of the known high-risk viral families. This anticipatory approach will increase the knowledge base needed for preparedness and enable rapid development and translation of candidate vaccines and mAbs into clinical trials and large-scale production.

Research Objectives and Scope

The objective of this FOA is to establish multidisciplinary research Centers to be part of the highly collaborative ReVAMPP Network focused on in-depth basic and translational research on prototype members of certain virus families that have the potential to emerge as pandemic pathogens, namely Paramyxoviridae, Picornaviridae and Bunyavirales including Arenaviridae, Hantaviridae, Nairoviridae, Phenuiviridae, and Peribunyaviridae. The major goal of these Centers will be to develop vaccine strategies for prototype pathogens that can be applied to other closely related family members based on shared functional and structural properties. Additionally, these Centers may perform basic research to expand foundational knowledge of virology, pathology, and immunology, or perform early development activities for mAbs for prototype viruses. Towards these goals, each Center will encompass a multi-project multidisciplinary research program that employs innovative virology, structural biology, and immunology to identify strategies for vaccine design. Each Center will collaborate across the ReVAMPP Network through the sharing of data, reagents, protocols, and animal models to facilitate advancement toward pandemic preparedness for all virus families.

Priority Viral Families for Pandemic Preparedness

A prototype pathogen is a representative virus from which research on the virology, pathology and immunology of the prototype will generate generalizable knowledge, and in turn vaccine and mAb strategies, which can be applied to other members of the virus family. For this FOA, each ReVAMPP Center will support a multi-project research program directed towards enabling basic and translational research and development of vaccines, and optionally early development of mAbs, against prototype viruses from one or more of the following selected virus families of pandemic potential, and Centers are encouraged to work on more than one virus family (listed alphabetically, all of equivalent priority):

- Bunyavirales
  - Arenaviridae (e.g., Lassa virus, Junin virus)
  - Hantaviridae (e.g., Andes virus, Sin Nombre virus, Hantaan orthohantavirus virus)
  - Nairoviridae (e.g., Crimean-Congo Hemorrhagic Fever virus (CCHF), Hazara virus)
  - Phenuiviridae (e.g., Rift Valley Fever virus, Severe Fever with thrombocytopenia syndrome virus, Punta Toro virus)
  - Peribunyaviridae (e.g., LaCrosse virus (LAC), Cache Valley virus)
- Paramyxoviridae (e.g., Menangle virus, HPIV1, HPIV3, Canine Distemper virus, Cedar virus)
- Picornaviridae (e.g., Enterovirus A71, Enterovirus D68, Echovirus B29, Rhinovirus C)

Centers are encouraged to include research projects focused on multiple virus families and may include a single prototype or multiple prototypes from a family. The investigators will determine which virus should serve as a prototype for a given family, but prototype selection must be justified. Considerations may include, but are not limited to, how well the prototype reflects properties shared by other members of the family, whether a single prototype is sufficient or whether multiple prototypes are needed to address differences amongst family members, the ease of working with the prototype virus, and whether vaccines against the prototype would have potential value for current public health needs.

These selected priority virus families of pandemic potential were identified by NIAID based on the ability to infect humans, the potential to cause a pandemic, and the current resources invested. The Coronaviridae and Orthomyxovirdae virus families were not included in this list as vaccine development and preparedness research for these families is supported through other mechanisms such as NOT-AI-21-002: Emergency Awards: Notice of Special Interest (NOSI) on Pan-Coronavirus Vaccine Development Program Projects (https://grants.nih.gov/grants/guide/notice-files/NOT-AI-21-002.html) and NIAID Centers of Excellence for Influenza Research and Surveillance (https://www.niaidceirs.org/), among others. In November 2021, NIAID convened a workshop titled "NIAID Workshop on Pandemic Preparedness: The Prototype Pathogen Approach to Accelerate Medical Countermeasures - Vaccines and Monoclonal Antibodies" where experts summarized current knowledge of the basic and translational research landscape, described research and intervention gaps, and proposed suitable prototype pathogens for further study and medical countermeasure development (Graham BS and Corbett KS J Clin Invest. 2020; 130(7):3348-3349 (https://www.jci.org/articles/view/139601), Cassetti MC, et al. JID. 2022;jiac296. (https://academic.oup.com/jid/advance-article/doi/10.1093/infdis/jiac296/6649664)) This workshop highlighted the critical need to continue to expand basic research efforts and advance translational science for nine of

the selected virus families. NIAID's intent for the ReVAMPP Network is to promote focused and coordinated research needed for the development of vaccines, and optionally early development of mAbs, for prototype-pathogens from virus families known to infect humans.

Research Areas

This FOA will support basic research such as virology studies to better understand cell tropism and receptor/entry requirements and to determine replication mechanisms, pathogenesis, and capacity for antigenic diversity. It will also support structural biology research to define the atomic-level details of surface proteins likely to be antigenic/immunologic targets. It will support research to assess the immune response in humans to natural infections and existing vaccines as well as determine correlates of immune protection, establish robust animal models, and develop reagents and new immunological assays.

This FOA will also support early and Investigational New Drug Application (IND)-enabling translational research for vaccines. Translational activities may include antigen/immunogen design and evaluation, screening technology platforms or adjuvants for immunogenicity and efficacy in animal models, development of assays and reagents, identification of correlates or surrogate markers of protection, lead optimization, stability, and manufacturability testing, and/or early process development. Once lead vaccine candidates/strategies have been identified, the same generalizable approach will be applied to other viruses within in the same family to validate the overall strategy for the virus family. Given the need to respond rapidly to emerging threats, the vaccine strategies should incorporate technologies that are amendable to antigen interchange and rapid manufacturing such as "plug and play" platforms.

The development of prototype vaccine strategies and structure-function characterization of the immune response to infection and vaccination is likely to result in the identification of mAbs with therapeutic potential. Thus, this FOA will support functional characterization of candidate mAbs and early translational activities for mAb development for prototype viruses. These activities may include discovery, *in vitro* characterization including epitope identification, neutralization potency, effector function analysis and structural studies, mAb optimization, determination of mechanism of action, *in vivo* evaluation including efficacy, dose titration, and route of administration studies in animal models, and candidate down-selection. If early mAb development is included, following the identification of lead mAb candidates for the prototype virus, it should be determined if antibodies with similar epitopes and/or properties are effective against other viruses within the same family.

Milestones

This FOA will utilize a bi-phasic, milestone-driven cooperative agreement award mechanism with Phase I consisting of the first 3 years, and Phase II consisting of years 4 and 5. Although applicants will apply for five years of funding, near the end of year 3 grantees will submit a transition package which will be evaluated by NIAID program staff for progress of research towards development of a generalizable vaccine strategy for the proposed viral family(ies), and if applicable early mAb development, and contribution to the ReVAMPP Network through data sharing and collaboration. The administrative review for funding of years 4 and 5 will be based on successful achievement of milestones included in the application and negotiated with the recipient prior to award, overall feasibility of program advancement,compliance with the ReVAMPP Network data sharing and CDSC requests, evidence of collaboration with other ReVAMPP Centers, programmatic priorities, and the availability of funding.

Industry Partnership

Each ReVAMPP Center is expected to have an established, or have plans to establish when appropriate, collaboration with an industry partner which will provide access to vaccine expertise in manufacturing, clinical development, and regulatory pathways. For Centers proposing IND-enabling translational research, an industry partnership is required. For the purpose of this FOA, "industry" is defined as a large or small, domestic, or foreign, pharmaceutical, biotechnology, or bioengineering company, or a related non-profit entity. The establishment of these public-private-partnerships is expected to extend the reach of the Center's comprehensive translational efforts, helping to ensure a focused, critical path through early-phase clinical trials for the most promising candidates. Centers will be encouraged to create partnerships/in-licensing opportunities and intellectual property strategies in compliance with NIH Intellectual Property Policy (https://grants.nih.gov/policy/intell-property.htm) to support advancing promising vaccine, and if applicable mAb, candidates into the clinic and to allow for hand-off to industry for advanced development. Centers are encouraged to develop and use intellectual property strategies that promote accessibility, similar to efforts from the World Health Organization (WHO), NIAID, and Bill & Melinda Gates Foundation (BMGF) to make COVID-19 vaccine and mAbs technologies accessible for the developing world (COVID-19 technology access pool (https://www.who.int/initiatives/covid-19-technology-access-pool)).

NIAID Resources

Each ReVAMPP Center is expected to provide lead candidates for comparative studies using NIAID's preclinical services or other gap filling mechanisms. It is anticipated that after award NIAID's comprehensive suite of preclinical services (Resources for Researchers | NIH: National Institute of Allergy and Infectious Diseases (https://www.niaid.nih.gov/research/resources)), NIAID's Division of Allergy, Immunology, and Transplantation (DAIT) programs (Adjuvant Discovery Program (https://www.niaid.nih.gov/research/adjuvant-discovery-program), B cell and T cell Epitope Discovery program, etc.) and repositories could be leveraged as needed to support ReVAMPP objectives including reagent storage/development, assay and animal model harmonization and evaluation, and development of lead vaccine and mAb candidates developed under this Network. NIAID program officials will connect ReVAMPP investigators to these services.

ReVAMPP Network interactions

Collaboration and data sharing among ReVAMPP Centers and with external partners is key to successful achievement of the ReVAMPP network goals. Therefore, each ReVAMPP Center must adopt FAIR data principles (https://www.go-fair.org/fair-principles/) as per the NIH Data Management Sharing Plan (DMSP (https://sharing.nih.gov/data-management-and-sharing-policy)) and manage and rapidly share data within the Network of ReVAMPP Centers under the direction of the ReVAMPP CDSC. This data will be shared confidentially within the Network to harmonize reagents, assays, and animal models and exchange knowledge on structure/function-based vaccine solutions and antigen/immunogen designs as well as assess the utility of vaccine technology platforms for virus families. To assist in the administration and management of information exchange, a separate ReVAMPP CDSC will be directing these efforts for the Network. The ReVAMPP CDSC will facilitate collaboration within and outside the Network, and each ReVAMPP Center will be expected to comply in accordance with network-wide timelines. As such, the CDSC will develop network-wide data sharing platforms and templates for all types of data generated by the ReVAMPP Centers. This may include information/data related to reagents, tools, assays, models, vaccine technology platforms, immune epitope design and/or correlates of protection. The CDSC will also develop, in conjunction with the Centers, a network wide ReVAMPP governance structure, provide guidance as to engagement with stakeholders within and outside the research centers, and collate information and facilitate exchange with other NIAID Programs, U.S. Government partners and other key stakeholders, including the WHO, BMGF, and Coalition for Epidemic Preparedness Innovations (CEPI), among others as appropriate.

ReVAMPP Center Structure

Each Center in the ReVAMPP Network will be organized around a multidisciplinary research program with interrelated projects focused on the prototype member(s) of virus families that have the potential to emerge as pandemic pathogens to inform a strategy for development of vaccines for the prototype pathogen and other closely related family members based on shared functional and structural properties, with the objective of translating research results to product development. Each Center is expected to include the following components:

Administrative Core

An Administrative Core will manage, coordinate, and supervise all Center activities under the direction of the Program Director(s)/Principal Investigator(s) (PD(s)/PI(s). The Administrative Core will also ensure seamless communication across the projects through regular meetings of Center participants and the ReVAMPP network as directed by the ReVAMPP CDSC. In addition, the Administrative Core will coordinate detailed communication of Center efforts and progress with NIAID program staff, including organizing annual ReVAMPP Center progress meetings with NIAID, and participating and assisting with ReVAMPP Network meetings as necessary virtually and/or at NIAID. The Administrative Core will also be responsible for leading coordination and collaboration efforts with the CDSC and other Centers within the Network and ensuring the Center program complies with requests from the CDSC and NIAID program staff.

Scientific Advisory Board

Each Center will include a Scientific Advisory Board (SAB) that will act as an independent, external advisory body for the PD(s)/PI(s) but will not be involved in the day-to-day activities of the Center. The SAB will facilitate Go/No-Go decision making and recommend new research directions as appropriate. The SAB will participate in annual ReVAMPP Center progress meetings at NIAID to review Center activities and evaluate progress, adherence to milestones and timelines, and the continued relevance of each Research Project, including those within industry partnerships, to the overall Center objective(s). If requested by the PD(s)/PI(s) and NIAID Project Scientist, the SAB will provide a summary written evaluation of the group's activities and recommendations following the annual ReVAMPP Center progress meeting. The SAB will include at least 5 non-conflicted external advisors. Centers must have at least 2 of the external advisors who have demonstrated and relevant industry-level expertise. SAB membership will be established in consultation with NIAID program staff. *Potential external SAB members MUST NOT be named in the application or contacted prior to completion of review activities.*

Data Management Core

A Data Management Core will be responsible for housing data generated by the Center and managing the transfer of data within the Center and to the ReVAMPP Network as directed by the ReVAMPP CDSC, including the upload of data to the ReVAMPP Network data sharing platforms. The Data Management Core will also be responsible for collating and collecting information from the Center as requested by the ReVAMPP CDSC or NIAID program staff in accordance with network-wide timelines, and ensuring the Center complies with network data sharing policies.

Scientific Cores

A Center may include up to three Scientific Cores to support resources and/or facilities that are essential for the activities of two or more Research Projects, but inclusion of Scientific Cores is not required. Scientific Cores are intended to only serve the needs of Center project researchers and they may not conduct research independent of the Research Projects. In lieu of a Scientific Core, use of existing institutional core facilities may be included in specific Research Projects.

Research Projects

Each Center must include at least 2 and no more than 5 interdependent Research Projects focused on prototype members of the priority virus families of pandemic potential listed above. At least one Research Project must focus on development of vaccines, and other Research Projects may focus on additional vaccine development, early mAb development or foundational research in virology, immunology, pathology, and structural biology necessary for such development. Each Research Project must clearly and directly contribute to the Center's approach and objective(s). The Center PD(s)/PI(s) will monitor all Research Projects and actively promote efforts that foster integration, collaboration, and synergy across the program. Research Project Leaders may be affiliated with either an academic organization or industry.

Research Projects are expected to incorporate state-of-the-art technology and approaches and may include consortium arrangements for required activities. Applicants are encouraged to carefully consider the scope and range of research proposed and develop a Center that is coherent overall and consistent with available resources and personnel.

Example ReVAMPP Centers:

Center programs and objectives may range from research and development of single or multiple prototype vaccines targeting one or more of the priority virus families, and activities may range from early basic research aimed at gaining the foundational knowledge needed to design a generalizable vaccine strategy, to mAb discovery and characterization to late-stage preclinical vaccine development with industry participation. Examples of hypothetical ReVAMPP Centers follow:

Example 1:

Center for Countermeasure Discovery for Bunyaviruses

Administrative Core

Data Management Core

Scientific Core: Virology, Reagents, and Assays

Scientific Core: Structural Biology of Viral Proteins, Vaccines, and Monoclonal Antibodies

Research Project 1: Viral receptor discovery and characterization for Nairoviruses and Peribunyaviruses

Research Project 2: Characterization of the human immune response and antibody discovery

Research Project 3: Antigen design and immunogenicity evaluation of Nairovirus vaccines

Research Project 4: Animal model development and determination of CCHF and LAC pathogenesis

Research Project 5: Antigen design and immunogenicity evaluation of Peribunyavirus vaccines

Example 2:

Paramyxovirus and Arenavirus Vaccine and Monoclonal Antibody Development Center

Administrative Core

Data Management Core

Scientific Core: Animal model development and candidate vaccine and monoclonal antibody evaluation

Research Project 1: Antigen design and immunogenicity testing of Paramyxovirus vaccine candidates

Research Project 2: Antigen design and immunogenicity testing of Arenavirus vaccine candidates

Research Project 3: Determination of correlates of protection for different vaccine platforms

Research Project 4: Preclinical evaluation of monoclonal antibodies targeting paramyxoviruses and arenaviruses

Applications including the following types of studies will be considered non-responsive and will not be reviewed:

- Clinical trials: Clinical research may be supported but not clinical trials, as defined by the NIH (https://grants.nih.gov/policy/clinical-trials/definition.htm (https://grants.nih.gov/policy/clinical-trials/definition.htm)).
- Centers proposing only monoclonal antibody discovery and development without vaccine development.
- Projects proposing toxicology studies or GMP manufacturing for vaccines.
- Projects proposing later-stage development of monoclonal antibodies including process development, tissue cross-reactivity studies, toxicology studies, or GMP manufacturing.
- Centers proposing research on Coronaviridae, Orthomyxoviridae or other virus families not listed under *Priority Viral Families for Pandemic Preparedness*.
- Centers proposing research on Flaviviridae or Togaviridae families which are covered in the companion RFA-AI-22-019 (https://grants.nih.gov/grants/guide/rfa-files/RFA-AI-22-019.html)

- Applications that do not include a clear section on Milestones with Go/No-Go criteria for the Overall Program and each individual project.

This FOA supports research on virus families from Bunyavirales, Paramyxoviridae and Picornaviridae. For ReVAMPP Center programs proposing research on virus families from Flaviviridae and Togaviridae see the companion FOA, RFA-AI-23-019 (https://grants.nih.gov/grants/guide/rfa-files/RFA-AI-23-019.html). The ReVAMPP Coordinating and Data Sharing Center (CDSC) will oversee data coordination and sharing for the ReVAMPP Centers in the ReVAMPP Network (companion FOA, RFA-AI-23-021 (https://grants.nih.gov/grants/guide/rfa-files/RFA-AI-23-021.html)).

For additional information about the Research and Development of Vaccines and Monoclonal Antibodies for Pandemic Preparedness (ReVAMPP) Centers), see the "Frequently Asked Questions (FAQ)" link here: https://www.niaid.nih.gov/grants-contracts/questions-and-answers-revampp-funding-opportunities (https://www.niaid.nih.gov/grants-contracts/questions-and-answers-revampp-funding-opportunities).

Webinar Announcement

NIAID plans to hold a pre-application informational webinar for this FOA. Details about webinar registration will be available at this same FAQ link shortly after FOA publication. Participation in the webinar is not required to submit an application in response to this FOA.
See Section VIII. Other Information for award authorities and regulations.

Section II. Award Information

Funding Instrument

Cooperative Agreement: A support mechanism used when there will be substantial Federal scientific or programmatic involvement. Substantial involvement means that, after award, NIH scientific or program staff will assist, guide, coordinate, or participate in project activities. See Section VI.2 for additional information about the substantial involvement for this FOA.

Application Types Allowed

New

The OER Glossary (//grants.nih.gov/grants/guide/url_redirect.htm?id=11116) and the SF424 (R&R) Application Guide provide details on these application types. Only those application types listed here are allowed for this FOA.

Clinical Trial?

Not Allowed: Only accepting applications that do not propose clinical trials.

Need help determining whether you are doing a clinical trial? (https://grants.nih.gov/grants/guide/url_redirect.htm?id=82370)

Funds Available and Anticipated Number of Awards

NIAID intends to commit ~$70-85M in FY2024 to fund 5-6 awards. Funding in subsequent years is subject to the availability of funds.

Award Budget

Application budgets are not expected to exceed $10M direct costs/year and need to reflect the actual needs of the proposed project.

Award Project Period

The scope of the proposed project should determine the project period. The maximum period is 5 years.

NIH grants policies as described in the NIH Grants Policy Statement (//grants.nih.gov/grants/guide/url_redirect.htm?id=11120) will apply to the applications submitted and awards made from this FOA.

Section III. Eligibility Information

1. Eligible Applicants

Eligible Organizations

Higher Education Institutions

- Public/State Controlled Institutions of Higher Education
- Private Institutions of Higher Education

The following types of Higher Education Institutions are always encouraged to apply for NIH support as Public or Private Institutions of Higher Education:

- Hispanic-serving Institutions
- Historically Black Colleges and Universities (HBCUs)
- Tribally Controlled Colleges and Universities (TCCUs)
- Alaska Native and Native Hawaiian Serving Institutions
- Asian American Native American Pacific Islander Serving Institutions (AANAPISIs)

Nonprofits Other Than Institutions of Higher Education

- Nonprofits with 501(c)(3) IRS Status (Other than Institutions of Higher Education)
- Nonprofits without 501(c)(3) IRS Status (Other than Institutions of Higher Education)

For-Profit Organizations

- Small Businesses
- For-Profit Organizations (Other than Small Businesses)

Local Governments

- State Governments
- County Governments
- City or Township Governments
- Special District Governments
- Indian/Native American Tribal Governments (Federally Recognized)
- Indian/Native American Tribal Governments (Other than Federally Recognized)

Federal Governments

- Eligible Agencies of the Federal Government
- U.S. Territory or Possession

Foreign Institutions

Non-domestic (non-U.S.) Entities (Foreign Institutions) **are** eligible to apply.

Non-domestic (non-U.S.) components of U.S. Organizations **are** eligible to apply.

Foreign components, as defined in the *NIH Grants Policy Statement* (//grants.nih.gov/grants/guide/url_redirect.htm?id=11118), **are** allowed.

Required Registrations

Applicant organizations

Applicant organizations must complete and maintain the following registrations as described in the SF 424 (R&R) Application Guide to be eligible to apply for or receive an award. All registrations must be completed prior to the application being submitted. Registration can take 6 weeks or more, so applicants should begin the registration process as soon as possible. The NIH Policy on Late Submission of Grant Applications (//grants.nih.gov/grants/guide/notice-files/NOT-OD-15-039.html) states that failure to complete registrations in advance of a due date is not a valid reason for a late submission.

- System for Award Management (SAM) (https://grants.nih.gov/grants/guide/url_redirect.htm?id=82390) – Applicants must complete and maintain an active registration, **which requires renewal at least annually**. The renewal process may require as much time as the initial registration. SAM registration includes the assignment of a Commercial and Government Entity (CAGE) Code for domestic organizations which have not already been assigned a CAGE Code.
  - NATO Commercial and Government Entity (NCAGE) Code (//grants.nih.gov/grants/guide/url_redirect.htm?id=11176) – Foreign organizations must obtain an NCAGE code (in lieu of a CAGE code) in order to register in SAM.
  - Unique Entity Identifier (UEI) - A UEI is issued as part of the SAM.gov registration process. The same UEI must be used for all registrations, as well as on the grant application.
- eRA Commons (https://era.nih.gov/) - Once the unique organization identifier is established, organizations can register with eRA Commons in tandem with completing their Grants.gov registration; all registrations must be in place by time of submission. eRA Commons requires organizations to identify at least one Signing Official (SO) and at least one Program Director/Principal Investigator (PD/PI) account in order to submit an application.
- Grants.gov (//grants.nih.gov/grants/guide/url_redirect.htm?id=82300) – Applicants must have an active SAM registration in order to complete the Grants.gov registration.

Program Directors/Principal Investigators (PD(s)/PI(s))

All PD(s)/PI(s) must have an eRA Commons account. PD(s)/PI(s) should work with their organizational officials to either create a new account or to affiliate their existing account with the applicant organization in eRA Commons. If the PD/PI is also the organizational Signing Official, they must have two distinct eRA Commons accounts, one for each role. Obtaining an eRA Commons account can take up to 2 weeks.

Eligible Individuals (Program Director/Principal Investigator)

Any individual(s) with the skills, knowledge, and resources necessary to carry out the proposed research as the Program Director(s)/Principal Investigator(s) (PD(s)/PI(s)) is invited to work with his/her organization to develop an application for support. Individuals from diverse backgrounds, including underrepresented racial and ethnic groups, individuals with disabilities, and women are always encouraged to apply for NIH support. See, Reminder: Notice of NIH's Encouragement of Applications Supporting Individuals from Underrepresented Ethnic and Racial Groups as well as Individuals with Disabilities, NOT-OD-22-019 (https://grants.nih.gov/grants/guide/notice-files/NOT-OD-22-019.html).

For institutions/organizations proposing multiple PDs/PIs, visit the Multiple Program Director/Principal Investigator Policy and submission details in the Senior/Key Person Profile (Expanded) Component of the SF424 (R&R) Application Guide.

Applicants listed as a PD(s)/PI(s) for this FOA will not be eligible to be listed as PD(s)/PI(s) on applications submitted to the companion FOA (RFA-AI-23-019) (https://grants.nih.gov/grants/guide/rfa-files/RFA-AI-23-019.html) ReVAMPP Centers for Flaviviridae and Togaviridae but can participate as collaborators on subcomponents of those Centers. Applicants to this FOA will not be eligible to submit to or participate in the companion ReVAMPP CDSC FOA (RFA-AI-23-021 (https://grants.nih.gov/grants/guide/rfa-files/RFA-AI-23-021.html)) due to the centralized role of the CDSC in the Network coordination and communication.

2. Cost Sharing

This FOA does not require cost sharing as defined in the NIH Grants Policy Statement (//grants.nih.gov/grants/guide/url_redirect.htm?id=11126).

3. Additional Information on Eligibility

Number of Applications

Applicant organizations may submit more than one application, provided that each application is scientifically distinct.

The NIH will not accept duplicate or highly overlapping applications under review at the same time, per 2.3.7.4 Submission of Resubmission Application (https://grants.nih.gov/grants/policy/nihgps/HTML5/section_2/2.3.7_policies_affecting_applications.htm#Submissi). This means that the NIH will not accept:

- A new (A0) application that is submitted before issuance of the summary statement from the review of an overlapping new (A0) or resubmission (A1) application.
- A resubmission (A1) application that is submitted before issuance of the summary statement from the review of the previous new (A0) application.
- An application that has substantial overlap with another application pending appeal of initial peer review (see 2.3.9.4 Similar, Essentially Identical, or Identical Applications (https://grants.nih.gov/grants/policy/nihgps/HTML5/section_2/2.3.9_application_receipt_information_and_deadlines.htm#Similar,)).

Section IV. Application and Submission Information

1. Requesting an Application Package

The application forms package specific to this opportunity must be accessed through ASSIST or an institutional system-to-system solution. A button to apply using ASSIST is available in Part 1 of this FOA. See your administrative office for instructions if you plan to use an institutional system-to-system solution.

2. Content and Form of Application Submission

It is critical that applicants follow the Multi-Project (M) Instructions in the SF424 (R&R) Application Guide (https://grants.nih.gov/grants/guide/url_redirect.htm?id=82400), except where instructed in this funding opportunity announcement to do otherwise and where instructions in the Application Guide are directly related to the Grants.gov downloadable forms currently used with most NIH opportunities. Conformance to the requirements in the Application Guide is required and strictly enforced. Applications that are out of compliance with these instructions may be delayed or not accepted for review.

Letter of Intent

Although a letter of intent is not required, is not binding, and does not enter into the review of a subsequent application, the information that it contains allows IC staff to estimate the potential review workload and plan the review.

By the date listed in Part 1. Overview Information, prospective applicants are asked to submit a letter of intent that includes the following information:

- Descriptive title of proposed activity
- Name(s), address(es), and telephone number(s) of the PD(s)/PI(s)
- Names of other key personnel
- Participating institution(s)
- Number and title of this funding opportunity

The letter of intent should be sent to:

Frank De Silva, Ph.D.
Telephone: 240-669-5023
Email: fdesilva@niaid.nih.gov (mailto:fdesilva@niaid.nih.gov)

Page Limitations

All page limitations described in the SF424 Application Guide and the Table of Page Limits (//grants.nih.gov/grants/guide/url_redirect.htm?id=11133) must be followed.

Component	Component Type for Submission	Page Limit	Required/Optional	Minimum	Maximum
Overall	Overall	12	Required	1	1
Admin Core	Admin Core	12	Required	1	1
Data Management Core	Data Management Core	6	Required	1	1
Core	Core	6	Optional	0	3
Project	Project	12	Required	2	5

Instructions for the Submission of Multi-Component Applications

The following section supplements the instructions found in the SF424 (R&R) Application Guide and should be used for preparing a multi-component application.

The application should consist of the following components:

Overall: required

Administrative Core: required

Data Management Core: required

Scientific Cores: optional, maximum 3, each Scientific Core must support at least two Research Projects

Projects: required, minimum 2, maximum 5

Overall Component

When preparing your application, use Component Type ‘Overall’.

All instructions in the SF424 (R&R) Application Guide must be followed, with the following additional instructions, as noted.

SF424(R&R) Cover (Overall)

Complete entire form.

PHS 398 Cover Page Supplement (Overall)

Note: Human Embryonic Stem Cell lines from other components should be repeated in cell line table in Overall component.

Research & Related Other Project Information (Overall)

Follow standard instructions.

Facilities & Other Resources

Describe any unique features in the environment and/or resources that make this a strong research program.

In the " Facilities & Other Resources" attachment include a clearly marked section titled “BSL3/4 facilities” detailing availability of adequate access to BSL3/4 biocontainment facilities to support the proposed Center program, if applicable. Applicants must identify all Research Projects within the application that will require BSL3/4 containment facilities and provide a description of facilities including that are available currently or planned at either the applicant institution or though consortium institutions. A table format may be used to list each activity that requires BSL3/4 access and the likely facilities to be used. All information on BSL3/4 facilities should be contained in the Overall and not within individual Research Projects.

If institutional core facilities will be utilized, in a clearly marked section titled "Institutional Core Facilities" describe how institutional core facilities will be used to support the Research Projects.

Project/Performance Site Locations (Overall)

Enter primary site only.

A summary of Project/Performance Sites in the Overall section of the assembled application image in eRA Commons compiled from data collected in the other components will be generated upon submission.

Research and Related Senior/Key Person Profile (Overall)

Include only the Project Director/Principal Investigator (PD/PI) and any multi-PDs/PIs (if applicable to this FOA) for the entire application.

A summary of Senior/Key Persons followed by their Biographical Sketches in the Overall section of the assembled application image in eRA Commons will be generated upon submission.

Budget (Overall)

The only budget information included in the Overall component is the Estimated Project Funding section of the SF424 (R&R) Cover.

A budget summary in the Overall section of the assembled application image in eRA Commons compiled from detailed budget data collected in the other components will be generated upon submission.

PHS 398 Research Plan (Overall)

Specific Aims: List in priority order, the broad, long-range objectives, and goals of the proposed Center. Concisely describe the Center objectives.

Research Strategy: This narrative section summarizes the overall research plan for the multi-project application. The multi-project application should be viewed as a confederation of interrelated research projects, each capable of standing on its own scientific merit, but complementary to one another. This is an important section for it provides the group of investigators an opportunity to give conceptual wholeness to the overall program – by giving a statement of the general problem area and by laying out a broad strategy for attacking the problems.

Applicants should clearly define the Center program and its significance regarding the scientific approach in terms of discovery and development of vaccines, and if applicable early development of mAbs, against prototype viruses from families with high pandemic potential. Discuss the rationale behind the overall approach and prototype selected for research and development including the public health need and benefit of a successful effort, and the range of activities being pursued. For the prototype selection justification, considerations may include, but are not limited to, how well the prototype reflects properties shared by other members of the family, whether a single prototype is sufficient or whether multiple prototypes are needed to address differences amongst family members, the ease of working with the prototype virus, and whether vaccines against the prototype would have potential value for current public health needs. Include a discussion of the current state of foundational knowledge and maturity of product development for the proposed virus families, and how this Center program will advance vaccine development, and if applicable, early development of mAbs, and enhance the ability to respond rapidly to an emerging virus from the prototype virus's family. Additionally, each application must detail how each Research Project and Core contributes to the Center program, objectives, and project interdependence. Applications should outline expected synergies provided by the proposed Center structure and any other special features that make this application strong or unique.

Milestone Plan: In a clearly labeled section titled "Program Milestones and Timelines", applicants should describe specific quantifiable milestones for the overall program, including detailed quantitative and qualitative criteria for Go/No-Go decisions by annum and include annual timelines for the overall research program and for tracking progress from individual research projects and Cores. This plan must include Go/No-Go criteria to be met by the end of Year 3 of the award for continuation to Phase II. Milestones must specify the outcome(s) for each activity. Milestones should be quantifiable and scientifically justified, and include major milestones from the individual research projects and Cores. Include any milestones that are integrated from independent research projects or Cores. Milestone criteria should not simply be a restatement of the specific aims. Using a Gantt chart or equivalent tool, describe the associated timelines and identified outcomes for the research Center.

Resource Sharing Plan:
Individuals are required to comply with the instructions for the Resource Sharing Plans as provided in the SF424 (R&R) Application Guide.

Other Plan(s):

Note: Effective for due dates on or after January 25, 2023, the Data Management and Sharing Plan will be attached in the Other Plan(s) attachment in FORMS-H application forms packages. If required, the Data Management and Sharing (DMS) Plan must be provided in the Overall component.

All instructions in the SF424 (R&R) Application Guide must be followed, with the following additional instructions:

- All applicants planning research (funded or conducted in whole or in part by NIH) that results in the generation of scientific data are required to comply with the instructions for the Data Management and Sharing Plan. All applications, regardless of the amount of direct costs requested for any one year, must address a Data Management and Sharing Plan.
- Investigators must develop data structures that are FAIR (FAIR Principles - GO FAIR (go-fair.org) (https://gcc02.safelinks.protection.outlook.com/?url=https%3A%2F%2Fwww.go-fair.org%2Ffair-principles%2F&data=05%7C01%7Cchelsea.boyd%40nih.gov%7C8247f78a75c04c5ea99208db1b643066%7C14b77578977342d58507251ca2dc2b06%7C0%7C0%7C638133 This will produce data sets that are harmonized and facilitate progressive data sharing models. Applications must provide a well-thought-out plan for how data will be shared using the FAIR principles.

Appendix:

Only limited items are allowed in the Appendix. Follow all instructions for the Appendix as described in the SF424 (R&R) Application Guide; any instructions provided here are in addition to the SF424 (R&R) Application Guide instructions.

PHS Human Subjects and Clinical Trials Information (Overall)

When involving human subjects research, clinical research, and/or NIH-defined clinical trials follow all instructions for the PHS Human Subjects and Clinical Trials Information form in the SF424 (R&R) Application Guide, with the following additional instructions:

If you answered "Yes" to the question "Are Human Subjects Involved?" on the R&R Other Project Information form, there must be at least one human subjects study record using the **Study Record: PHS Human Subjects and Clinical Trials Information** form or a **Delayed Onset Study** record within the application. The study record(s) must be included in the component(s) where the work is being done, unless the same study spans multiple components. To avoid the creation of duplicate study records, a single study record with sufficient information for all involved components must be included in the Overall component when the same study spans multiple components.

Study Record: PHS Human Subjects and Clinical Trials Information

All instructions in the SF424 (R&R) Application Guide must be followed.

Delayed Onset Study

Note: Delayed onset (https://grants.nih.gov/grants/glossary.htm#DelayedOnsetStudy) does NOT apply to a study that can be described but will not start immediately (i.e., delayed start).All instructions in the SF424 (R&R) Application Guide must be followed.

PHS Assignment Request Form (Overall)

All instructions in the SF424 (R&R) Application Guide must be followed.

Administrative Core

When preparing your application, use Component Type 'Admin Core.'

All instructions in the SF424 (R&R) Application Guide must be followed, with the following additional instructions, as noted.

Note: Effective for due dates on or after January 25, 2023, the Data Management and Sharing Plan will be attached in the Other Plan(s) attachment in FORMS-H application forms packages. If required, the Data Management and Sharing (DMS) Plan must be provided in the Overall component.

SF424 (R&R) Cover (Administrative Core)

Complete only the following fields:

- Applicant Information
- Type of Applicant (optional)
- Descriptive Title of Applicant's Project
- Proposed Project Start/Ending Dates

PHS 398 Cover Page Supplement (Administrative Core)

Enter Human Embryonic Stem Cells in each relevant component.

Research & Related Other Project Information (Administrative Core)

Human Subjects: Answer only the 'Are Human Subjects Involved?' and 'Is the Project Exempt from Federal regulations?' questions.

Vertebrate Animals: Answer only the 'Are Vertebrate Animals Used?' question.

Project Narrative: Do not complete. Note: ASSIST screens will show an asterisk for this attachment indicating it is required. However, eRA systems only enforce this requirement in the Overall component and applications will not receive an error if omitted in other components.

Project /Performance Site Location(s) (Administrative Core)

List all performance sites that apply to the specific component.

Note: The Project Performance Site form allows up to 300 sites, prior to using additional attachment for additional entries.

Research & Related Senior/Key Person Profile (Administrative Core)

- In the Project Director/Principal Investigator section of the form, use Project Role of 'Other' with Category of 'Project Lead' and provide a valid eRA Commons ID in the Credential field.
- In the additional Senior/Key Profiles section, list Senior/Key persons that are working in the component.
- Include a single Biographical Sketch for each Senior/Key person listed in the application regardless of the number of components in which they participate. When a Senior/Key person is listed in multiple components, the Biographical Sketch can be included in any one component.
- If more than 100 Senior/Key persons are included in a component, the Additional Senior Key Person attachments should be used.

Budget (Administrative Core)

Budget forms appropriate for the specific component will be included in the application package.

- The Core Lead must commit at least 0.6 person months effort per year to these responsibilities.
- Include funds for the overall administrative effort, collaborative activities, communications, and publications.
- Include costs related to Regulatory expertise as defined effort or periodic consultation.
- Include funds for the PD(s)/PI(s), Project Leaders, additional Center Key Personnel and postdocs/researchers/students (at the discretion of the PD(s)/PI(s)), to travel and attend annual ReVAMPP Network-wide review meetings to be held over an approximately 1-3 full days in the Rockville, MD area or other NIAID-approved site for data presentation, progress evaluation and related activities.
- Include funds for the PD(s)/PI(s), Project Leaders, external SAB members, and additional Center Key Personnel (at the discretion of the PD/PI) to travel and attend annual mandatory ReVAMPP Center progress meetings at NIAID in Years 1-4 of the project period.

Note: The R&R Budget form included in many of the component types allows for up to 100 Senior/Key Persons in section A and 100 Equipment Items in section C prior to using attachments for additional entries. All other SF424 (R&R) instructions apply.

PHS 398 Research Plan (Administrative Core)

Specific Aims: List in priority order, the broad, long-range objectives, and goals of the proposed Core. In addition, state the Core's relationship to the Center's program and how it relates to the individual Research Projects or other Cores in the application.

Research Strategy: The Administrative Core must include a Management Plan that identifies and discusses: The Administrative Core organizational structure, the roles of Administrative Core personnel, the facilitation of communications throughout the Center, including with industry partners and how a strong collaborative environment will be established within the Center. The plan should specifically address continual evaluation of research and development progress, communications, group meetings and teleconferences, the identification and proposed resolution of problems and engagement of the NIAID staff as appropriate. A description of how consortia (subcontracts) will be managed should be provided and should include how communications such as periodic meetings and conference calls will be organized, managed, and documented. The plan should also detail how Center and research-related travel will be managed.

Describe how the Center will coordinate communication and collaborations with the CDSC, NIAID Staff, and other Centers within the Network. Describe how the Administrative Core will ensure the Center program complies in a timely manner with requests from the CDSC and NIAID program staff.

Each Administrative Core must include the following:

Scientific Advisory Board: Describe the composition and duties of the Scientific Advisory Board (SAB), including the categories of expertise to be represented on the SAB and how the SAB will be utilized to guide Center activities. The description should include a discussion of how the proposed expertise of the SAB will be integrated into the operations of the Center. Describe the procedures and approaches for obtaining SAB input via teleconferences, ad hoc and annual meetings, review of written materials/data, etc. The SAB must include at least two members with relevant industry-level experience and procedures for identification and selection of the SAB should be included. *Candidates for the SAB MUST NOT be named in the application or contacted prior to completion of review activities.*

Resource Sharing Plan: Individuals are required to comply with the instructions for the Resource Sharing Plans as provided in the SF424 (R&R) Application Guide.

Appendix:

Only limited items are allowed in the Appendix. Follow all instructions for the Appendix as described in the SF424 (R&R) Application Guide; any instructions provided here are in addition to the SF424 (R&R) Application Guide instructions.

PHS Human Subjects and Clinical Trials Information (Administrative Core)

When involving human subjects research, clinical research, and/or NIH-defined clinical trials follow all instructions for the PHS Human Subjects and Clinical Trials Information form in the SF424 (R&R) Application Guide, with the following additional instructions:

If you answered "Yes" to the question "Are Human Subjects Involved?" on the R&R Other Project Information form, you must include at least one human subjects study record using the **Study Record: PHS Human Subjects and Clinical Trials Information form** or a **Delayed Onset Study** record.

Study Record: PHS Human Subjects and Clinical Trials Information

All instructions in the SF424 (R&R) Application Guide must be followed

Delayed Onset Study

Note: Delayed onset (https://grants.nih.gov/grants/glossary.htm#DelayedOnsetHumanSubjectStudy) does NOT apply to a study that can be described but will not start immediately (i.e., delayed start). All instructions in the SF424 (R&R) Application Guide must be followed.

Data Management Core

When preparing your application, use Component Type 'Data Management Core.'

All instructions in the SF424 (R&R) Application Guide must be followed, with the following additional instructions, as noted.

Note: Effective for due dates on or after January 25, 2023, the Data Management and Sharing Plan will be attached in the Other Plan(s) attachment in FORMS-H application forms packages. If required, the Data Management and Sharing (DMS) Plan must be provided in the Overall component.

SF424 (R&R) Cover (Data Management Core)

Complete only the following fields:

- Applicant Information
- Type of Applicant (optional)
- Descriptive Title of Applicant's Project
- Proposed Project Start/Ending Dates

PHS 398 Cover Page Supplement (Data Management Core)

Enter Human Embryonic Stem Cells in each relevant component.

Research & Related Other Project Information (Data Management Core)

Human Subjects: Answer only the 'Are Human Subjects Involved?' and 'Is the Project Exempt from Federal regulations?' questions.

Vertebrate Animals: Answer only the 'Are Vertebrate Animals Used?' question.

Project Narrative: Do not complete. Note: ASSIST screens will show an asterisk for this attachment indicating it is required. However, eRA systems only enforce this requirement in the Overall component and applications will not receive an error if omitted in other components.

Project /Performance Site Location(s) (Data Management Core)

List all performance sites that apply to the specific component.

Note: The Project Performance Site form allows up to 300 sites, prior to using additional attachment for additional entries.

Research & Related Senior/Key Person Profile (Data Management Core)

- In the Project Director/Principal Investigator section of the form, use Project Role of 'Other' with Category of 'Core Lead' and provide a valid eRA Commons ID in the Credential field.
- In the additional Senior/Key Profiles section, list Senior/Key persons that are working in the component.
- Include a single Biographical Sketch for each Senior/Key person listed in the application regardless of the number of components in which they participate. When a Senior/Key person is listed in multiple components, the Biographical Sketch can be included in any one component.
- If more than 100 Senior/Key persons are included in a component, the Additional Senior Key Person attachments should be used.

Budget (Data Management Core)

Budget forms appropriate for the specific component will be included in the application package.

The Core Leader should commit to the Core at least 1.2 person months of effort.

Note: The R&R Budget form included in many of the component types allows for up to 100 Senior/Key Persons in section A and 100 Equipment Items in section C prior to using attachments for additional entries. All other SF424 (R&R) instructions apply.

PHS 398 Research Plan (Data Management Core)

Specific Aims: List in priority order, the broad, long-range objectives, and goals of the proposed Core. In addition, state the Core's relationship to the Center program and how it relates to the individual Research Projects or other Cores in the application.

Research Strategy: Describe the organizational structure and role of the Data Management Core in the overall Center research activities and include a Strategy for Management of Data Activities Plan that describes internal and external data acquisition strategies to achieve harmonization of systems and procedures for data management, data quality, data analyses, and dissemination for all data and data-related materials generated by the Center to the ReVAMPP CDSC. Describe the quality control procedures for the data, and how to identify and resolve issues with quality control that maintains data integrity. Describe how the Data Management Core will ensure compliance with the ReVAMPP network-wide data sharing.

Resource Sharing Plan: Individuals are required to comply with the instructions for the Resource Sharing Plans as provided in the SF424 (R&R) Application Guide.

Appendix:

Only limited items are allowed in the Appendix. Follow all instructions for the Appendix as described in the SF424 (R&R) Application Guide; any instructions provided here are in addition to the SF424 (R&R) Application Guide instructions.

PHS Human Subjects and Clinical Trials Information (Data Management Core)

When involving human subjects research, clinical research, and/or NIH-defined clinical trials follow all instructions for the PHS Human Subjects and Clinical Trials Information form in the SF424 (R&R) Application Guide, with the following additional instructions:

If you answered "Yes" to the question "Are Human Subjects Involved?" on the R&R Other Project Information form, you must include at least one human subjects study record using the **Study Record: PHS Human Subjects and Clinical Trials Information** form or a **Delayed Onset Study** record.

Study Record: PHS Human Subjects and Clinical Trials Information

All instructions in the SF424 (R&R) Application Guide must be followed

Delayed Onset Study

Note: Delayed onset (https://grants.nih.gov/grants/glossary.htm#DelayedOnsetStudy) does NOT apply to a study that can be described but will not start immediately (i.e., delayed start). All instructions in the SF424 (R&R) Application Guide must be followed.

Scientific Core

When preparing your application, use Component Type 'Core.'

All instructions in the SF424 (R&R) Application Guide must be followed, with the following additional instructions, as noted.

Note: Effective for due dates on or after January 25, 2023, the Data Management and Sharing Plan will be attached in the Other Plan(s) attachment in FORMS-H application forms packages. If required, the Data Management and Sharing (DMS) Plan must be provided in the Overall component.

SF424 (R&R) Cover (Scientific Core)

Complete only the following fields:

- Applicant Information
- Type of Applicant (optional)
- Descriptive Title of Applicant's Project
- Proposed Project Start/Ending Dates

PHS 398 Cover Page Supplement (Scientific Core)

Enter Human Embryonic Stem Cells in each relevant component.

Research & Related Other Project Information (Scientific Core)

Human Subjects: Answer only the 'Are Human Subjects Involved?' and 'Is the Project Exempt from Federal regulations?' questions.

Vertebrate Animals: Answer only the 'Are Vertebrate Animals Used?' question.

Project Narrative: Do not complete. Note: ASSIST screens will show an asterisk for this attachment indicating it is required. However, eRA systems only enforce this requirement in the Overall component and applications will not receive an error if omitted in other components.

Project /Performance Site Location(s) (Scientific Core)

List all performance sites that apply to the specific component.

Note: The Project Performance Site form allows up to 300 sites, prior to using additional attachment for additional entries.

Research & Related Senior/Key Person Profile (Scientific Core)

- In the Project Director/Principal Investigator section of the form, use Project Role of 'Other' with Category of 'Core Lead' and provide a valid eRA Commons ID in the Credential field.
- In the additional Senior/Key Profiles section, list Senior/Key persons that are working in the component.
- Include a single Biographical Sketch for each Senior/Key person listed in the application regardless of the number of components in which they participate. When a Senior/Key person is listed in multiple components, the Biographical Sketch can be included in any one component.
- If more than 100 Senior/Key persons are included in a component, the Additional Senior Key Person attachments should be used.

Budget (Scientific Core)

Budget forms appropriate for the specific component will be included in the application package.

The Core Leader should commit to the Core at least 1.2 person months of effort.

Note: The R&R Budget form included in many of the component types allows for up to 100 Senior/Key Persons in section A and 100 Equipment Items in section C prior to using attachments for additional entries. All other SF424 (R&R) instructions apply.

PHS 398 Research Plan (Scientific Core)

Specific Aims: List in priority order, the broad, long-range objectives, and goals of the proposed Core. In addition, state the Core's relationship to the Center program and how it relates to the individual Research Projects or other Cores in the application.

Research Strategy: Describe and justify the role of the Core in the overall Center research activities, describe how the proposed Core activities will contribute to meeting the Center's goals and objectives, specify how a proposed scientific Core provides a unique service that cannot be obtained through institutional or commercial means, and explain the rationale for selection of the general methods and approaches proposed to accomplish the specific aims. Describe the facilities or services that will be provided by the Core including procedures, techniques, and quality control to ensure high quality outputs. In addition, this section should indicate the relevance of the Core to the primary objectives of the application. Provide details of the services or resources provided by the optional Cores to at least two Research Projects and clarify how the optional Cores are not duplicative of other services or facilities. Additionally, plans for staffing, managing, and prioritizing use of the Cores must be provided, as well as plans for determining fees to users if charging fees is necessary.

Resource Sharing Plan: Individuals are required to comply with the instructions for the Resource Sharing Plans as provided in the SF424 (R&R) Application Guide.

Appendix:

Only limited items are allowed in the Appendix. Follow all instructions for the Appendix as described in the SF424 (R&R) Application Guide; any instructions provided here are in addition to the SF424 (R&R) Application Guide instructions.

PHS Human Subjects and Clinical Trials Information (Scientific Core)

When involving human subjects research, clinical research, and/or NIH-defined clinical trials follow all instructions for the PHS Human Subjects and Clinical Trials Information form in the SF424 (R&R) Application Guide, with the following additional instructions:

If you answered "Yes" to the question "Are Human Subjects Involved?" on the R&R Other Project Information form, you must include at least one human subjects study record using the **Study Record: PHS Human Subjects and Clinical Trials Information** form or a **Delayed Onset Study** record.

Study Record: PHS Human Subjects and Clinical Trials Information

All instructions in the SF424 (R&R) Application Guide must be followed

Delayed Onset Study

Note: Delayed onset (https://grants.nih.gov/grants/glossary.htm#DelayedOnsetStudy) does NOT apply to a study that can be described but will not start immediately (i.e., delayed start). All instructions in the SF424 (R&R) Application Guide must be followed.

Research Project

When preparing your application, use Component Type 'Project.'

All instructions in the SF424 (R&R) Application Guide must be followed, with the following additional instructions, as noted.

Note: Effective for due dates on or after January 25, 2023, the Data Management and Sharing Plan will be attached in the Other Plan(s) attachment in FORMS-H application forms packages. If required, the Data Management and Sharing (DMS) Plan must be provided in the Overall component.

SF424 (R&R) Cover (Research Project)

Complete only the following fields:

- Applicant Information
- Type of Applicant (optional)
- Descriptive Title of Applicant's Project
- Proposed Project Start/Ending Dates

PHS 398 Cover Page Supplement (Research Project)

Enter Human Embryonic Stem Cells in each relevant component.

Research & Related Other Project Information (Research Project)

Human Subjects: Answer only the 'Are Human Subjects Involved?' and 'Is the Project Exempt from Federal regulations? questions.

Vertebrate Animals: Answer only the 'Are Vertebrate Animals Used?' question.

Project Narrative: Do not complete. Note: ASSIST screens will show an asterisk for this attachment indicating it is required. However, eRA systems only enforce this requirement in the Overall component and applications will not receive an error if omitted in other components.

Project /Performance Site Location(s) (Research Project)

List all performance sites that apply to the specific component.

Note: The Project Performance Site form allows up to 300 sites, prior to using additional attachment for additional entries.

Research & Related Senior/Key Person Profile (Research Project)

- In the Project Director/Principal Investigator section of the form, use Project Role of 'Other' with Category of 'Project Lead' and provide a valid eRA Commons ID in the Credential field.
- In the additional Senior/Key Profiles section, list Senior/Key persons that are working in the component.
- Include a single Biographical Sketch for each Senior/Key person listed in the application regardless of the number of components in which they participate. When a Senior/Key person is listed in multiple components, the Biographical Sketch can be included in any one component.
- If more than 100 Senior/Key persons are included in a component, the Additional Senior Key Person attachments should be used.

Budget (Research Project)

Budget forms appropriate for the specific component will be included in the application package.

Each project leader must commit at least 1.2 person months effort to their project per year.

Note: The R&R Budget form included in many of the component types allows for up to 100 Senior/Key Persons in section A and 100 Equipment Items in section C prior to using attachments for additional entries. All other SF424 (R&R) instructions apply.

PHS 398 Research Plan (Research Project)

Specific Aims: List, in priority order, the broad long-range objectives and goals of the proposed project. Concisely describe the research activities to be performed. In addition, concisely state the individual Research Project's relationship to the Center program and how it relates to other projects or cores.

Research Strategy: Use this section to describe how the proposed research will contribute to meeting the Center objectives and explain the rationale for selecting the methods to accomplish the specific aims, and the biological significance of the research.

At least one Research Project must focus on development of vaccines, and other Research Projects may focus on additional vaccine development, early mAb development or foundational research in virology, immunology, pathology, and structural biology necessary for such development.

Describe the research design, conceptual procedures, and analyses to be used to accomplish the specific aims of the project. Describe any new methodology and its advantage over existing methodologies. Describe any novel concepts, approaches, techniques, methodologies, tools, or technologies for the proposed studies that will fundamentally advance how vaccines, and if applicable mAbs, will be developed. Discuss whether traditional approaches will be used in a new, novel way and how reliable, validated methods that mitigate risk will be balanced with innovative new approaches to expand foundational knowledge and advance product development. Describe plans for how the vaccine strategies and other research findings from the prototype virus research will be applied broadly to other viruses within the family to assess the breadth of the approach within and outside of the virus family to validate the generalizability of the approach. Discuss how the vaccine technologies or platforms used can be rapidly adapted

for response to known or novel emerging viruses within the same family. If proposing early mAb development, describe plans for the assessment of whether antibodies with similar epitopes and/or properties of identified lead mAb candidates for the prototype virus are effective against other viruses within the same family. Discuss the potential difficulties and limitations of the proposed procedures and alternative approaches to achieve the aims.

Milestone Plan: In a clearly labeled section titled “Project Milestones and Timelines" include a clear delineation of goals with measurable milestones, including detailed quantitative and qualitative criteria for Go/No-Go decision-making, and a timeline for the attainment of each goal and milestone and should be reflected in the Milestone Plan for the overall Program. This plan must include Go/No-Go criteria to be met by the end of Year 3 of the award for continuation to Phase II. Milestones must specify the outcome(s) for each activity. Milestones should be quantifiable and scientifically justified, and include the completion of major research study activities, for example, identification of protective epitopes, animal model development, vaccine or mAb candidate down-selection, identification of correlates of protection, validation of vaccine or mAb strategies for other family members, and analysis, sharing and publication of final data. Milestone criteria should not simply be a restatement of the specific aims. Using a Gantt chart or equivalent tool, describe the associated timelines and identified outcomes for the research Center.

Industry Expertise and Regulatory Considerations: For projects proposing early vaccine development, describe how industry partners will be identified and incorporated into the proposed project including a timeline for inclusion. For projects proposing IND-enabling later stage vaccine development, NIAID requires Centers to include active participation of an industry partner to ensure access to vaccine technology platforms, expertise in manufacturing, clinical development, and regulatory pathways. Applicants should describe the role of this partner in the proposed project and/or team to facilitate discovery, candidate evaluation and/or product development. For the purpose of this FOA, "industry" is defined as a large or small, domestic or foreign, pharmaceutical, biotechnology, bioengineering, or chemical company, or a related non-profit entity.

Additionally, each Center is expected to consider anticipated regulatory barriers for the targeted vaccine technology, particularly for new technology platforms for which there are no precedents for FDA approval. Describe anticipated regulatory barriers and propose research and/or strategies to resolve or overcome these barriers. For a project where multiple and/or complementary expertise is required, discuss plans for coordination among investigators and other collaborators including industry partners and the process to overcome obstacles to achieve the Center aims.

Letter of Support: For projects proposing later stage vaccine development where an industry partner is required, provide a Letter of Support from the Industry partner(s). For projects without later stage vaccine development, a Letter of Support from Industry partners may be included. Provide any additional letters of support that are specific to this project.

Resource Sharing Plan: Individuals are required to comply with the instructions for the Resource Sharing Plans as provided in the SF424 (R&R) Application Guide.

Appendix:

Only limited items are allowed in the Appendix. Follow all instructions for the Appendix as described in the SF424 (R&R) Application Guide; any instructions provided here are in addition to the SF424 (R&R) Application Guide instructions.

PHS Human Subjects and Clinical Trials Information (Research Project)

When involving human subjects research, clinical research, and/or NIH-defined clinical trials follow all instructions for the PHS Human Subjects and Clinical Trials Information form in the SF424 (R&R) Application Guide, with the following additional instructions:

If you answered “Yes” to the question “Are Human Subjects Involved?” on the R&R Other Project Information form, you must include at least one human subjects study record using the **Study Record: PHS Human Subjects and Clinical Trials Information** form or a **Delayed Onset Study** record.

Study Record: PHS Human Subjects and Clinical Trials Information

All instructions in the SF424 (R&R) Application Guide must be followed

Delayed Onset Study

Note: Delayed onset (https://grants.nih.gov/grants/glossary.htm#DelayedOnsetStudy) does NOT apply to a study that can be described but will not start immediately (i.e., delayed start). All instructions in the SF424 (R&R) Application Guide must be followed.

Foreign Institutions

Foreign (non-U.S.) institutions must follow policies described in the *NIH Grants Policy Statement* (//grants.nih.gov/grants/guide/url_redirect.htm?id=11137), and procedures for foreign institutions described throughout the SF424 (R&R) Application Guide.

3. Unique Entity Identifier and System for Award Management (SAM)

See Part 1. Section III.1 for information regarding the requirement for obtaining a unique entity identifier and for completing and maintaining active registrations in System for Award Management (SAM), NATO Commercial and Government Entity (NCAGE) Code (if applicable), eRA Commons, and Grants.gov

4. Submission Dates and Times

Part I. Overview Information contains information about Key Dates and times. Applicants are encouraged to submit applications before the due date to ensure they have time to make any application corrections that might be necessary for successful submission. When a submission date falls on a weekend or Federal holiday (https://grants.nih.gov/grants/guide/url_redirect.htm?id=82380), the application deadline is automatically extended to the next business day.

Organizations must submit applications to Grants.gov (//grants.nih.gov/grants/guide/url_redirect.htm?id=11128) (the online portal to find and apply for grants across all Federal agencies) using ASSIST or other electronic submission systems. Applicants must then complete the submission process by tracking the status of the application in the eRA Commons (//grants.nih.gov/grants/guide/url_redirect.htm?id=11123), NIH’s electronic system for grants administration. NIH and Grants.gov systems check the application against many of the application instructions upon submission. Errors must be corrected and a changed/corrected application must be submitted to Grants.gov on or before the application due date and time. If a Changed/Corrected application is submitted after the deadline, the application will be considered late. Applications that miss the due date and time are subjected to the NIH Policy on Late Application Submission.

Applicants are responsible for viewing their application before the due date in the eRA Commons to ensure accurate and successful submission.

Information on the submission process and a definition of on-time submission are provided in the SF424 (R&R) Application Guide.

5. Intergovernmental Review (E.O. 12372)

This initiative is not subject to intergovernmental review (https://grants.nih.gov/grants/policy/nihgps/html5/section_10/10.10.1_executive_orders.htm).

6. Funding Restrictions

All NIH awards are subject to the terms and conditions, cost principles, and other considerations described in the *NIH Grants Policy Statement* (//grants.nih.gov/grants/guide/url_redirect.htm?id=11120).

Pre-award costs are allowable only as described in the *NIH Grants Policy Statement* (//grants.nih.gov/grants/guide/url_redirect.htm?id=11143).

7. Other Submission Requirements and Information

Applications must be submitted electronically following the instructions described in the SF424 (R&R) Application Guide. Paper applications will not be accepted.

For information on how your application will be automatically assembled for review and funding consideration after submission go to: http://grants.nih.gov/grants/ElectronicReceipt/files/Electronic_Multi-project_Application_Image_Assembly.pdf (//grants.nih.gov/grants/ElectronicReceipt/files/Electronic_Multi-project_Application_Image_Assembly.pdf).

Applicants must complete all required registrations before the application due date. Section III. Eligibility Information contains information about registration.

For assistance with your electronic application or for more information on the electronic submission process, visit How to Apply – Application Guide (https://grants.nih.gov/grants/how-to-apply-application-guide.html). If you encounter a system issue beyond your control that threatens your ability to complete the submission process on-time, you must follow the Dealing with System Issues (https://grants.nih.gov/grants/how-to-apply-application-guide/due-dates-and-submission-policies/dealing-with-system-issues.htm) guidance. For assistance with application submission, contact the Application Submission Contacts in Section VII.

Important reminders:

All PD(s)/PI(s) and component Project Leads must include their eRA Commons ID in the Credential field of the Senior/Key Person Profile form**.** Failure to register in the Commons and to include a valid PD/PI Commons ID in the credential field will prevent the successful submission of an electronic application to NIH.

The applicant organization must ensure that the unique entity identifier provided on the application is the same identifier used in the organization's profile in the eRA Commons and for the System for Award Management. Additional information may be found in the SF424 (R&R) Application Guide (https://grants.nih.gov/grants/guide/url_redirect.htm?id=82400).

See more tips (//grants.nih.gov/grants/guide/url_redirect.htm?id=11146) for avoiding common errors.

Upon receipt, applications will be evaluated for completeness and compliance with application instructions by the Center for Scientific Review and responsiveness by NIAID, NIH. Applications that are incomplete, non-compliant and/or nonresponsive will not be reviewed.

Post Submission Materials

Applicants are required to follow the instructions for post-submission materials, as described in the policy (//grants.nih.gov/grants/guide/url_redirect.htm?id=82299).

Section V. Application Review Information

1. Criteria

Only the review criteria described below will be considered in the review process. Applications submitted to the NIH in support of the NIH mission (//grants.nih.gov/grants/guide/url_redirect.htm?id=11149) are evaluated for scientific and technical merit through the NIH peer review system.

Overall Impact - Overall

Reviewers will provide an overall impact score to reflect their assessment of the likelihood for the Program to exert a sustained, powerful influence on the research field(s) involved, in consideration of the following review criteria and additional review criteria (as applicable for the Program proposed).

Scored Review Criteria - Overall

Reviewers will consider each of the review criteria below in the determination of scientific merit and give a separate score for each. An application does not need to be strong in all categories to be judged likely to have major scientific impact. For example, a project that by its nature is not innovative may be essential to advance a field.

Significance

Does the project address an important problem or a critical barrier to progress in the field? Is the prior research that serves as the key support for the proposed project rigorous? If the aims of the project are achieved, how will scientific knowledge, technical capability, and/or clinical practice be improved? How will successful completion of the aims change the concepts, methods, technologies, treatments, services, or preventative interventions that drive this field?

Specific to this FOA:

How well does the application describe a single clearly defined and scientifically justified program that supports the development of vaccine strategies for prototype pathogens that can be applied to other family members? Understanding that the different virus families have different levels of existing foundational knowledge and different states of maturity for product development, how appropriate is the research to increase knowledge to advance vaccine, and if applicable mAb, development for the proposed virus family? If successful, how likely will the proposed Center program enhance the ability to rapidly respond to an emerging known or currently unknown virus from the prototype virus's family?

How well does the Center as a whole leverage scientific gains and synergy by combining the component projects into a multi-project program beyond the gains achievable if each project were pursued independently? To what extent is the program cohesive and do the Research Projects and Cores relate to a common objective demonstrating cohesion, multidisciplinary interactions, and coordination? How well are the scientific cores justified relative to the Overall?

Investigator(s)

Are the PD(s)/PI(s), collaborators, and other researchers well suited to the project? If Early Stage Investigators or those in the early stages of independent careers, do they have appropriate experience and training? If established, have they demonstrated an ongoing record of accomplishments that have advanced their field(s)? If the project is collaborative or multi-PD/PI, do the investigators have complementary and integrated expertise; are their leadership approach, governance and organizational structure appropriate for the project?

Specific to this FOA:

To what extent is there an appropriate balance between investigators with expertise studying the virus families and investigators with product development experience? To what extent is there appropriate and adequate representation of investigators with the necessary vaccine discovery and development experience and expertise? For projects involving IND-enabling later stage activities, how appropriate are industry partnerships proposed to support this effort? For early development projects, how appropriate is the plan and timeline for identification and incorporation of industry partnership to facilitate rapid transition of vaccines into clinical development? How sufficiently are the academic and industry partners coordinated to facilitate discovery, candidate evaluation and/or product development? How adequate is the level of commitment of the PD(s)/PI(s) and key personnel to manage the overall Program?

Innovation

Does the application challenge and seek to shift current research or clinical practice paradigms by utilizing novel theoretical concepts, approaches or methodologies, instrumentation, or interventions? Are the concepts, approaches or methodologies, instrumentation, or interventions novel to one field of research or novel in a broad sense? Is a refinement, improvement, or new application of theoretical concepts, approaches or methodologies, instrumentation, or interventions proposed?

Specific to this FOA:

To what extent is there an effective balance between reliable, validated methods that mitigate risk with innovative, new approaches that could expand the field? How well have the investigators used traditional approaches in new, novel ways? To what extent are there techniques, methodologies, concepts, or processes which fundamentally advance how vaccines, and if applicable mAbs, are developed?

Approach

Are the overall strategy, methodology, and analyses well-reasoned and appropriate to accomplish the specific aims of the project? Have the investigators included plans to address weaknesses in the rigor of prior research that serves as the key support for the proposed project? Have the investigators presented strategies to ensure a robust and unbiased approach, as appropriate for the work proposed? Are potential problems, alternative strategies, and benchmarks for success presented? If the project is in the early stages of development, will the strategy establish feasibility and will particularly risky aspects be managed? Have the investigators presented adequate plans to address relevant biological variables, such as sex, for studies in vertebrate animals or human subjects?

If the project involves human subjects and/or NIH-defined clinical research, are the plans to address:

1) the protection of human subjects from research risks, and
2) inclusion (or exclusion) of individuals on the basis of sex/gender, race, and ethnicity, as well as the inclusion or exclusion of individuals of all ages (including children and older adults), justified in terms of the scientific goals and research strategy proposed?

Specific to this FOA:

How well is the selection of the prototype(s) justified, and likely to generate generalizable knowledge that can be applied to develop vaccines for related viruses? To what extent do the investigators propose a well-reasoned plan to evaluate their vaccine approach broadly to other viruses within a given family to validate the generalizability of the approach? If proposing early mAb development, to what extent do the investigators propose a well-reasoned plan to validate the generalizability of the approach by assessing whether mAbs with similar epitopes and/or properties of lead candidate mAbs against the prototype virus are effective against other viruses within a given family?

To what extent does the approach use technologies and platforms that could be rapidly adapted for response to known or novel emerging viruses within the same family? How appropriate is the proposed project given the current level of knowledge and vaccine development landscape for the virus family? For programs that include IND-enabling translational research, how well does the approach take into consideration the anticipated regulatory process and any anticipated regulatory barriers and resolutions?

How well are the overall Timelines and proposed Milestones defined with quantifiable measures and criteria that are appropriate for enabling clear Go/No-Go decisions and assessing the success of the overall program? How well do the overall milestones support the goal of advancing generalizable vaccine, and if applicable, mAb solutions? How realistic are the timelines proposed for achieving these overall milestones?

How well do the individual projects contribute, either directly or through generation of essential resources or foundational knowledge, to the identification of generalizable vaccine, and if applicable mAb, approaches for a given virus family? How well are the Research Project milestones defined with quantifiable measures and appropriate for enabling clear Go/No-Go decisions and assessing the success of the individual Research Projects? How well do the investigators provide a clear plan for achieving defined Research Project milestones and timelines? To what extent are the timelines proposed for achieving these Research Project milestones realistic or inclusive of necessary steps?

Environment

Will the scientific environment in which the work will be done contribute to the probability of success? Are the institutional support, equipment and other physical resources available to the investigators adequate for the project proposed? Will the project benefit from unique features of the scientific environment, subject populations, or collaborative arrangements?

Specific to this FOA: To what extent do the investigators have access to facilities with the appropriate biocontainment and capacity and resources for the proposed research?

Additional Review Criteria - Overall, Administrative Core, Data Management Core, Scientific Core, and Research Projects

As applicable for the project proposed, reviewers will evaluate the following additional items while determining scientific and technical merit, and in providing an overall impact score, but will not give separate scores for these items.

Administrative Core

How appropriate is the administrative and organizational structure and adequate to achieve the goals of the proposed program? How appropriate is the Management Plan for fiscal accountability and communication within the program? How appropriate are the plans for coordination and the establishment of a strong collaborative environment for the program? How adequate are the plans for communication among the Centers and with the CDSC to facilitate collaborative activities? How appropriate is the plan for collaboration with industry partners clear given the state of the program? How sufficient is the time and effort committed by the PD/PI and Key Personnel to adequately manage the Program? To what extent do the investigators provide a well-thought-out plan for coordination, communication, and collaborations with the CDSC, NIAID Staff, and other Centers within the Network? How well have the applicants developed a plan for ensuring timely communication and collaboration with the CDSC, NIAID Staff, and other Centers within the Network?

Data Management Core

How appropriate and adequate is the organizational structure to achieve the goals of the proposed program? How appropriate is the Strategy for Management of Data Activities Plan for the type of data generated by the research program? How sufficient are the described data management activities sufficient? To what extent do the investigators provide a well-thought-out plan for network-wide sharing of the data generated by the Center?

Scientific Core

How sufficiently is the Core justified? To what extent does it support at least two Research Projects? How well is the core connected to the central focus of the overall program? To what extent are the facilities or services provided by the core (including procedures, techniques, and quality control) high quality and well-justified? How effectively will the services be used? To what extent are the core leader and key personnel well qualified and is there an adequate commitment of time?

Protections for Human Subjects

For research that involves human subjects but does not involve one of the categories of research that are exempt under 45 CFR Part 46, the committee will evaluate the justification for involvement of human subjects and the proposed protections from research risk relating to their participation according to the following five review criteria: 1) risk to subjects, 2) adequacy of protection against risks, 3) potential benefits to the subjects and others, 4) importance of the knowledge to be gained, and 5) data and safety monitoring for clinical trials.

For research that involves human subjects and meets the criteria for one or more of the categories of research that are exempt under 45 CFR Part 46, the committee will evaluate: 1) the justification for the exemption, 2) human subjects involvement and characteristics, and 3) sources of materials. For additional information on review of the Human Subjects section, please refer to the Guidelines for the Review of Human Subjects (//grants.nih.gov/grants/guide/url_redirect.htm?id=11175).

Inclusion of Women, Minorities, and Individuals Across the Lifespan

When the proposed project involves human subjects and/or NIH-defined clinical research, the committee will evaluate the proposed plans for the inclusion (or exclusion) of individuals on the basis of sex/gender, race, and ethnicity, as well as the inclusion (or exclusion) of individuals of all ages (including children and older adults) to determine if it is justified in terms of the scientific goals and research strategy proposed. For additional information on review of the Inclusion section, please refer to the Guidelines for the Review of Inclusion in Clinical Research (//grants.nih.gov/grants/guide/url_redirect.htm?id=11174).

Vertebrate Animals

The committee will evaluate the involvement of live vertebrate animals as part of the scientific assessment according to the following criteria: (1) description of proposed procedures involving animals, including species, strains, ages, sex, and total number to be used; (2) justifications for the use of animals versus alternative models and for the appropriateness of the species proposed; (3) interventions to minimize discomfort, distress, pain and injury; and (4) justification for euthanasia method if NOT consistent with the AVMA Guidelines for the Euthanasia of Animals. Reviewers will assess the use of chimpanzees as they would any other application proposing the use of vertebrate animals. For additional information on review of the Vertebrate Animals section, please refer to the Worksheet for Review of the Vertebrate Animals Section (//grants.nih.gov/grants/guide/url_redirect.htm?id=11150).

Biohazards

Reviewers will assess whether materials or procedures proposed are potentially hazardous to research personnel and/or the environment, and if needed, determine whether adequate protection is proposed.

Resubmissions

Not Applicable

Renewals

Not Applicable

Revisions

Not Applicable

Additional Review Considerations - Overall, Administrative Core, Data Management Core, Scientific Core, and Research Projects

As applicable for the project proposed, reviewers will consider each of the following items, but will not give scores for these items, and should not consider them in providing an overall impact score.

Applications from Foreign Organizations

Reviewers will assess whether the project presents special opportunities for furthering research programs through the use of unusual talent, resources, populations, or environmental conditions that exist in other countries and either are not readily available in the United States or augment existing U.S. resources.

Select Agent Research

Reviewers will assess the information provided in this section of the application, including 1) the Select Agent(s) to be used in the proposed research, 2) the registration status of all entities where Select Agent(s) will be used, 3) the procedures that will be used to monitor possession use and transfer of Select Agent(s), and 4) plans for appropriate biosafety, biocontainment, and security of the Select Agent(s).

Resource Sharing Plans

Reviewers will comment on whether the Resource Sharing Plan(s) (e.g., Sharing Model Organisms (https://sharing.nih.gov/other-sharing-policies/model-organism-sharing-policy#policy-overview)) or the rationale for not sharing the resources, is reasonable.

Authentication of Key Biological and/or Chemical Resources:

For projects involving key biological and/or chemical resources, reviewers will comment on the brief plans proposed for identifying and ensuring the validity of those resources.

Budget and Period of Support

Reviewers will consider whether the budget and the requested period of support are fully justified and reasonable in relation to the proposed research.

2. Review and Selection Process

Applications will be evaluated for scientific and technical merit by (an) appropriate Scientific Review Group(s) convened by the National Institute of Allergy and Infectious Diseases, in accordance with NIH peer review policy and procedures (//grants.nih.gov/grants/guide/url_redirect.htm?id=11154), using the stated review criteria. Assignment to a Scientific Review Group will be shown in the eRA Commons.

As part of the scientific peer review, all applications will receive a written critique.

Applications may undergo a selection process in which only those applications deemed to have the highest scientific and technical merit (generally the top half of applications under review) will be discussed and assigned an overall impact score.

Appeals (https://grants.nih.gov/grants/policy/nihgps/html5/section_2/2.4.2_appeals_of_initial_scientific_review.htm) of initial peer review will not be accepted for applications submitted in response to this FOA

Applications will be assigned to the appropriate NIH Institute or Center. Applications will compete for available funds with all other recommended applications submitted in response to this FOA. Following initial peer review, recommended applications will receive a second level of review by the National Advisory Allergy and Infectious Diseases Council. The following will be considered in making funding decisions:

- Scientific and technical merit of the proposed project as determined by scientific peer review.
- Availability of funds.
- Relevance of the proposed project to program priorities.

3. Anticipated Announcement and Award Dates

After the peer review of the application is completed, the PD/PI will be able to access his or her Summary Statement (written critique) via the eRA Commons (//grants.nih.gov/grants/guide/url_redirect.htm?id=11123). Refer to Part 1 for dates for peer review, advisory council review, and earliest start date.

Information regarding the disposition of applications is available in the NIH Grants Policy Statement (//grants.nih.gov/grants/guide/url_redirect.htm?id=11120).

Section VI. Award Administration Information

1. Award Notices

If the application is under consideration for funding, NIH will request "just-in-time" information from the applicant as described in the NIH Grants Policy Statement (https://grants.nih.gov/grants/policy/nihgps/HTML5/section_2/2.5.1_just-in-time_procedures.htm).

A formal notification in the form of a Notice of Award (NoA) will be provided to the applicant organization for successful applications. The NoA signed by the grants management officer is the authorizing document and will be sent via email to the recipient's business official.

Recipients must comply with any funding restrictions described in Section IV.6. Funding Restrictions. Selection of an application for award is not an authorization to begin performance. Any costs incurred before receipt of the NoA are at the recipient's risk. These costs may be reimbursed only to the extent considered allowable pre-award costs.

Any application awarded in response to this FOA will be subject to terms and conditions found on the Award Conditions and Information for NIH Grants (https://grants.nih.gov/grants/policy/nihgps/HTML5/part_ii_subpart_b.htm) website. This includes any recent legislation and policy applicable to awards that is highlighted on this website.

Institutional Review Board or Independent Ethics Committee Approval: Grantee institutions must ensure that protocols are reviewed by their IRB or IEC. To help ensure the safety of participants enrolled in NIH-funded studies, the recipient must provide NIH copies of documents related to all major changes in the status of ongoing protocols.

2. Administrative and National Policy Requirements

All NIH grant and cooperative agreement awards include the NIH Grants Policy Statement (//grants.nih.gov/grants/guide/url_redirect.htm?id=11120) as part of the NoA. For these terms of award, see the NIH Grants Policy Statement Part II: Terms and Conditions of NIH Grant Awards, Subpart A: General (//grants.nih.gov/grants/guide/url_redirect.htm?id=11157) and Part II: Terms and Conditions of NIH Grant Awards, Subpart B: Terms and Conditions for Specific Types of Grants, Recipients, and Activities (//grants.nih.gov/grants/guide/url_redirect.htm?id=11159), including of note, but not limited to:

- Federal wide Research Terms and Conditions (https://grants.nih.gov/grants/policy/nihgps/HTML5/section_3/3.1_federalwide_standard_terms_and_conditions_for_research_grants.htm)
- Prohibition on Certain Telecommunications and Video Surveillance Services or Equipment (https://grants.nih.gov/grants/guide/notice-files/NOT-OD-21-041.html)
- Acknowledgment of Federal Funding (https://grants.nih.gov/grants/policy/nihgps/HTML5/section_4/4.2.1_acknowledgement_of_federal_funding.htm)

If a recipient is successful and receives a Notice of Award, in accepting the award, the recipient agrees that any activities under the award are subject to all provisions currently in effect or implemented during the period of the award, other Department regulations and policies in effect at the time of the award, and applicable statutory provisions.

Should the applicant organization successfully compete for an award, recipients of federal financial assistance (FFA) from HHS will be required to complete an HHS Assurance of Compliance form (HHS 690) (https://ocrportal.hhs.gov/ocr/aoc/instruction.jsf) in which the recipient agrees, as a term and condition of receiving the grant, to administer their programs in compliance with federal civil rights laws that prohibit discrimination on the basis of race, color, national origin, age, sex and disability, and agreeing to comply with federal conscience laws, where applicable. This includes ensuring that entities take meaningful steps to provide meaningful access to persons with limited English proficiency; and ensuring effective communication with persons with disabilities. Where applicable, Title XI and Section 1557 prohibit discrimination on the basis of sexual orientation, and gender identity. The HHS Office for Civil Rights provides guidance on complying with civil rights laws enforced by HHS. Please see https://www.hhs.gov/civil-rights/for-providers/provider-obligations/index.html (https://www.hhs.gov/civil-rights/for-providers/provider-obligations/index.html) and https://www.hhs.gov/civil-rights/for-individuals/nondiscrimination/index.html (https://www.hhs.gov/civil-rights/for-individuals/nondiscrimination/index.html)

HHS recognizes that research projects are often limited in scope for many reasons that are nondiscriminatory, such as the principal investigator's scientific interest, funding limitations, recruitment requirements, and other considerations. Thus, criteria in research protocols that target or exclude certain populations are warranted where nondiscriminatory justifications establish that such criteria are appropriate with respect to the health or safety of the subjects, the scientific study design, or the purpose of the research. For additional guidance regarding how the provisions apply to NIH grant programs, please contact the Scientific/Research Contact that is identified in Section VII under Agency Contacts of this FOA.

- Recipients of FFA must ensure that their programs are accessible to persons with limited English proficiency. For guidance on meeting the legal obligation to take reasonable steps to ensure meaningful access to programs or activities by limited English proficient individuals see https://www.hhs.gov/civil-rights/for-individuals/special-topics/limited-english-proficiency/fact-sheet-guidance/index.html (https://www.hhs.gov/civil-rights/for-individuals/special-topics/limited-english-proficiency/fact-sheet-guidance/index.html)andhttps://www.lep.gov (https://www.lep.gov/).
- For information on an institution's specific legal obligations for serving qualified individuals with disabilities, including providing program access, reasonable modifications, and to provide effective communication, see https://www.hhs.gov/civil-rights/for-individuals/disability/index.html (https://www.hhs.gov/civil-rights/for-individuals/disability/index.html).
- HHS funded health and education programs must be administered in an environment free of sexual harassment, see https://www.hhs.gov/civil-rights/for-individuals/sex-discrimination/index.html (https://www.hhs.gov/civil-rights/for-individuals/sex-discrimination/index.html). For information about NIH's commitment to supporting a safe and respectful work environment, who to contact with questions or concerns, and what NIH's expectations are for institutions and the individuals supported on NIH-funded awards, please see https://grants.nih.gov/grants/policy/harassment.htm (https://grants.nih.gov/grants/policy/harassment.htm).
- For guidance on administering programs in compliance with applicable federal religious nondiscrimination laws and applicable federal conscience protection and associated anti-discrimination laws see https://www.hhs.gov/conscience/conscience-protections/index.html (https://www.hhs.gov/conscience/conscience-protections/index.html) and https://www.hhs.gov/conscience/religious-freedom/index.html (https://www.hhs.gov/conscience/religious-freedom/index.html).

Please contact the HHS Office for Civil Rights for more information about obligations and prohibitions under federal civil rights laws at https://www.hhs.gov/ocr/about-us/contact-us/index.html (https://www.hhs.gov/ocr/about-us/contact-us/index.html) or call 1-800-368-1019 or TDD 1-800-537-7697.

In accordance with the statutory provisions contained in Section 872 of the Duncan Hunter National Defense Authorization Act of Fiscal Year 2009 (Public Law 110-417), NIH awards will be subject to the Federal Awardee Performance and Integrity Information System (FAPIIS) requirements. FAPIIS requires Federal award making officials to review and consider information about an applicant in the designated integrity and performance system (currently FAPIIS) prior to making an award. An applicant, at its option, may review information in the designated integrity and performance systems accessible through FAPIIS and comment on any information about itself that a federal agency previously entered and is currently in FAPIIS. The Federal awarding agency will consider any comments by the applicant, in addition to other information in FAPIIS, in making a judgement about the applicant's integrity, business ethics, and record of performance under Federal awards when completing the review of risk posed by applicants as described in 45 CFR Part 75.205 and 2 CFR Part 200.206 "Federal awarding agency review of risk posed by applicants." This provision will apply to all NIH grants and cooperative agreements except fellowships."

Cooperative Agreement Terms and Conditions of Award

The following special terms of award are in addition to, and not in lieu of, otherwise applicable U.S. Office of Management and Budget (OMB) administrative guidelines, U.S. Department of Health and Human Services (DHHS) grant administration regulations at 45 CFR Part 75 and 2 CFR Part 200, and other HHS, PHS, and NIH grant administration policies.

The administrative and funding instrument used for this program will be the cooperative agreement, an "assistance" mechanism (rather than an "acquisition" mechanism), in which substantial NIH programmatic involvement with the recipients is anticipated during the performance of the activities. Under the cooperative agreement, the NIH purpose is to support and stimulate the recipients' activities by involvement in and otherwise working jointly with the recipients in a partnership role; it is not to assume direction, prime

responsibility, or a dominant role in the activities. Consistent with this concept, the dominant role and prime responsibility resides with the recipients for the project as a whole, although specific tasks and activities may be shared among the recipients and the NIH as defined below.

The PD(s)/PI(s) will have the primary responsibility for:

PD(s)/PI(s) will have the primary responsibility for coordinating the Projects and Cores within the overall Program. Specifically, the PD(s)/PI(s) have primary responsibility as described below.

- The PD(s)/PI(s) will be responsible for defining the research objectives, approaches, and details of the projects within the guidelines of the FOA and retains primary responsibility for the planning, directing, and executing the proposed scientific activities
- The PD(s)/PI(s) will monitor all Research Projects and actively promote efforts that foster integration, collaboration, and synergy across the projects.
- The PD(s)/PI(s) will be responsible for ensuring timely compliance with ReVAMPP Network policies for template usage, data sharing, and collaboration.
- The PD(s)/PI(s) are responsible for ensuring that appropriate systems are in place to provide for biosafety and security of materials, data, facilities and resources, including compliance with regard to Select Agent Regulations, Biosafety in Microbiology and Biomedical Laboratories (BMBL) Guidelines, Centers for Disease Control and Prevention and the National Institutes of Health, sixth Edition; U.S. Code of Federal Regulations 42 C.F.R. Part 73, 7 C.F.R. Part 331, and 9 C.F.R. Part 121.

In addition, the PD(s)/PI(s) will be responsible for:

- Organizing and chairing annual ReVAMPP Center Progress meeting activities. The annual ReVAMPP Center progress meetings are anticipated to be held at a location at/near Rockville, MD or at another NIAID-approved site and will last up to 2 days.
- Advertising the availability of the Program generated resources through outreach activities.

Recipients will retain custody of and have primary rights to the data and software developed under these awards, subject to Government rights of access consistent with current HHS, PHS, and NIH policies.

NIH staff have substantial programmatic involvement that is above and beyond the normal stewardship role in awards, as described below:

The role of the NIAID Project Scientist is to support and encourage the recipient's activities by substantial involvement as partners and facilitators in the process without assuming responsibilities that remain with the PDs/PIs.

- The NIAID Project Scientist will work closely with the PD(s)/PI(s) and other Program member scientists to facilitate collaborations and to leverage the resources available to the ReVAMPP Network.
- The NIAID Project Scientist will monitor the progress of the Center, help coordinate research approaches among all Centers funded through the FOA and contribute to the shaping of research projects or approaches as warranted. The NIAID Project Scientist will support and facilitate this process but will not direct it.
- Near the end of year 3 of the award, Program Staff will assess the progress towards development of generalizable approaches for vaccine, and if applicable early development of mAbs, for virus families of pandemic concern through the accomplishment of the milestones and overall feasibility of program advancement. The assessment will be based on the first three annual reports, the milestones included in the application and negotiated with the recipient prior to award, any additional information that the PD/PI elects to submit, evidence of collaboration with other ReVAMPP Centers, compliance with the ReVAMPP Network data sharing and CDSC requests, programmatic priorities, and the availability of funding.
- The NIAID Project Scientist will keep the ReVAMPP Centers informed about other ongoing studies supported by NIAID to avoid duplication of effort and encourage sharing/collaboration in infectious diseases research.
- The NIAID Project Scientist will coordinate access for the recipients to other NIAID resources, as well as assist the research efforts of the Program by facilitating access to fiscal and intellectual resources provided by industry, private foundations, NIH intramural scientists and other federal government agencies as appropriate.

In addition to the NIAID Project Scientist, an agency program official or IC program director will be responsible for the normal scientific and programmatic stewardship of the award and will be named in the award notice.

Areas of Joint Responsibility include:

- The NIAID Project Scientist and the PD(s)/PI(s) will hold regular program-wide discussions to facilitate the achievement of program goals.
- The PD(s)/PI(S) and the NIAID Project Scientist will collaborate in the establishment of the Scientific Advisory Board
- The NIAID Project Scientist and the PD/PI will collaborate during the course of the award to revise and/or update project milestones as appropriate.

Dispute Resolution

Any disagreements that may arise in scientific or programmatic matters (within the scope of the award) between award recipients and the NIH may be brought to Dispute Resolution. A Dispute Resolution Panel composed of three members will be convened. It will have three members: a designee of the Steering Committee chosen without NIH staff voting, one NIH designee, and a third designee with expertise in the relevant area who is chosen by the other two; in the case of individual disagreement, the first member may be chosen by the individual recipient. This special dispute resolution procedure does not alter the recipient's right to appeal an adverse action that is otherwise appealable in accordance with PHS regulation 42 CFR Part 50, Subpart D and DHHS regulation 45 CFR Part 16.

3. Data Management and Sharing

Note: The NIH Policy for Data Management and Sharing is effective for due dates on or after January 25, 2023.

Consistent with the NIH Policy for Data Management and Sharing, when data management and sharing is applicable to the award, recipients will be required to adhere to the Data Management and Sharing requirements as outlined in the NIH Grants Policy Statement (https://grants.nih.gov/grants/policy/nihgps/HTML5/section_8/8.2.3_sharing_research_resources.htm). Upon the approval of a Data Management and Sharing Plan, it is required for recipients to implement the plan as described.

Recipients will be required to adhere to the FAIR Principles (FAIR Principles - GO FAIR (go-fair.org) (https://gcc02.safelinks.protection.outlook.com/?url=https%3A%2F%2Fwww.go-fair.org%2Ffair-principles%2F&data=05%7C01%7Cchelsea.boyd%40nih.gov%7C8247f78a75c04c5ea99208db1b643066%7C14b77578977342d58507251ca2dc2b06%7C0%7C0%7C638133889348 for data management and sharing.

4. Reporting

When multiple years are involved, recipients will be required to submit the Research Performance Progress Report (RPPR) (//grants.nih.gov/grants/rppr/index.htm) annually and financial statements as required in the NIH Grants Policy Statement. (https://grants.nih.gov/grants/policy/nihgps/HTML5/section_8/8.4.1_reporting.htm)

A final RPPR, invention statement, and the expenditure data portion of the Federal Financial Report are required for closeout of an award, as described in the NIH Grants Policy Statement (https://grants.nih.gov/grants/policy/nihgps/HTML5/section_8/8.6_closeout.htm). NIH FOAs outline intended research goals and objectives. Post award, NIH will review and measure performance based on the details and outcomes that are shared within the RPPR, as described at 45 CFR Part 75.301 and 2 CFR Part 200.301.

The Federal Funding Accountability and Transparency Act of 2006 (Transparency Act), includes a requirement for recipients of Federal grants to report information about first-tier subawards and executive compensation under Federal assistance awards issued in FY2011 or later. All recipients of applicable NIH grants and cooperative agreements are required to

report to the Federal Subaward Reporting System (FSRS) available at www.fsrs.gov (//grants.nih.gov/grants/guide/url_redirect.htm?id=11170) on all subawards over the threshold. See the NIH Grants Policy Statement (https://grants.nih.gov/grants/policy/nihgps/HTML5/section_4/4.1.8_federal_funding_accountability_and_transparency_act__ffata_.htm) for additional information on this reporting requirement.

In accordance with the regulatory requirements provided at 45 CFR 75.113and 2 CFR Part 200.113 and Appendix XII to 45 CFR Part 75 and 2 CFR Part 200, recipients that have currently active Federal grants, cooperative agreements, and procurement contracts from all Federal awarding agencies with a cumulative total value greater than $10,000,000 for any period of time during the period of performance of a Federal award, must report and maintain the currency of information reported in the System for Award Management (SAM)about civil, criminal, and administrative proceedings in connection with the award or performance of a Federal award that reached final disposition within the most recent five-year period. The recipient must also make semiannual disclosures regarding such proceedings. Proceedings information will be made publicly available in the designated integrity and performance system (currently FAPIIS). This is a statutory requirement under section 872 of Public Law 110-417, as amended (41 U.S.C. 2313). As required by section 3010 of Public Law 111-212, all information posted in the designated integrity and performance system on or after April 15, 2011, except past performance reviews required for Federal procurement contracts, will be publicly available. Full reporting requirements and procedures are found in Appendix XII to 45 CFR Part 75and 2 CFR Part 200 – Award Term and Condition for Recipient Integrity and Performance Matters.

Section VII. Agency Contacts

We encourage inquiries concerning this funding opportunity and welcome the opportunity to answer questions from potential applicants.

Application Submission Contacts

eRA Service Desk (Questions regarding ASSIST, eRA Commons, application errors and warnings, documenting system problems that threaten submission by the due date, and post-submission issues)

Finding Help Online: https://www.era.nih.gov/need-help (https://www.era.nih.gov/need-help) (preferred method of contact)
Telephone: 301-402-7469 or 866-504-9552 (Toll Free)

General Grants Information (Questions regarding application instructions, application processes, and NIH grant resources)
Email: GrantsInfo@nih.gov (mailto:GrantsInfo@nih.gov) (preferred method of contact)
Telephone: 301-637-3015

Grants.gov Customer Support (Questions regarding Grants.gov registration and Workspace)
Contact Center Telephone: 800-518-4726
Email: support@grants.gov (mailto:support@grants.gov)

Scientific/Research Contact(s)

Kaitlyn Morabito, Ph.D.
National Institute of Allergy and Infectious Diseases (NIAID)
Telephone: 301-204-3248
Email: Kaitlyn.dambach@nih.gov (mailto:Kaitlyn.dambach@nih.gov)

Peer Review Contact(s)

Frank De Silva, Ph.D.
National Institute of Allergy and Infectious Diseases (NIAID)
Telephone: 240-669-5023
Email: fdesilva@niaid.nih.gov (mailto:fdesilva@niaid.nih.gov)

Financial/Grants Management Contact(s)

Elizabeth Sihombing
National Institute of Allergy and Infectious Diseases (NIAID)
Telephone: 240-669-5530
Email: elizabeth.sihombing@nih.gov (mailto:elizabeth.sihombing@nih.gov)

Section VIII. Other Information

Recently issued trans-NIH policy notices (//grants.nih.gov/grants/guide/url_redirect.htm?id=11163) may affect your application submission. A full list of policy notices published by NIH is provided in the NIH Guide for Grants and Contracts (//grants.nih.gov/grants/guide/url_redirect.htm?id=11164). All awards are subject to the terms and conditions, cost principles, and other considerations described in the NIH Grants Policy Statement (//grants.nih.gov/grants/guide/url_redirect.htm?id=11120).

Authority and Regulations

Awards are made under the authorization of Sections 301 and 405 of the Public Health Service Act as amended (42 USC 241 and 284) and under Federal Regulations 42 CFR Part 52 and 45 CFR Part 75 and 2 CFR Part 200.

Weekly TOC for this Announcement (/grants/guide/WeeklyIndex.cfm?03-17-23)
NIH Funding Opportunities and Notices (/grants/guide/index.html)

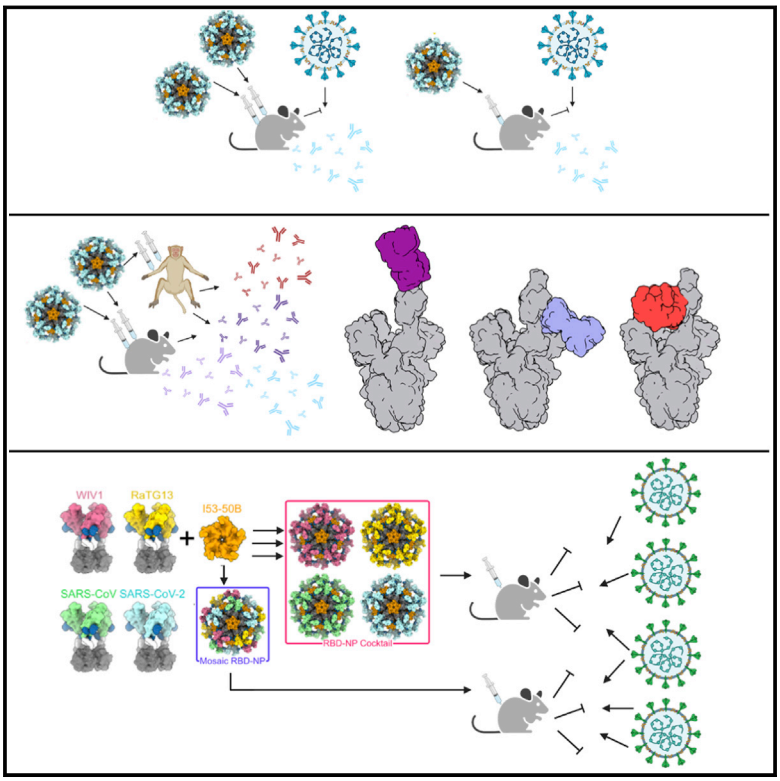
Note: For help accessing PDF, RTF, MS Word, Excel, PowerPoint, Audio or Video files, see Help Downloading Files (/grants/edocs.htm).

Cell

Article

Elicitation of broadly protective sarbecovirus immunity by receptor-binding domain nanoparticle vaccines

Graphical abstract



Authors

Alexandra C. Walls, Marcos C. Miranda, Alexandra Schäfer, ..., Ralph S. Baric, Neil P. King, David Veesler

Correspondence

neil@ipd.uw.edu (N.P.K.), dveesler@uw.edu (D.V.)

In brief

A clinical stage multivalent SARS-CoV-2 spike receptor-binding domain nanoparticle (RBD-NP) vaccine is protective in mice after a single immunization and elicits strong antibody responses across circulating mutants and some sarbecoviruses. Multivalent sarbecovirus RBD-NP vaccines elicit heterotypic protection against sarbecoviruses.

Highlights

- RBD-NP enables dose-sparing and protects mice after only one dose
- RBD-NP elicits diverse polyclonal antibody responses in non-human primates
- RBD-NP and HexaPro confer similar resilience to antigenic drift
- Mosaic and cocktail RBD-NPs protect against heterotypic SARS-CoV challenge

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Article

Elicitation of broadly protective sarbecovirus immunity by receptor-binding domain nanoparticle vaccines

Alexandra C. Walls,[1] Marcos C. Miranda,[1,2] Alexandra Schäfer,[3] Minh N. Pham,[1,2] Allison Greaney,[4,5] Prabhu S. Arunachalam,[6] Mary-Jane Navarro,[1] M. Alejandra Tortorici,[1,7] Kenneth Rogers,[8] Megan A. O'Connor,[9,10] Lisa Shirreff,[8] Douglas E. Ferrell,[8] John Bowen,[1] Natalie Brunette,[1,2] Elizabeth Kepl,[1,2] Samantha K. Zepeda,[1] Tyler Starr,[4] Ching-Lin Hsieh,[11] Brooke Fiala,[1,2] Samuel Wrenn,[1,2] Deleah Pettie,[1,2] Claire Sydeman,[1,2] Kaitlin R. Sprouse,[1] Max Johnson,[1,2] Alyssa Blackstone,[1,2] Rashmi Ravichandran,[1,2] Cassandra Ogohara,[1,2] Lauren Carter,[1,2] Sasha W. Tilles,[12] Rino Rappuoli,[13] Sarah R. Leist,[3] David R. Martinez,[3] Matthew Clark,[14] Roland Tisch,[15] Derek T. O'Hagan,[16] Robbert Van Der Most,[17] Wesley C. Van Voorhis,[12] Davide Corti,[18] Jason S. McLellan,[11] Harry Kleanthous,[19] Timothy P. Sheahan,[3] Kelly D. Smith,[20] Deborah H. Fuller,[9,10] Francois Villinger,[8] Jesse Bloom,[4,5] Bali Pulendran,[6] Ralph S. Baric,[3] Neil P. King,[1,2,*] and David Veesler[1,21,*]

[1]Department of Biochemistry, University of Washington, Seattle, WA 98195, USA
[2]Institute for Protein Design, University of Washington, Seattle, WA 98195, USA
[3]Department of Epidemiology, University of North Carolina at Chapel Hill, Chapel Hill, NC 27514, USA
[4]Basic Sciences and Computational Biology, Fred Hutchinson Cancer Research Center, Seattle, WA 98109, USA
[5]Department of Genome Sciences, University of Washington, Seattle, WA 98109, USA
[6]Institute for Immunity, Transplantation and Infection, Stanford University School of Medicine, Stanford University, Stanford, CA, USA
[7]Institut Pasteur and CNRS UMR 3569, Unite de Virologie Structurale, Paris, France
[8]New Iberia Research Center and Department of Biology, University of Louisiana at Lafayette, New Iberia, LA 70560, USA
[9]Washington National Primate Research Center, Seattle, WA 98121, USA
[10]Department of Microbiology, University of Washington, Seattle, WA 98195, USA
[11]Department of Molecular Biosciences, The University of Texas at Austin, Austin, TX 78712, USA
[12]Division of Allergy and Infectious Diseases, Department of Medicine, University of Washington School of Medicine, Seattle, WA 98195, USA
[13]GSK, Siena, Italy
[14]Department of Microbiology and Immunology, University of North Carolina at Chapel Hill, Chapel Hill, NC 27514, USA
[15]Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill, Chapel Hill, NC 27514, USA
[16]GSK, Rockville, MD, USA
[17]GSK, Rixensart, Belgium
[18]Humabs Biomed SA, a subsidiary of Vir Biotechnology, 6500 Bellinzona, Switzerland
[19]Bill & Melinda Gates Foundation, Seattle, WA 98109, USA
[20]UW Medicine Department of Laboratory Medicine and Pathology, Seattle, WA 98195, USA
[21]Lead contact
*Correspondence: neil@ipd.uw.edu (N.P.K.), dveesler@uw.edu (D.V.)
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SUMMARY

Understanding vaccine-elicited protection against SARS-CoV-2 variants and other sarbecoviruses is key for guiding public health policies. We show that a clinical stage multivalent SARS-CoV-2 spike receptor-binding domain nanoparticle (RBD-NP) vaccine protects mice from SARS-CoV-2 challenge after a single immunization, indicating a potential dose-sparing strategy. We benchmarked serum neutralizing activity elicited by RBD-NPs in non-human primates against a lead prefusion-stabilized SARS-CoV-2 spike (HexaPro) using a panel of circulating mutants. Polyclonal antibodies elicited by both vaccines are similarly resilient to many RBD residue substitutions tested, although mutations at and surrounding position 484 have negative consequences for neutralization. Mosaic and cocktail nanoparticle immunogens displaying multiple sarbecovirus RBDs elicit broad neutralizing activity in mice and protect mice against SARS-CoV challenge even in the absence of SARS-CoV RBD in the vaccine. This study provides proof of principle that multivalent sarbecovirus RBD-NPs induce heterotypic protection and motivates advancing such broadly protective sarbecovirus vaccines to the clinic.

INTRODUCTION

The emergence of SARS-CoV-2 in late 2019 resulted in the COVID-19 pandemic that brought the world to a standstill. Moreover, the recurrent spillovers of coronaviruses in humans along with detection of SARS-CoV-2-, SARS-CoV-, and MERS-CoV-related coronaviruses in bats suggest that future zoonotic transmission events may continue to occur (Menachery et al., 2015, 2016; Zhou et al., 2020a). SARS-CoV-2 infects host cells through the attachment of the viral transmembrane spike (S) glycoprotein to angiotensin-converting enzyme 2 (ACE2), followed by fusion of the viral and host membranes (Hoffmann et al., 2020; Lan et al., 2020; Letko et al., 2020; Shang et al., 2020; Walls et al., 2020a; Wang et al., 2020; Wrapp et al., 2020; Yan et al., 2020; Zhou et al., 2020b). The SARS-CoV-2 S protein is the primary target of neutralizing antibodies (Abs), and the immunodominant receptor-binding domain (RBD) accounts for more than 90% of the neutralizing activity in COVID-19 convalescent sera and vaccinated individuals (Greaney et al., 2021a, 2021b; Piccoli et al., 2020). Numerous monoclonal Abs (mAbs) recognizing distinct antigenic sites on the RBD were isolated and shown to neutralize viral entry and protect small animals and non-human primates (NHPs) from SARS-CoV-2 challenge (Barnes et al., 2020; Baum et al., 2020a; Brouwer et al., 2020; Piccoli et al., 2020; Pinto et al., 2020; Rogers et al., 2020; Starr et al., 2021; Tortorici et al., 2020; Wec et al., 2020; Zost et al., 2020). As a result, SARS-CoV-2 S is the focus of nucleic acid, vectored, and protein subunit vaccines currently being developed and deployed (Corbett et al., 2020a, 2020b; Jackson et al., 2020; Mercado et al., 2020; Polack et al., 2020; Tostanoski et al., 2020; Yu et al., 2020).

Worldwide sequencing of SARS-CoV-2 clinical isolates has led to the identification of numerous mutations in the >1,500,000 genome sequences available to date (https://www.gisaid.org/). The SARS-CoV-2 S D614G mutation has become globally dominant and is associated with enhanced viral transmission and replication but does not significantly affect Ab-mediated neutralization (Hou et al., 2020; Korber et al., 2020; Plante et al., 2020; Yurkovetskiy et al., 2020). Conversely, some mutations found in circulating SARS-CoV-2 isolates were shown to promote escape from mAbs and to reduce neutralization by immune sera (Baum et al., 2020b; Collier et al., 2021; Li et al., 2020; McCallum et al., 2021a, 2021b; Wang et al., 2021a; Weisblum et al., 2020; Wibmer et al., 2021). As a result, formulation of mAb cocktails or the use of mAbs targeting conserved epitopes and neutralizing a broader spectrum of circulating SARS-CoV-2 variants emerged as a promising strategy to overcome this issue (Baum et al., 2020b; Cathcart et al., 2021; Dong et al., 2021; Greaney et al., 2020; Jette et al., 2021; Martinez et al., 2021a; Pinto et al., 2020; Tortorici et al., 2020, 2021). The recent emergence of several variants and variants of concern (VOCs) with numerous S mutations is especially worrisome, including B.1.1.7 (alpha), B.1.351 (beta), B.1.427/B.1.429 (epsilon), P.1 (gamma), and B.1.617.2 (delta) that originated in the UK, South Africa, the USA, Brazil, and India, respectively (Davies et al., 2020; Deng et al., 2021; Faria et al., 2021; McCallum et al., 2021b; Tegally et al., 2020). Some of these mutations lead to significant reductions in the neutralization potency of N-terminal domain (NTD)- and RBD-specific mAbs, convalescent sera, and Pfizer/BioNTech BNT162b2- or Moderna mRNA-1273-elicited sera (Collier et al., 2021; McCallum et al., 2021a, 2021b; Wang et al., 2021a).

We recently described a multivalent subunit vaccine displaying the SARS-CoV-2 RBD nanoparticle (RBD-NP) in a highly immunogenic array using a computationally designed self-assembling protein nanoparticle (Bale et al., 2016; Walls et al., 2020b). Vaccination with RBD-NP resulted in 10-fold higher neutralizing Ab titers in mice than the prefusion-stabilized S2P trimer (which is used in most current vaccines) despite a 5-fold lower dose and protected mice against mouse-adapted SARS-CoV-2 (SARS-CoV-2-MA) challenge (Dinnon et al., 2020; Walls et al., 2020b). We demonstrated that the RBD-NP vaccine elicited robust neutralizing Ab and CD4 T cell responses in NHPs when formulated with several clinic-ready adjuvants and conferred protection against SARS-CoV-2 infection in the nose, pharynges, and bronchioles (Arunachalam et al., 2021). The RBD-NP vaccine is currently being evaluated in two phase I/II clinical trials (ClinicalTrials.gov: NCT04742738 and NCT04750343) and recently received funding from the coalition for epidemic preparedness innovations for phase III clinical trials.

Although the S fusion machinery (S_2 subunit) has higher sequence conservation than the RBD (Pinto et al., 2021; Sauer et al., 2021; Tortorici and Veesler, 2019; Walls et al., 2016, 2020a), the breadth of neutralization and protection provided by RBD-based vaccines remains unknown. The isolation of RBD-specific cross-reactive mAbs neutralizing SARS-CoV-2 and SARS-CoV suggests that RBD-based vaccines could, in principle, elicit Abs that neutralize distantly related sarbecoviruses, which have future pandemic potential (Cathcart et al., 2021; Jette et al., 2021; Martinez et al., 2021a; Pinto et al., 2020; Rappazzo et al., 2021; Starr et al., 2021; Tortorici et al., 2021; Wec et al., 2020). RBD-based vaccines are also unaffected by S mutations outside of the RBD, especially in the highly variable NTD that is heavily mutated in many VOCs (Andreano et al., 2020; Avanzato et al., 2020; Choi et al., 2020; Collier et al., 2021; Wang et al., 2021a; McCallum et al., 2021a, 2021b; McCarthy et al., 2021). Here, we explored dose-sparing strategies for the RBD-NP vaccine and evaluated the impact of genetic diversity among SARS-CoV-2 clinical isolates and sarbecoviruses on vaccine-elicited Abs. We further designed mosaic and cocktail sarbecovirus RBD-NPs that elicit broad and protective Ab responses against heterologous sarbecovirus challenge, which could represent the next generation of pan-sarbecovirus vaccines.

RESULTS

Dose-sparing RBD-NP vaccination protects mice against SARS-CoV-2 challenge

Considering the unprecedented need for rapid global distribution of SARS-CoV-2 vaccines, we investigated the ability of RBD-NP to induce neutralizing and protective Ab titers at lower doses than previously evaluated. Specifically, we set out to test whether vaccine-elicited neutralizing Ab titers are altered by lowering the immunogen dose 10-fold (two 0.1 μg immunizations, RBD antigen dose) or reducing the number of doses

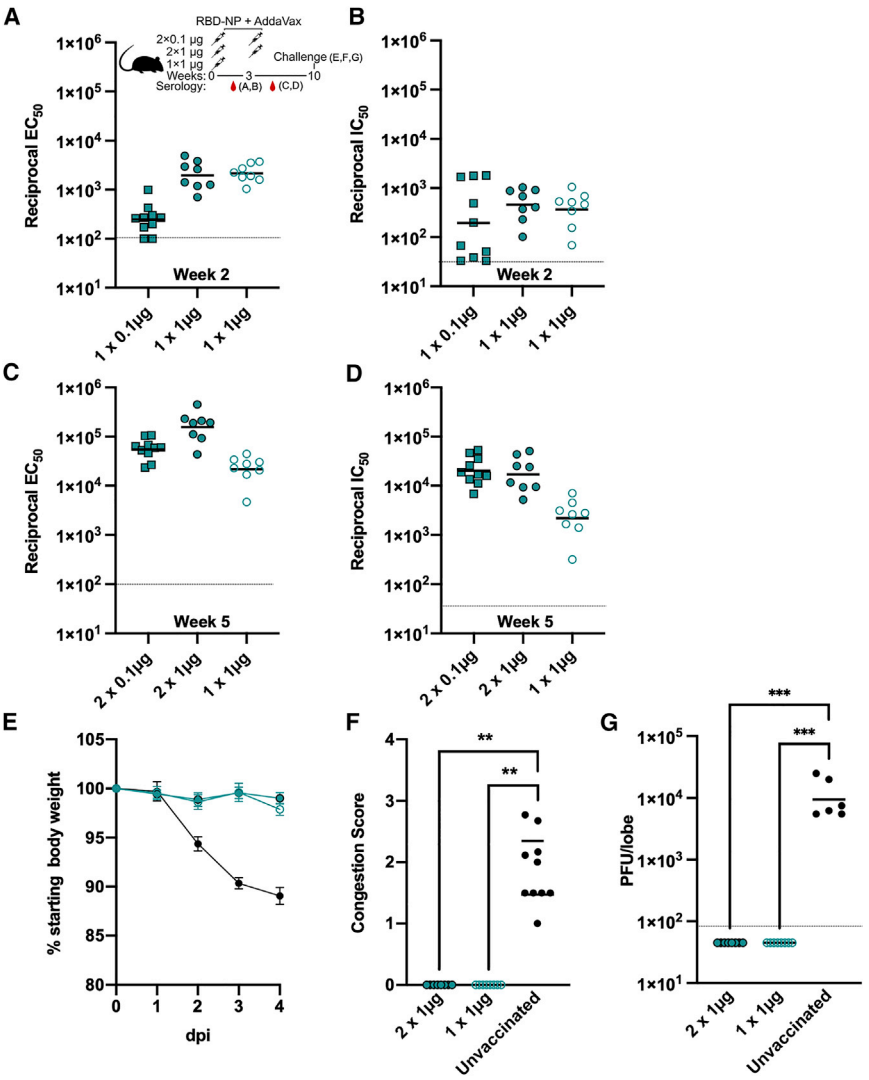


Figure 1. A single immunization with RBD-NP protects BALB/c cByJ mice from SARS-CoV-2 MA10 challenge

(A and C) S2P-binding Abs were measured 2 (A) or 5 (C) weeks post-prime; teal squares: 2 doses of 0.1 μg (n = 10); filled teal circles: 2 doses of 1 μg (n = 8); open teal circles: one dose of 1 μg (n = 8) with a limit of detection (LOD) of 1×10^2.

(B and D) Serum neutralizing Ab titers at 2 (B) or 5 (D) weeks post-prime determined using an MLV pseudotyping system with an LOD of 3.3×10^1.

(E) Weight loss following SARS-CoV-2 MA10 challenge up to 4 days post infection (n = 8 vaccinated; n = 6 naive mice shown as black filled circles).

(F) Congestion score following SARS-CoV-2 MA10 challenge with a score of 0 indicating unchanged lung color and 4 indicating a darkened and diseased lung.

(G) Viral titers in the mice lungs (expressed in plaque forming units [PFUs] per lobe) following challenge with an LOD of 9×10^1. Statistical significance was determined by Kruskal-Wallis test and shown only when significant. $^{**}p < 0.01$. LODs are shown as gray horizontal dotted lines.

Raw data curves shown in Data S1.

(a single 1 μg immunization, RBD antigen dose) compared to our initial work (Walls et al., 2020b). To test this, BALB/c cByJ mice were immunized intramuscularly at week 0 with either 0.1 μg (ten mice) or 1 μg (two groups of eight mice or sixteen mice total) of AddaVax-adjuvanted RBD-NP. Two weeks post-prime, serum binding and neutralizing Ab titers were roughly comparable for the two vaccine doses (neutralization geometric mean titer [GMT] ~2–4 × 10^2) measured using a mouse leukemia virus (MLV) pseudotyping system (Millet and Whittaker, 2016; Walls et al., 2020a) (Figures 1A and 1B; Data S1). Three weeks post prime, we boosted the mice that were immunized with 0.1 μg of the vaccine and eight of the sixteen mice immunized with 1 μg of the vaccine with the same respective dose. Two weeks post-boost, serum binding and neutralizing Ab titers were comparable for the 0.1 μg and 1 μg groups (neutralization GMT ~2 × 10^4) (Figures 1C and 1D; Data S1), suggesting that a 10-fold reduction in the RBD-NP dose does not affect serum neutralizing responses. Furthermore, we observed that the magnitude of binding and neutralizing Ab titers increased over time for the mice that received a single immunization (neutralization GMT 2 × 10^3). These results suggest that lowering the vaccine dose or reducing the number of immunizations represent possible dose-sparing strategies that did not compromise elicitation of high levels of neutralizing Ab titers using RBD-NPs.

To further evaluate vaccine efficacy, eight mice that received one dose and eight mice that received two doses of the 1-μg RBD-NP vaccine along with six unvaccinated mice were challenged ten weeks post-prime with 10^5 plaque-forming units (PFUs) of mouse-adapted SARS-CoV-2 MA10 (Leist et al., 2020). The SARS-CoV-2 MA10 model causes weight loss and lung pathology when 10^5 PFUs (the highest titer tested [Leist et al., 2020]) are used in young BALB/c mice. The mice were followed for four days to assess protection from disease. All mice in the RBD-NP-vaccinated groups were protected from weight loss throughout the duration of the experiment regardless of the number of doses, whereas control mice lost ~10% of their weight by day four (Figure 1E). Analysis of lung pathology and viral titers in lung tissues indicated that the vaccinated mice were not affected by SARS-CoV-2 MA10 challenge, whereas the control mice showed lung discoloration four days post-infection and a high viral load (~1 × 10^4 PFUs) (Figures 1F and 1G). These results indicate that one or two immunizations with 1 μg RBD-NPs results in protection against SARS-CoV-2 MA10-induced disease. Furthermore, mice vaccinated twice with a 10-fold lower dose of RBD-antigen (0.1 μg) had higher

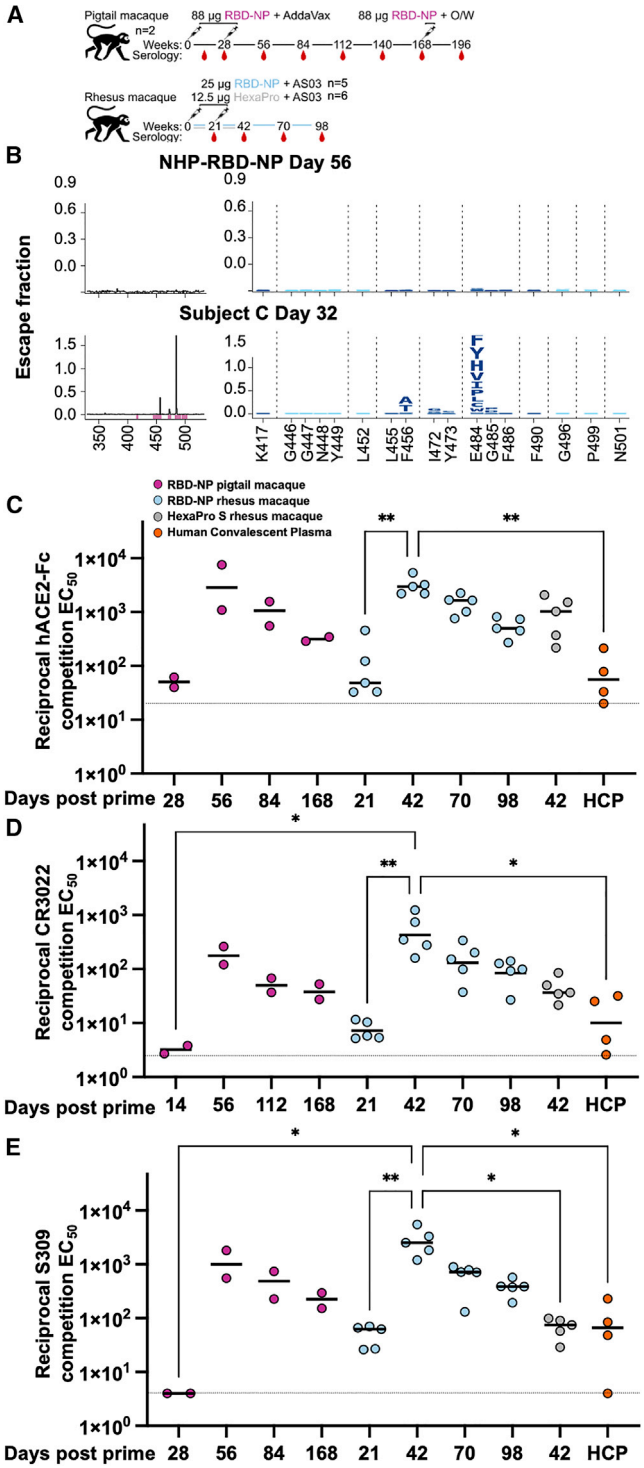


Figure 2. RBD-NP vaccination elicits high titers of Abs targeting diverse RBD antigenic sites in NHPs

(A) NHP study designs.

(B) Effect of RBD mutations on polyclonal Ab binding measured by DMS analysis of serum obtained 8 weeks post-prime from an immunized pigtail macaque (n = 1) compared to a previously reported DMS measurement from a representative COVID-19 HCP sample (reproduced here for comparison; Greaney et al., 2021b). The line plots on the left show the summed effect of all mutations at a site in the RBD on serum or plasma binding, with RBD residues on the x axis and Ab escape on the y axis (Table S1). Due to the use of sample-specific FACS gates (Figure S1), the y axes are scaled independently. Sites in the logo plots are colored dark blue if located in the receptor-binding ridge or cyan if located in the RBD 443–450 loop. Larger values indicate more Ab escape.

(C–E) Competition ELISA between 0.2-nM hACE2-Fc (LOD of 2 × 10^{1}) (C), 2-nM CR3022 mAb (LOD of 2 × 10^{0}) (D), or 0.01-nM S309 mAb (LOD of 4 × 10^{0}) (E), and RBD-NP-elicited sera in pigtail macaques (n = 2), RBD-NP-elicited sera in rhesus macaques (n = 5), or HexaPro S-elicited sera in rhesus macaques (n = 5) at various time points following vaccination, benchmarked against HCP (n = 4) (Table S2). Each plot shows the magnitude of inhibition of hACE2/mAb binding to immobilized SARS-CoV-2 S2P, expressed as reciprocal serum dilution blocking 50% of the maximum binding response. Statistical significance was determined by Kruskal-Wallis test and shown only when significant. **p < 0.01. LODs are shown as gray horizontal dotted lines.

Raw data curves shown in Data S1.

serum neutralizing Ab titers than mice receiving a single 1-µg dose, suggesting that they would likely also be protected since neutralizing Ab titers are a correlate of protection against SARS-CoV-2 (Arunachalam et al., 2021; McMahan et al., 2021). These data suggest that multiple RBD-NP dose-sparing regimens may be possible.

RBD-NP vaccination induces Abs targeting diverse antigenic sites in NHPs

To characterize the epitopes targeted by RBD-NP-elicited polyclonal Abs, we used deep mutational scanning (DMS) with sera obtained 56 days post-prime from one of two pigtail macaques immunized at days 0 and 28 with a dose of AddaVax-adjuvanted RBD-NP containing 88 µg of the SARS-CoV-2 RBD (with a linker of twelve glycine/serine (GS) residues between the RBD and the NP) (Walls et al., 2020b) (Figures 2A and 2B). These experiments rely on yeast surface display of RBD libraries covering nearly all possible amino acid mutations coupled with fluorescence-activated cell sorting (FACS) to identify RBD mutants with attenuated Ab binding compared to the wild-type (Wuhan-Hu-1) SARS-CoV-2 RBD (Greaney et al., 2020; Starr et al., 2020a). No single mutation had more than a marginal effect on serum Ab (IgG/IgM/IgA) binding, indicating broad targeting of distinct RBD epitopes, whereas several previously described COVID-19 human convalescent plasmas (HCPs) analyzed by DMS displayed greater sensitivity to individual mutations and are shown for comparison (Greaney et al., 2021b) (Figures 2B and S1; Table S1). These results show that RBD-NP vaccination elicits highly diverse polyclonal Ab responses that target multiple distinct antigenic sites and are more resilient to escape mutations reducing polyclonal Ab binding than HCP (Barnes et al., 2020; Greaney et al., 2021b; Piccoli et al., 2020; Starr et al., 2021).

To measure the magnitude of vaccine-elicited Abs against distinct RBD antigenic sites, we used quantitative competition ELISAs with human ACE2-Fc (hACE2-Fc), which binds to the receptor-binding motif (RBM) corresponding to antigenic sites Ia and Ib, as well as with structurally characterized mAbs recognizing antigenic sites II (CR3022) and IV (S309) (Huo et al., 2020; Piccoli et al., 2020; Pinto et al., 2020; Yuan et al., 2020) (Figures 2C–2E; Data S1). These experiments

used sera from the aforementioned pigtail macaques (RBD GMT EC_{50} 5×10^4) followed for 168 days and sera from 5 rhesus macaques immunized twice on days 0 and 21 with AS03-adjuvanted RBD-NPs containing 25 μg of the SARS-CoV-2 RBD (with a 16-GS linker between the RBD and the NP; RBD GMT EC_{50} 1×10^4) and followed for 98 days (Arunachalam et al., 2021; Walls et al., 2020b; Figures 2A and S2A). These two sets of RBD-NP sera were compared to sera from 5 rhesus macaques immunized twice on days 0 and 21 with 12.5 μg of AS03-adjuvanted prefusion-stabilized HexaPro S trimer (RBD EC_{50} 1×10^4) and followed for 42 days (Arunachalam et al., 2021; Hsieh et al., 2020), as well as a panel of COVID-19 HCP (Table S2) (RBD EC_{50} 2×10^4) (Figures 2A and S2A). All NHP sera post boost had high titers of Abs targeting sites Ia and Ib in the immunodominant RBM (day 42 hACE2 competition RBD-NP GMT 3×10^3 and HexaPro GMT 7×10^2) (Figure 2C; Data S1), a correlate of neutralization potency (Piccoli et al., 2020), in agreement with the potent immunogenicity and protective efficacy of RBD-NP and HexaPro S in NHPs (Arunachalam et al., 2021). We also observed strong Ab responses against antigenic sites II (RBD-NP day 42 GMT 4×10^2 and HexaPro GMT 4×10^1) with CR3022 competition and IV (RBD-NP day 42 GMT 2.5×10^3 and HexaPro GMT 6.5×10^1) (Figures 2D and 2E; Data S1), which comprise conserved sarbecovirus epitopes recognized by broadly neutralizing sarbecovirus mAbs such as S2X259 (site II) and S309 (site IV) (Piccoli et al., 2020; Pinto et al., 2020; Tortorici et al., 2021). Ab responses were durable against all 3 antigenic sites regardless of dose or adjuvant, with a decrease of competition titers by roughly half an order to one order of magnitude 168 days (pigtail macaque) or 98 days (rhesus macaque) post-prime. RBD-NP elicited much higher peak binding titers toward all RBD antigenic sites evaluated compared to HexaPro S and HCP, showcasing the potency, durability, and multi-specificity of Ab responses induced by multivalent display of the RBD, possibly as a result of enhanced accessibility of the antigen.

RBD-NP vaccine elicits potent neutralizing Ab responses in NHPs against a panel of SARS-CoV-2 S variants

To assess the neutralization breadth of RBD-NP- and HexaPro S-elicited Abs in NHPs at peak titer (42 days post-prime), we evaluated serum neutralizing activity against a panel of pseudotyped viruses comprising wild-type (D614G) SARS-CoV-2 S and twelve single-residue SARS-CoV-2 RBD mutants detected in clinical isolates (N439K, L452R, Y453F, L455F, S477N, E484A/K/Q, F486L, S494P, N501Y, and G504D) as well as the B.1.1.7 (H69-V70 deletion, Y144 deletion, N501Y, A570D, D614G, P681H, T716I, S982A, D1118H); B.1.1.7 + E484K (H69-V70 deletion, Y144 deletion, E484K, N501Y, A570D, D614G, P681H, T716I, S982A, D1118H); B.1.351 (L18F, D80A, D215G, L242-L244 deletion, R246I, K417N, E484K, N501Y, D614G, A701V); and P.1 (L18F, T20N, P26S, D138Y, R190S, K417T, E484K, N501Y, D614G, H655Y, T1027I). Several single-residue RBD mutations did not affect neutralizing titers compared to wild-type D614G SARS-CoV-2 S, for which GMTs of 1.8×10^3 and 2.4×10^3 were determined with RBD-NP- and HexaPro S-elicited sera, respectively, using a vesicular stomatitis virus (VSV) pseudotyped virus (Figure 3A; Data S1). For example, the N439K- (GMT 2.5×10^3 for RBD-NP and 2.7×10^3 for HexaPro S) and mink-associated Y453F (GMT 4×10^3 for RBD-NP and 5×10^3 for HexaPro S) mutation did not dampen the neutralization potency of either RBD-NP- or HexaPro S-elicited sera (Figure 3A; Data S1). However, the N501Y substitution (present in the B.1.1.7, P.1, and B.1.351 lineages) reduced the neutralization potency of RBD-NP- (GMT 5×10^2) and HexaPro S (GMT 8×10^2)-elicited sera 3-to-4-fold and the L452R mutation (present in the B.1.427/B.1.429, B.1.617.1, and B.1.617.22 variant) led to a 3.4-to-4.4-fold drop in neutralization potency (GMT 4×10^2 for RBD-NP and 7×10^2 for HexaPro S). These substitutions have been associated with the loss of binding and neutralization for vaccine-elicited Abs and mAbs (Collier et al., 2021; McCallum et al., 2021b; Wang et al., 2021b; Thomson et al., 2021; Wang et al., 2021c). The E484A mutation reduced serum neutralization activity up to 2-fold (GMT $1.1x10^3$ for RBD-NP and $1.1x10^3$ for HexaPro S) whereas E484K, present in B.1.351 and P1 (GMT 2×10^2 for RBD-NP and 2×10^2 for HexaPro S), and E484Q, found in B.1.617.1 (GMT 5×10^2 for RBD-NP and 3.6×10^2 for HexaPro S), decreased it 9-fold (E484K) and 4-fold (E484Q) for RBD-NP and 12-fold (484K) and 7-fold (484Q) for HexaPro S compared to D614G SARS-CoV-2 S. These experiments suggest that a substantial fraction of the neutralizing activity elicited by both RBD-NP and HexaPro S is focused on the RBM, especially near position 484, as further supported by the ~5-fold decrease in neutralization resulting from the F486L (GMT 3.7×10^2 for RBD-NP and 5×10^2 for HexaPro S) substitution.

To understand the impact of the full constellation of mutations present in the S proteins of the aforementioned VOCs, we evaluated RBD-NP- and HexaPro S-elicited serum neutralizing activity against corresponding HIV and VSV pseudotyped variants (D614G GMT RBD-NP HIV 5×10^3 and VSV RBD-NP 6×10^2, respectively, and D614G GMT HexaPro S HIV 3.5×10^3 and VSV 1.2×10^3) (Figures 3B and 3C). Although we did not observe major reductions in neutralization titers (up to to 2-fold) toward the B.1.1.7 VOC (GMT RBD-NP HIV 2.5×10^3, VSV 4.7×10^2 and HexaPro S HIV 1.8×10^3, VSV 8.8×10^2) (Figures 3B and 3C), addition of the E484K mutation to the B.1.1.7 background reduced neutralization 4-to-6-fold for RBD-NP- (GMT HIV 8.8×10^2) and HexaPro S-elicited sera (GMT HIV 8.6×10^2), confirming the importance of the 484 position (Figure 3B). Neutralization of B.1.351 was 10-fold lower using HIV pseudovirus (GMT RBD-NP 5×10^2 and HexaPro S $3.6x10^2$) (Figure 3B) and ~3-fold lower using VSV pseudovirus (GMT RBD-NP 2×10^2 and HexaPro S $4x10^2$) (Figure 3C) for both RBD-NP- and HexaPro-elicited sera, in agreement with authentic virus neutralization data (Arunachalam et al., 2021). Interestingly, RBD-specific binding Ab titers did not differ significantly between the B.1.351 RBD (GMT RBD-NP 6.5×10^3 and HexaPro S 3×10^3) and Wuhan-Hu-1 RBD (GMT RBD-NP 1.4×10^4 and HexaPro S 1.7×10^4) (Figure S2A), indicating that neutralizing activity is accounted for by a portion of total binding Abs. Reductions in neutralizing Ab titers were also observed against P.1 S VSV pseudoviruses, with a 6-fold drop for RBD-NP (GMT 1×10^2) and 8-fold dampening for HexaPro S (GMT 1.5×10^2) (Figure 3C). Furthermore, we observed a 10-fold reduction in plasma

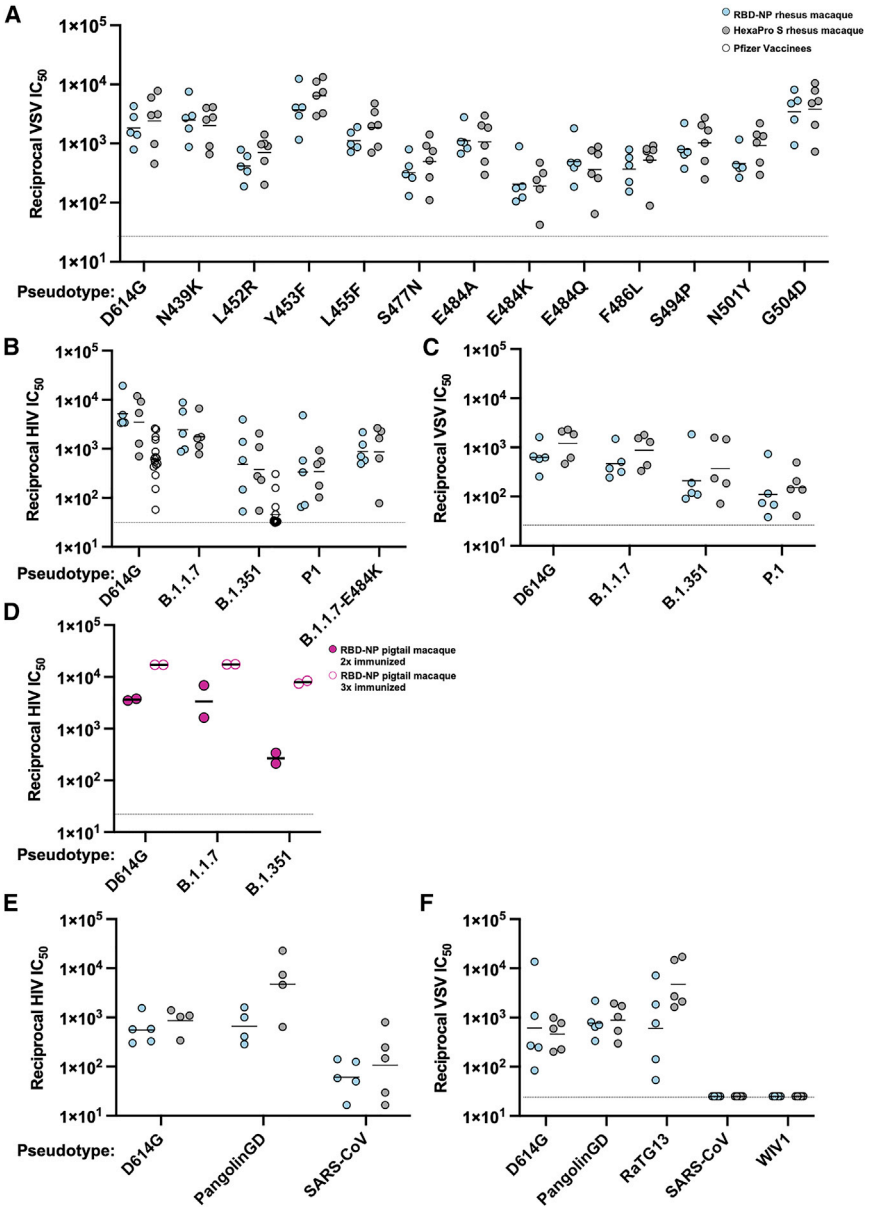


Figure 3. RBD-NP and HexaPro S elicit Abs with similar neutralization breadth toward SARS-CoV-2 variants

(A) Neutralizing Ab titers against wild-type (D614G) SARS-CoV-2 S and RBD point mutants determined using RBD-NP-elicited sera in rhesus macaques (blue, n = 5) and HexaPro S-elicited sera in rhesus macaques (gray, n = 6) with an VSV pseudotyping system with an LOD of 3.3 × 10^1. Neutralization performed once.

(B) Neutralizing Ab titers against HIV pseudotyped viruses harboring wild-type (D614G) SARS-CoV-2 S, B.1.1.7 S, B.1.1.7-E484K S, B.1.351 S, or P.1 S, determined using RBD-NP-elicited sera in rhesus macaques (blue), HexaPro S-elicited sera in rhesus macaques (gray), or plasma from individuals who received two doses of Pfizer mRNA vaccine (open circles) with an LOD of 3.3 × 10^1. Neutralization performed twice and a representative shown.

(C) Neutralizing Ab titers against VSV pseudotyped viruses harboring wild-type (D614G) SARS-CoV-2 S, B.1.1.7 S, B.1.351 S, or P.1 S, determined using RBD-NP-elicited sera in rhesus macaques (blue) or HexaPro S-elicited sera in rhesus macaques (gray) with an LOD of 2.5 × 10^1. Neutralization performed twice and a representative shown.

(D) Neutralizing Ab titers against D614G SARS-CoV-2 S, B.1.1.7, and B.1.351 S HIV pseudoviruses in pigtail macaque sera collected 28 days after a second (filled symbols, n = 2) or third (open symbols, n = 2) immunization with 88-μg RBD-NP (RBD antigen dose) with an LOD of 1 × 10^2. Neutralization performed twice and a representative shown.

(E) Neutralizing Ab titers against HIV pseudotyped viruses harboring wild-type (D614G) SARS-CoV-2 S, Pangolin-GD S, or SARS-CoV S, determined using RBD-NP-elicited sera in in rhesus macaques (blue) or HexaPro S-elicited sera in rhesus macaques (gray) with an LOD of 1 × 10^1. Neutralization performed twice and a representative shown.

(F) Neutralizing Ab titers against VSV pseudotyped viruses harboring wild-type (D614G) SARS-CoV-2 S, Pangolin-GD S, RaTG13 S, SARS-CoV S, or WIV1 S determined using SARS-CoV-2 RBD-NP-elicited sera in rhesus macaques (blue) or HexaPro S-elicited sera in rhesus macaques (gray) with an LOD of 2.5 × 10^1. Neutralization performed twice and a representative shown. Statistical significance was determined by Kruskal-Wallis test and shown in Table S3. LODs are shown as gray horizontal dotted lines. Raw data curves shown in Data S1 and GMTs in Table S4. The various pseudovirus backbones were benchmarked against NIBSC standard and are shown in Table S5.

neutralizing potency for individuals who received two doses of the Pfizer-BioNTech BNT162b2 mRNA vaccine against B.1.351 S pseudotyped virus (GMT 6 × 10^1) relative to the wild-type (D614G) SARS-CoV-2 S (GMT 6 × 10^2) pseudotyped virus (Figure 3B; Data S1). These findings show that, as is the case for many convalescent individuals (Greaney et al., 2021b; Liu et al., 2021; Piccoli et al., 2020), an important fraction of vaccine-elicited neutralizing Abs in NHPs and humans is focused on the RBM (specifically around position 484), independently of the immunogen (RBD-NP, HexaPro S, or 2P-stabilized S [Pallesen et al., 2017]) or the vaccine modality (protein subunit or mRNA). However, we note that neutralization assays may underestimate the contribution of NTD-specific or non-RBM RBD-targeted neutralizing Abs (Lempp et al., 2021; McCallum et al., 2021a; Suryadevara et al., 2021), and protection from challenge will be the ultimate readout.

To further investigate the relationships between neutralizing Ab titers and emerging SARS-CoV-2 variants, we immunized the two pigtail macaques a third time with RBD-NP, formulated with an oil-in-water (O/W) emulsion-based adjuvant, 168 days after the primary immunization (Figure 2A). This boost induced potent serum neutralizing activity against wild-type (D614G)

SARS-CoV-2 (GMT 2 × 10^4) (Figure 3D) as well as the B.1.1.7 (GMT 2 × 10^4) and B.1.351 (GMT 8 × 10^3) VOC pseudoviruses, suggesting that an overall increase in neutralizing Ab titers may be a suitable strategy to cope with emerging variants. These results are consistent with recent studies demonstrating that boosting COVID-19 convalescent individuals with a single mRNA vaccination elicited high (neutralizing) Ab titers, including against the B.1.351 variant (Abu Jabal et al., 2021; Krammer et al., 2021; Stamatatos et al., 2021), and suggest that a third vaccination of naive individuals could be a suitable strategy to limit the impact of emerging variants. This strategy is currently being evaluated for immunocompromised patients in several countries.

RBD-NP vaccine elicits cross-reactive sarbecovirus polyclonal Abs in NHPs

As RBD-NP- and HexaPro S-elicited polyclonal Abs exhibited similar resilience to a range of mutations, we next investigated cross-reactivity with a panel of sarbecovirus RBDs. RBD-NP-elicited polyclonal Abs purified from pigtail macaque serum obtained 70 days post-prime strongly cross-reacted with the SARS-CoV-2-related Pangolin-GD and RaTG13 RBDs and bound more weakly to distantly related RmYN02, SARS-CoV, WIV16, and ZXC21 RBDs (Figures S2B and S2C). Measurement of Ab binding titers using the SARS-CoV and SARS-CoV-2 S2P ectodomain trimers by ELISA showed that RBD-NPs and HexaPro S induced similar levels of cross-reactive Abs against each antigen, with responses roughly two orders of magnitude higher against SARS-CoV-2 S2P (detection antigen matching immunogen) compared to SARS-CoV S2P (Figure S2D; Data S1). The two immunogens also elicited similar peak levels of SARS-CoV S ACE2-competing Abs (GMT 2 × 10^2 RBD-NP and 5 × 10^1 HexaPro) suggesting that cross-neutralizing Abs might have been induced to similar extents between the two vaccines (Figure S2E).

Motivated by the cross-reactivity of RBD-NP-elicited polyclonal Abs with various sarbecovirus RBDs and the correlation between ACE2 competition and serum neutralization titers (Piccoli et al., 2020), we evaluated neutralization of a panel of HIV (D614G GMT 5.5 × 10^2) and VSV (D614G GMT 6 × 10^2) pseudoviruses harboring sarbecovirus S glycoproteins. RBD-NP-elicited sera efficiently neutralized pseudotyped viruses harboring the S glycoprotein of the Pangolin-GD isolate (GMT 6.6 × 10^2 and 7.7 × 10^2 VSV) and RaTG13 (GMT VSV 6 × 10^2) (Figures 3E and 3F) (Lam et al., 2020; Zhou et al., 2020b), in agreement with the close phylogenetic relationship of their RBDs with that of SARS-CoV-2 S (Figure S2B). HexaPro S-elicited NHP sera also efficiently neutralized Pangolin-GD S (GMT HIV 4.7 × 10^3 and VSV 9 × 10^2) and RaTG13 S pseudotypes (GMT VSV 4.7 × 10^3) (Figures 3E and 3F). Furthermore, we observed that both RBD-NP- and HexaPro S-induced polyclonal Abs that weakly neutralized HIV pseudovirus carrying SARS-CoV S (RBD-NP GMT 6 × 10^1 and HexaPro GMT 1 × 10^2) (Figures 3E and 3F). Collectively, these data demonstrate that both immunogens elicited comparable neutralization breadth and potency against the pseudoviruses tested, reinforcing the notion that most sarbecovirus S-directed neutralizing Abs target the RBD (Greaney et al., 2021a, 2021b; Piccoli et al., 2020).

Design, assembly, and characterization of mosaic and cocktail sarbecovirus RBD-NPs

We and others have recently evaluated nanoparticle immunogens that display multiple antigenic variants of viral glycoproteins as a potential route toward broadly protective vaccines (Boyoglu-Barnum et al., 2021; Cohen et al., 2021a, 2021b; Kanekiyo et al., 2019). Given the large number of coronaviruses circulating in zoonotic reservoirs, such vaccines could be important for understanding vaccine-induced neutralization/protection breadth and preventing future pandemics (Menachery et al., 2015, 2016). We expressed and purified four RBDs from the S proteins of SARS-CoV-2, SARS-CoV, and the bat coronaviruses WIV1 and RaTG13 genetically fused to the I53-50A trimer. An equimolar mixture of these four proteins was added to the I53-50B pentamer to assemble a mosaic RBD-NP (mRBD-NP) co-displaying the four RBDs on the same nanoparticle (Figure 4A and S3A). We also assembled a trivalent mosaic RBD-NP designated "drop out" lacking the SARS-CoV RBD (mRBD-NP-DO), as well as cocktail immunogens with three (cRBD-NP-DO; lacking the SARS-CoV RBD) or four (cRBD-NP) independently assembled nanoparticles, each displaying a single type of the aforementioned RBDs, mixed after independent assembly. Finally, we made a bivalent mosaic RBD-NP co-displaying the SARS-CoV and SARS-CoV-2 RBDs (Figure S3A) to directly confirm co-display on the nanoparticles using a sandwich binding assay. All of the nanoparticle immunogens formed the intended icosahedral architecture and retained native antigenicity, as shown by SDS-PAGE, dynamic light scattering, negative staining electron microscopy, and binding to hACE2-Fc (Figures S3B–S3E). We found that the vaccine candidates were stable for at least 4 weeks at several temperatures except the highest temperature evaluated (37°C), at which we observed a decrease in hACE2 recognition over time beginning after 7 to 14 days, presumably due to aggregation (Figures 4B–4E). Following immobilization using the SARS-CoV-2-specific mAb S2H14 (Piccoli et al., 2020), the quadrivalent, trivalent, and bivalent mRBD-NPs all bound the Fab of the SARS-CoV-specific Ab S230 (Piccoli et al., 2020; Rockx et al., 2008; Walls et al., 2019), confirming co-display, whereas the monovalent SARS-CoV-2 RBD-NP did not (Figure S3G). We determined that the reactivity of the trivalent mRBD-NP derived from the inclusion of the WIV1 RBD in this vaccine, as the monovalent WIV1 RBD-NP also bound the S230 Fab after immobilization with hACE2 (as expected [Menachery et al., 2016]), whereas the monovalent SARS-CoV-2 and RaTG13 RBD-NPs did not (Figure S3H). Collectively, these data demonstrate that all of the nanoparticles were stable and (co-)displayed the various RBDs as intended.

Cocktail and mosaic RBD-NP vaccines elicit cross-reactive and broadly neutralizing sarbecovirus Abs

The mosaic and cocktail nanoparticle immunogens were compared to monovalent RBD-NP vaccines and a non-assembling control vaccine comprising all four RBD-I53-50A trimeric components and a non-assembling I53-50B pentamer (Figure S3F) in an immunization study in BALB/c cByJ mice (Figures 4A and S3A). All immunizations comprised 1 μg of total RBD antigen, such that ~0.25 μg of each RBD was given in each administration of the quadrivalent vaccines

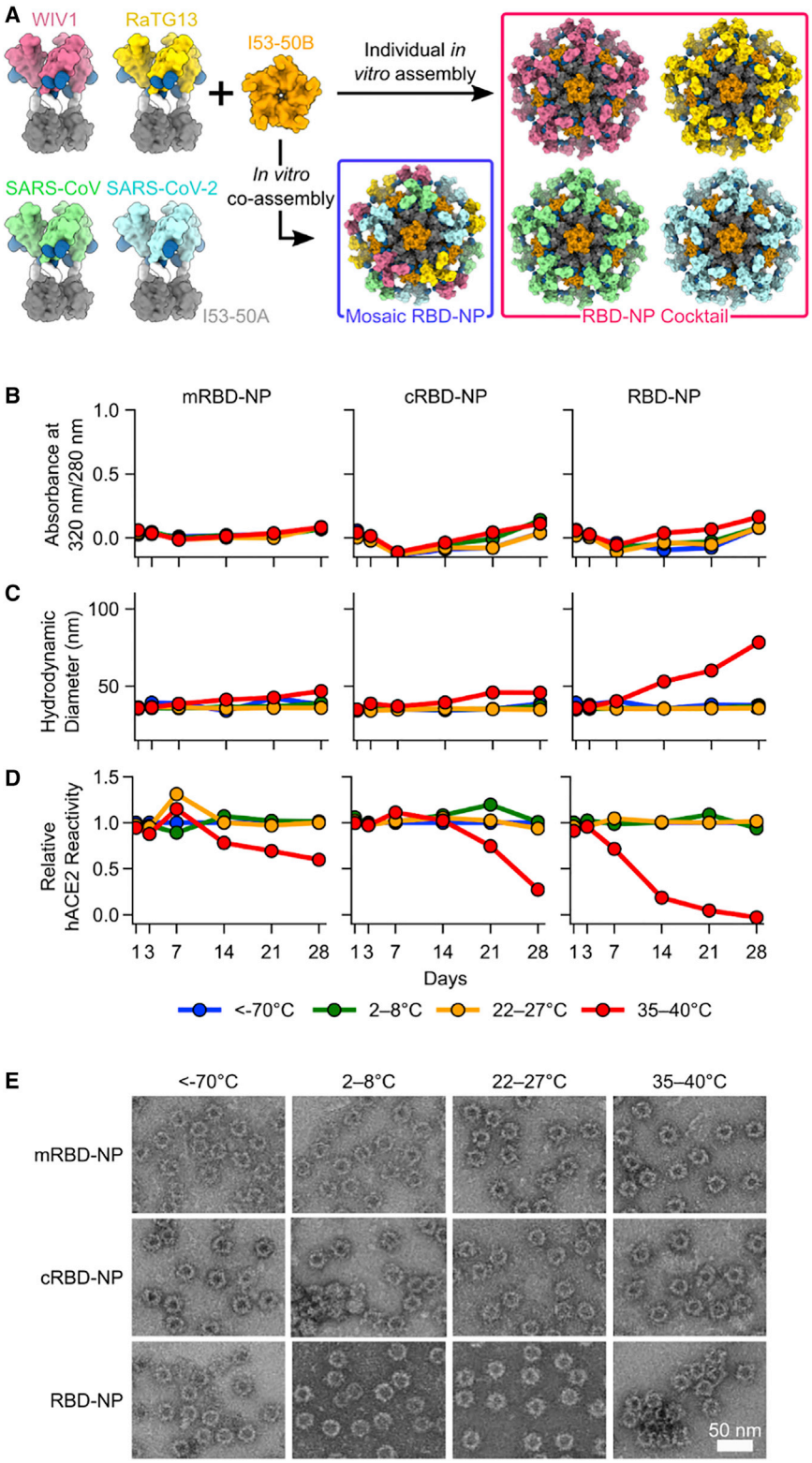


Figure 4. *In vitro* assembly and accelerated stability studies of mosaic and cocktail nanoparticle immunogens

(A) Schematic of *in vitro* assembly of mRBD-NPs and cRBD-NPs.

(B–E) The physical and antigenic stability of mRBD-NP, cRBD-NP, and (SARS-CoV-2) RBD-NP samples incubated at four different temperatures was followed for four weeks.

(B) The ratio of UV/vis absorbance at 320 nm/280 nm is a measure of turbidity (proxy for aggregation).

(C) Hydrodynamic diameter of the nanoparticles measured using dynamic light scattering.

(D) hACE2-Fc binding measured by comparing the peak amplitude of hACE2-Fc binding for each sample to a reference sample stored at < −70°C using biolayer interferometry.

(E) Electron micrographs of negatively stained samples after incubation for 28 days at the indicated temperatures. Scale bar, 50 nm.

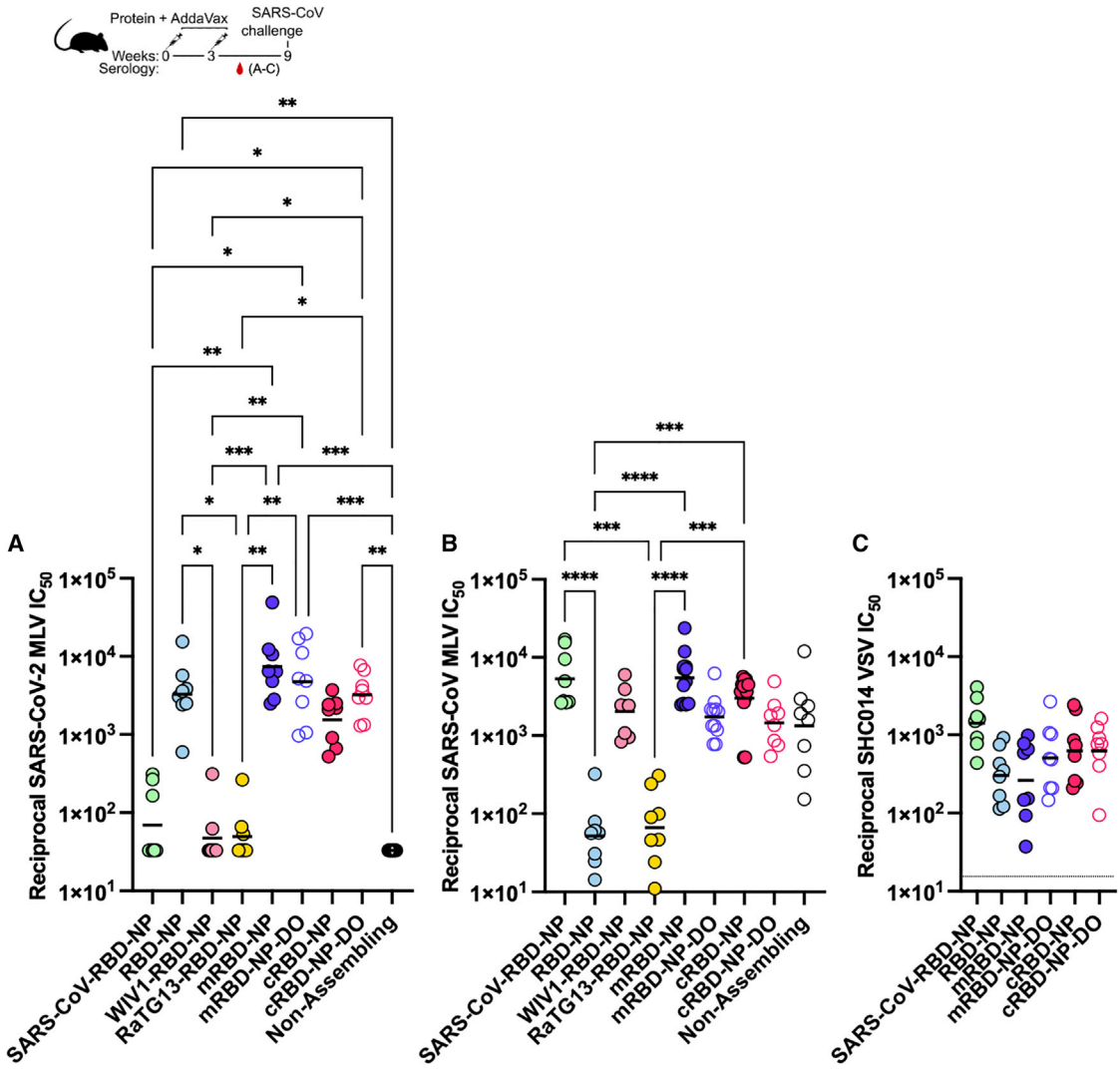


Figure 5. Mosaic and cocktail RBD-NP vaccines elicit neutralizing Abs against multiple sarbecoviruses

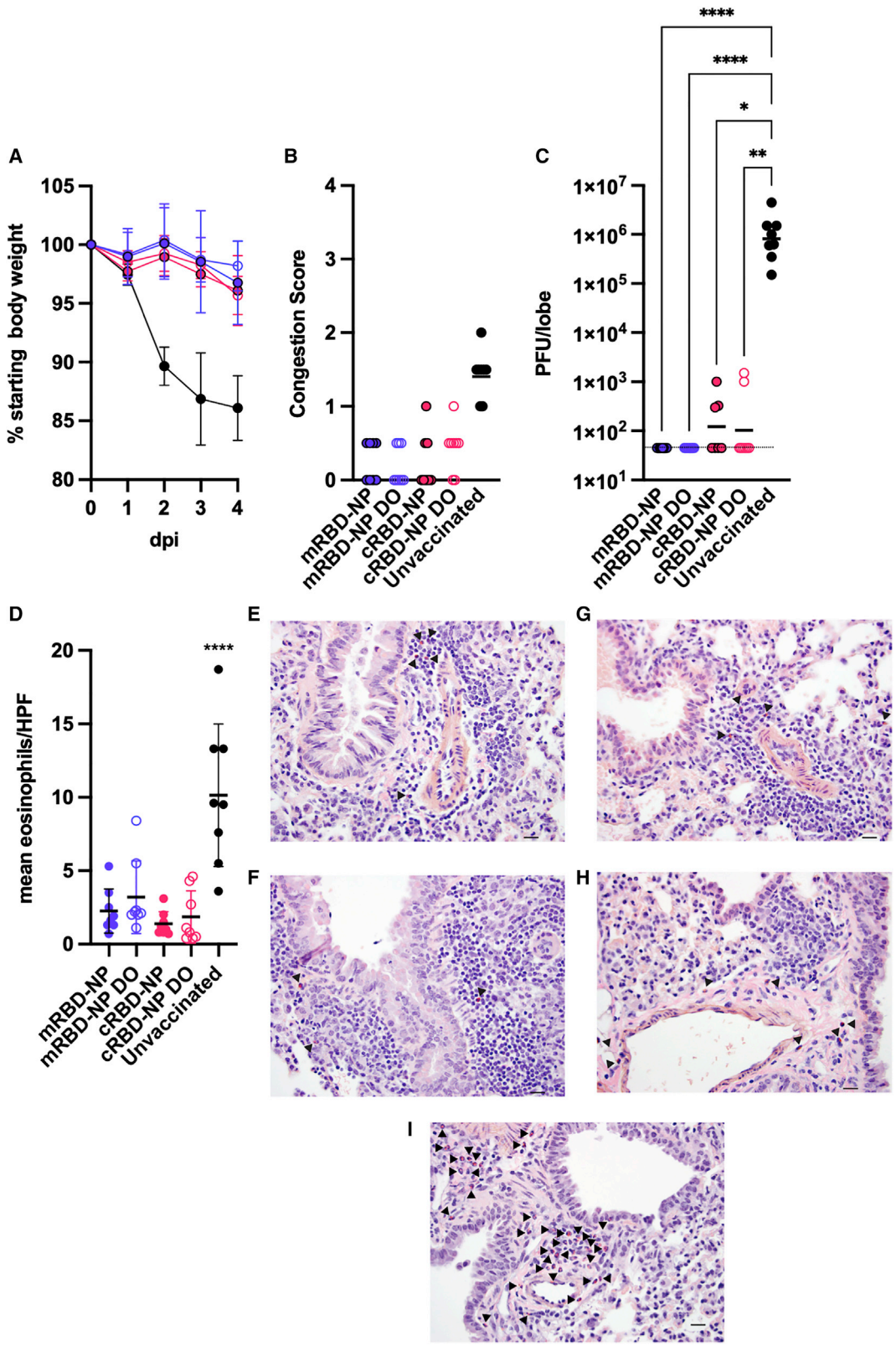
(A) Neutralizing Ab titers in mice (n = 8) against wild-type (D614G) SARS-CoV-2 S MLV pseudovirus five weeks post-prime elicited by monovalent, mosaic, and cocktail RBD-NPs with an LOD of 1×10^1.

(B) Neutralizing Ab titers in mice against SARS-CoV S MLV pseudovirus five weeks post-prime elicited by monovalent, mosaic, and cocktail RBD-NPs with an LOD of 1×10^1.

(C) Neutralizing Ab titers in mice against SHC014 VSV pseudovirus five weeks post-prime elicited by monovalent, mosaic, and cocktail RBD-NPs with an LOD of 1.7×10^1.

Raw data curves shown in Data S1. Statistical significance was determined by Kruskal-Wallis test and shown only when significant. **p<0.01. LOD is shown as a gray horizontal dotted line in (C).

and ~0.33 μg of each RBD in the trivalent vaccines. After two immunizations, all four mosaic or cocktail RBD-NP vaccines elicited strong binding (GMT ~1–5 × 10^4) (Figure S4A; Data S1) and potent serum neutralizing (GMT 2–8 × 10^3; Figure 5A; Data S1) Ab titers against wild-type (D614G) SARS-CoV-2 S pseudovirus. Competition ELISAs using hACE2, CR3022, S309, and S2X259 demonstrated that all mosaic and cocktail vaccine candidates elicited robust Ab titers targeting all antigenic sites tested, showcasing the diversity of RBD-specific Abs elicited (Figures S4B–S4E; Data S1). The neutralizing SARS-CoV-2 S (D614G) Ab responses were slightly higher for mRBD-NPs and slightly lower for cRBD-NPs than that of the monovalent RBD-NP (GMT 3 × 10^3) (Figure 5A), similar to the aforementioned low-dose RBD-NP immunization study (Figure 1D) and suggesting that the dose of strain-matched antigen in the multivalent vaccines is not a limiting factor of the magnitude of neutralizing Ab responses. The neutralizing activity against SARS-CoV-2 elicited by the other monovalent RBD-NPs and the non-assembling control were ~2 orders of magnitude lower (GMT < 5 × 10^1) than for the monovalent RBD-NP, indicating poor elicitation of cross-neutralizing Abs by these heterologous monovalent RBD-NPs.



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Although ELISA binding titers were comparable across mosaic and cocktail groups against SARS-CoV S2P (GMT 3–10 × 10^3), the corresponding pseudovirus neutralization titers showed more nuanced patterns (Figures 5B and S4F; Data S1). Tetravalent mosaic and cocktail RBD-NPs elicited potent neutralizing activity (GMT 3–5.5 × 10^3) with magnitudes roughly comparable to that of the monovalent SARS-CoV-RBD-NP (GMT 5 × 10^3) (Figure 5B). Strikingly, the trivalent nanoparticle immunogens (mRBD-NP-DO and cRBD-NP-DO)—which did not contain the SARS-CoV RBD—also elicited potent neutralization (GMT ~1–2 × 10^3). This cross-neutralization likely arose from the inclusion of the WIV1 RBD (Figure S4G; Data S1) in the trivalent immunogens, as WIV1 cross-reacts with a SARS-CoV-specific mAb (Figure S3H) (Menachery et al., 2016), and the monovalent WIV1-RBD-NP induced similar levels of pseudovirus neutralization (GMT 2 × 10^3) (Figure 5B; Data S1). The non-assembling control immunogen, which contains all four RBD-I53-50A trimeric components, also elicited substantial neutralizing activity against SARS-CoV (GMT 1 × 10^3) but not against SARS-CoV-2. Furthermore, we show that sera elicited by the monovalent, mRBD-NPs, and cRBD-NPs neutralize SHC014 pseudotyped virus, a distantly related sarbecovirus sharing 76%, 76%, 82%, and 84% amino acid sequence identity with the SARS-CoV-2, RaTG13, SARS-CoV, and WIV1 RBDs, respectively (Figure 5C; Data S1). These data show that both mRBD-NPs and cRBD-NPs are promising vaccine candidates for eliciting broad sarbecovirus immunity, in agreement with previous findings using a different nanoparticle platform (Cohen et al., 2021a) or chimeric sarbecovirus S glycoproteins (Martinez et al., 2021b) and recent results obtained with influenza virus hemagglutinin nanoparticle vaccines (Boyoglu-Barnum et al., 2021).

Mosaic RBD-NPs protect mice against heterotypic SARS-CoV challenge

To gauge the ability of the multivalent RBD-NPs to confer protection against vaccine-matched and heterotypic sarbecoviruses, we challenged groups of eight mice with a high dose (10^5 PFUs) of the mouse-adapted SARS-CoV MA15 virus (Roberts et al., 2007). In agreement with the pseudovirus neutralization data, animals immunized with mRBD-NP, mRBD-NP-DO, cRBD-NP, and cRBD-NP-DO were protected from weight loss (ranging between 1% and 5%) and serious lung pathology throughout the four days of the experiment (Figures 6A and 6B). Unvaccinated mice exhibited up to 15% average weight loss and signs of lung pathology (congestion score ~1.5) that were only minorly seen in all other vaccinated groups (Figures 6A and 6B). mRBD-NP- and mRBD-NP-DO-vaccinated mice were completely protected from viral replication in the lungs (Figure 6C). Overall, the trivalent mRBD-NP-DO provided protection that was indistinguishable from the tetravalent mRBD-NP, despite lacking the SARS-CoV RBD. Five out of eight and six out of eight cRBD-NP- and cRBD-NP-DO-vaccinated mice were completely protected from viral replication in the lungs, respectively, with viral burden ranging between 10^2 and 10^3 PFUs/lobe for the remaining animals (compared to unvaccinated mice with ~10^6 PFUs/lobe, Figure 6C). To assess whether the RBD-NP platform causes vaccine-induced immune pathology, which was previously described with double-inactivated SARS-CoV vaccines (Bolles et al., 2011), we investigated eosinophil infiltration and inflammation in the lungs following SARS-CoV MA15 challenge. Although infection induced inflammation and infiltration of eosinophils in the lungs of unvaccinated mice (Figures 6D, 6I, and S5A), no signs of eosinophilia were detected for any animals in the vaccinated groups using histological analysis of stained lung sections (Figures 6D–6I and S5A). These data show that mRBD-NP, mRBD-NP-DO, cRBD-NP, and cRBD-NP-DO protect against weight loss, lung injury, and viral replication following challenge, with no detectable vaccine-induced immune pathology.

To identify the gain in protective breadth provided by multivalent display of sarbecovirus RBD-NP, relative to monovalent RBD-NPs, we performed a second SARS-CoV MA15 challenge experiment including monovalent RBD-NP and SARS-CoV-RBD-NP (Figure S5B). In agreement with the pseudovirus neutralization data and first challenge (Figures 4B–4D and 6A–6C), animals immunized with the SARS-CoV-RBD-NP, mosaic, and cocktail RBD-NP formulations, as well as the non-assembling control immunogen, were protected from weight loss and serious lung pathology throughout the four days of the experiment. The animals receiving the monovalent RBD-NP vaccine experienced up to 12% average weight loss, whereas the unvaccinated mice exhibited further (up to 15%) average weight loss and signs of lung pathology (congestion score ~2) that were only minorly seen in all other vaccinated groups (Figures S5B and S5C). All mice vaccinated with SARS-CoV-RBD-NP, RBD-NP, and mRBD-NP were protected from viral replication in the lungs, whereas we detected ~$10^{2.5}$ and 10^6 PFUs/lobe for half of the mice receiving the non-assembling control immunogen and unvaccinated mice, respectively (Figure S5D). These results provide proof-of-principle that mosaic and cocktail RBD nanoparticle vaccines elicit broad protection against heterotypic sarbecovirus challenge and could represent the next generation of vaccines developed in anticipation of future spillovers.

Figure 6. Mosaic and cocktail RBD-NP vaccines protect against heterotypic SARS-CoV-MA15 challenge in 15-week-old BALB/c cByJ mice

(A) Weight loss following SARS-CoV MA15 challenge up to 4 days post infection (n = 8). Unvaccinated animals are shown as black circles.

(B) Congestion score following SARS-CoV MA15 infection with a score of 0 indicating unchanged lung color and 4 indicating a darkened and diseased lung (n = 8).

(C) Viral titers in mice lungs (expressed in PFUs per lobe) following challenge (n = 8) with an LOD of 9 × 10^1. LOD is shown as a gray horizontal dotted line. Statistical significance was determined by Kruskal-Wallis and shown when significant. **$p < 0.01$.

(D) Mean eosinophils per high power field (HPF) per sample run over 10 HPF per lung stained with congo red. Significance was determined using one-way ANOVA and shown where significant.

(E–I) Histological analysis of stained lung sections for mRBD-NP (E), mRBD-NP-DO (F), cRBD-NP (G), cRBD-NP-DO (H), and unvaccinated mice (I). Arrowheads indicate eosinophils. Scale bar, 20 μm.

DISCUSSION

The data presented here show that two ultra-low dose immunizations or a single immunization with RBD-NP produces potent neutralizing Ab responses in mice. The latter strategy confers protection against SARS-CoV-2 MA10 challenge, suggesting that the nanoparticle platform could enable dose-sparing regimens to achieve global vaccination, especially given the high shelf stability of this vaccine (Walls et al., 2020b). NHP vaccination with RBD-NP was concurrently shown to also elicit CD4 T cell responses and to protect from viral replication (Arunachalam et al., 2021). Moreover, RBD-NP vaccination, which is currently under phase I/II evaluation in the clinic, elicits diverse Ab responses neutralizing a broad spectrum of SARS-CoV-2 variants detected in clinical isolates with similar potency to those elicited by HexaPro S vaccination. Several RBM mutations, including at position E484, however, lead to reductions in neutralizing activity elicited by either RBD-NP or HexaPro S in NHPs. Although both RBD-NP- and HexaPro S-elicited sera robustly neutralize the B.1.1.7 S variant, which does not contain the E484 substitution, neutralization of the B.1.351, P.1, and B.1.1.7/E484K variants was dampened similarly to sera from individuals vaccinated twice with the Pfizer-BioNTech BNT162b2 mRNA. These findings are in agreement with reports showing that the serum neutralizing activity against the B.1.351 variant from mRNA-1273-vaccinated individuals was comparably reduced (Wu et al., 2021), as was also the case for neutralization of authentic SARS-CoV-2 B.1.351 by HCP (Wibmer et al., 2021). Collectively, these data indicate that a significant fraction of vaccine-elicited neutralizing activity is directed to the RBM, which is the target of potent neutralizing Abs, irrespective of the antigen design strategy (RBD- or S-based), the vaccine modality (protein subunit or mRNA), or species (NHPs or humans). We show that receiving a third immunization, even of an unaltered vaccine, improves the neutralizing Ab responses to VOCs, which is reminiscent of what has been shown in vaccinated humans previously infected with SARS-CoV-2 (Abu Jabal et al., 2021; Krammer et al., 2021; Stamatatos et al., 2021), suggesting that further booster immunizations may limit the impact of VOCs without vaccine update.

The ongoing global spread of SARS-CoV-2 and the circulation of a large number of sarbecoviruses in bats (Menachery et al., 2015, 2016) and other animal reservoirs strongly motivate the development of vaccines that protect against a broad spectrum of coronaviruses. We observed that vaccination of NHPs with RBD-NPs or HexaPro S induced comparable but moderate neutralization breadth against genetically distinct sarbecoviruses. We demonstrate here that co-display (mRBD-NP) and co-immunization (cRBD-NP) of multivalently arrayed sarbecovirus RBDs elicit robust neutralizing Ab responses against SARS-CoV-2, SARS-CoV, and SHC014 pseudotyped viruses and outperform their monovalent vaccine counterparts. The observation that mosaic and cocktail RBD-NPs elicit greater titers of Abs competing with the broadly neutralizing sarbecovirus mAb S2X259 than monovalent vaccines suggest that this vaccine design strategy could enhance neutralization breadth. Accordingly, mice vaccinated with multivalent vaccines were protected from disease upon SARS-CoV MA15 challenge, including with formulations that did not include the SARS-CoV RBD, whereas RBD-NP-vaccinated mice experienced weight loss. Previous studies showed that double-inactivated SARS-CoV vaccines could lead to eosinophil infiltration in the lungs and subsequent immune pathology (Bolles et al., 2011). Our data show that none of the multivalent vaccine candidates evaluated here induced eosinophilia whereas SARS-CoV MA15 challenge did, pointing to the safety and efficacy of these immunogens. Our data highlight the potential of this approach to achieve broad sarbecovirus immunity, overcoming both the emergence of SARS-CoV-2 variants and putative future zoonosis of genetically distinct sarbecoviruses not included in the vaccine. Based on the RBD-centric nature of neutralizing Ab responses resulting from infection and vaccination (Dejnirattisai et al., 2021; Greaney et al., 2021a, 2021b; Piccoli et al., 2020), irrespective of immunogen format or vaccine modality, and the enhanced elicitation of Abs targeting all RBD antigenic sites for multivalent RBD-NPs compared to S-based vaccines, this study paves the way for advancing RBD pan-sarbecovirus subunit vaccines to the clinic.

Limitations of study

Due to the cost of challenge and BSL-3 vivarium limitations, the mice vaccinated with a low dose of RBD-NP were not challenged, and therefore we can only infer the likelihood of protection from their sera's ability to neutralize SARS-CoV-2 pseudovirus better than other mice (i.e., those given a single vaccine dose) that were challenged. Neutralization is a correlate of protection in NHPs (Arunachalam et al., 2021), but we recognize that neutralization assays using HEK-ACE2 cell lines may underestimate the effects of non-RBM targeting Abs (Lempp et al., 2021; McCallum et al., 2021a; Suryadevara et al., 2021). Due to the limitations of the number of animals and the cost of challenge, we cannot challenge with all desired point mutant or variant strains and must use neutralization as the best correlate for interpreting the expected challenge outcome.

STAR★METHODS

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● QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.cell.2021.09.015.

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AUTHOR CONTRIBUTIONS

Conceptualization, A.C.W., P.S.A., H.K., B.P., N.P.K., D.V.; modeling and design, A.C.W., N.P.K., D.V.; formal analysis, A.C.W., M.C.M., A.S., A.G., M.A.T., J.B., B.F., M.J.N., S.W., T.S., R.R., C.S., L.C., N.P.K, D.V.; resources, M.A.O., P.S.A., S.W.T., R.R., D.T.O., R.V.D.M., R.S.B., W.V.W., D.C., C.-L.H., J.S.M., D.H.F., F.V., B.P.; writing – original draft, A.C.W., N.P.K., D.V.; writing – review & editing, all authors; visualization, A.C.W., M.C.M., A.S., A.G., N.B., N.P.K., R.S.B., D.V.; supervision, D.H.F., J.S.M., L.C., R.S.B., T.P.S., B.P., N.P.K., D.V.; funding acquisition, J.S.M., R.S.B., N.P.K., D.V.

DECLARATION OF INTERESTS

A.C.W., N.P.K., and D.V. are named as inventors on patent applications filed by the University of Washington based on the studies presented in this paper. N.P.K. is a co-founder, shareholder, paid consultant, and chair of the scientific advisory board of Icosavax, Inc. and has received an unrelated sponsored research agreement from Pfizer. D.C. is an employee of Vir Biotechnology and may hold shares in Vir Biotechnology. D.V. is a consultant for and has received an unrelated sponsored research agreement from Vir Biotechnology, Inc. R.R., D.T.O., and R.V.D.M. are employees of GlaxoSmithKline. C.-L.H. and J.S.M. are inventors on US patent application no. 63/032,502, "Engineered Coronavirus Spike (S) Protein and Methods of Use Thereof". R.S.B. has collaborative research agreements with Takeda, Pfizer, Eli Lily, Gilead, Ridgeback Biosciences, and VaxArt, unrelated to the current research. The other authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
CR3022	ter Meulen et al., 2006	N/A
S309	Pinto et al., 2020	N/A
S2X259	Tortorici et al., 2021	N/A
S2H14	Piccoli et al., 2020	N/A
S230	Rockx et al., 2008	N/A
Goat anti-mouse HRP	Invitrogen	Cat #626520; Lot #TG275230; AB_2533947
Goat anti-human HRP	Invitrogen	Cat #A18817; Lot #65-180-071919; AB_2535594
Streptavidin-HRP	Thermo Scientific	Cat #N100
CD45/LCA-A700-C61	BD Biosciences	Cat#5605010
CD11c-BV501	Biolegend	Cat#117338; AB_2562016
SiglecF-BV650	BD Biosceinces	Cat#740557; AB_2740258
I1-Hybridoma	ATCC	CRL-2700
Biological samples		
BALB/c mice	Jackson Laboratory	Cat#000651
Pigtail macaques (Macaca nemestrina)	WANPRC	
Rhesus macaques (Macaca mulatta)	NIRC	
20/130 COVID-19 plasma	NIBSC	Sample#20/130
VSV (G*ΔG-luciferase)	Kaname et al., 2010	N/A
Chemicals, peptides, and recombinant proteins		
AddaVax adjuvant	InvivoGen	Cat# vac-adx-10
AS03	GSK	N/A
O/W	VFI	N/A
TMB	SeraCare	Cat# 5120-0083
Thrombin	Sigma	Cat# T9326-150UN
Immobilized Papain	ThermoScientific	Cat# 20341
LysC-endoproteinase	NEB	Cat# P8109S
EZ-Link Sulfo-NHS-LC Biotinylation Kit	Thermo Fisher Scientific	Cat#21435
Experimental models: Cell lines		
Expi 293F	ThermoFisher	Cat #A14527
HEK-ACE2 adherent	BEI (Gift from Bloom lab)	Sample#NR-52511
HEK293T/17 Adherent	ATCC	Cat# CRL-11268
Recombinant DNA		
HexaPro S	Hsieh et al., 2020	N/A
RBD-16GS-50A	GenScript Walls et al., 2020b	N/A
RBD-12GS-50A	GenScript Walls et al., 2020b	N/A
SARS-COV-RBD-16GS-50A	GenScript	N/A
WIV1-RBD-16GS-50A	GenScript	N/A
RaTG13-RBD-16GS-50A	GenScript	N/A
SARS-CoV-2 S-2P trimer	GenScript Walls et al., 2020a	Vector# BEI NR-52421
SARS-CoV S-2P trimer	GeneArt Walls et al., 2019	N/A
SARS-CoV-2 RBD	GenScript Walls et al., 2020a	Vector# BEI NR-52422
SARS-CoV RBD	GenScript	N/A

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
WIV1 RBD	GenScript	N/A
RaTG13 RBD	GenScript	N/A/
SARS-CoV-2 S full-length D614G (YP 009724390.1)	Crawford et al., 2020	Vector# BEI NR-52514
SHC014 S full-length (AGZ48806.1)	GenScript	N/A
SARS-CoV S full-length (YP 009825051.1)	GeneArt (Walls et al., 2020)	N/A
RaTG13 S full-length (QHR63300.2)	GenScript	N/A
WIV1 full-length S (AGZ48831.1)	GenScript	N/A
PangolinGD full-length S (QLR06867.1)	GenScript	N/A
Murine leukemia virus gag-pol	Millet and Whittaker 2016	N/A
pTG-Luciferase	Millet and Whittaker 2016	N/A
HIV Hgpm2	Crawford et al., 2020	Vector# BEI NR-52517
HIV luciferase	Crawford et al., 2020	Vector# BEI NR-52516
HIV tat1b	Crawford et al., 2020	Vector# BEI NR-52518
HIV Rev1b	Crawford et al., 2020	Vector# BEI NR-52519
Software and algorithms		
UCSF ChimeraX	Goddard et al., 2018	https://www.rbvi.ucsf.edu/chimerax/
Prism	Graphpad	https://www.graphpad.com/scientific-software/prism/
FlowJo v10	FlowJo	https://www.flowjo.com
Dms-variants package	Greaney et al., 2020	https://jbloomlab.github.io/dms_variants/dms_variants.globalepistasis.html
Other		
Octet Biosensors: protein A	Sartorius (FortéBio)	Cat# 18-5010
Octet Biosensors: ARG2	Sartorius (FortéBio)	Cat# 18-5092
EM supplies 300 mesh grids	Ted Pella	Cat# 01843-F
Filter paper	Cytiva	Cat# 1004047
Uranyl formate	SPI Chem	Cat# 02545-AA
Unis Capillary Cassettes	Unchained Labs	Cat# 201-1010
PrismA Protein A resin	Cytiva	Cat# 17549802
Superdex 200 Increase SEC column	Cytiva	Cat# 28-9909-44
Superose 6 Increase SEC column	Cytiva	Cat# 29091596
Talon resin	TaKaRa	Cat# 635652
Excel resin	Cytiva	Cat# 17371203
Patterson Veterinary, Isoflurane, USP	Patterson	Cat# 07-893-1389
EndoSafe LAL Test Cartridges	Charles River Labs	Cat # PTS20005F
Lemo21(DE3)	New England BioLabs	Cat#C2528J
Isopropyl-B-D-thiogalactoside (IPTG)	Sigma Aldrich	Cat#I6758
Kanamycin Sulfate	Sigma-Aldrich	Cat#K1876
HiLoad S200 pg	Cytiva	Cat#28989336
Ni Sepharose 6 FF	Cytiva	Cat#17531808
HisTrap FF	Cytiva	Cat#17525501

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, David Veesler (dveesler@uw.edu).

Materials availability

All reagents will be made available on request after completion of a Materials Transfer Agreement.

Data and code availability

All data supporting the findings of this study are found within the paper and its Supplemental information, and are available from the Lead Contact author upon request. Additional supplemental items are available from Mendeley Data at https://doi.org/10.17632/5k989kb8t7.1.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cell lines

Expi293F (derived from 293 cells which are female) cells are derived from the HEK293F cell line (Life Technologies). Expi293F cells were grown in Expi293 Expression Medium (Life Technologies), cultured at 36.5°C with 8% CO_2 and shaking at 150 rpm. HEK293T/17 is a female human embryonic kidney cell line (ATCC). The HEK-ACE2 (derived from HEK293T cells which are female) adherent cell line was obtained through BEI Resources, NIAID, NIH: Human Embryonic Kidney Cells (HEK293T) Expressing Human Angiotensin-Converting Enzyme 2, HEK293T-hACE2 Cell Line, NR-52511. All adherent cells were cultured at 37°C with 8% CO_2 in flasks with DMEM + 10% FBS (Hyclone) + 1% penicillin-streptomycin. Cell lines other than Expi293F were not tested for mycoplasma contamination nor authenticated.

Mice

Female BALB/c mice (Stock # 000651, BALB/c cByJ mice) four weeks old were obtained from Jackson Laboratory, Bar Harbor, Maine, and maintained at the Comparative Medicine Facility at the University of Washington, Seattle, WA, accredited by the American Association for the Accreditation of Laboratory Animal Care International (AAALAC). Animal procedures were performed under the approvals of the Institutional Animal Care and Use Committee (IACUC) of University of Washington, Seattle, WA, and University of North Carolina, Chapel Hill, NC.

Pigtail macaques

Two adult male Pigtail macaques (*Macaca nemestrina*) were immunized in this study. All animals were housed at the Washington National Primate Research Center (WaNPRC), an AAALAC International accredited institution. All experiments were approved by The University of Washington's IACUC. Animals were singly housed in comfortable, clean, adequately-sized cages with ambient temperatures between 72–82°F. Animals received environmental enrichment for the duration of the study including grooming contact, perches, toys, foraging experiences and access to additional environment enrichment devices. Water was available through automatic watering devices and animals were fed a commercial monkey chow, supplemented daily with fruits and vegetables. Throughout the study, animals were checked twice daily by husbandry staff.

Rhesus macaques

Male Rhesus macaques (Macaca mulatta) of Indian origin, aged 3–7 years were assigned to the study (Arunachalam et al., 2021). Animals were distributed between the groups such that the age and weight distribution were comparable across the groups. Animals were housed and maintained at the New Iberia Research Center (NIRC) of the University of Louisiana at Lafayette, an AAALAC International accredited institution, in accordance with the rules and regulations of the Guide for the Care and Use of Laboratory Animal Resources. The entire study (protocol 2020-8808-15) was reviewed and approved by the University of Louisiana at Lafayette IACUC. All animals were negative for SIV, simian T cell leukemia virus, and simian retrovirus.

Convalescent human sera

Samples collected between 1–60 days post infection from individuals who tested positive for SARS-CoV-2 by PCR were profiled for anti-SARS-CoV-2 S antibody responses and those with anti-S Ab responses were maintained in the cohort (Walls et al., 2020b). Individuals were enrolled as part of the HAARVI study at the University of Washington in Seattle, WA. Baseline sociodemographic and clinical data for these individuals are summarized in Table S1. This study was approved by the University of Washington Human Subjects Division Institutional Review Board (STUDY00000959 and STUDY00003376). All experiments were performed in at least two replicates. One sample is the 20/130 COVID-19 plasma from NIBSC (https://www.nibsc.org/documents/ifu/20-130.pdf).

Pfizer vaccinated human sera

Blood samples were collected from participants who had received both doses of the Pfizer mRNA vaccine and were 7–30 days post second vaccine dose. Clinical data for these individuals are summarized in Table S1. Individuals were enrolled in the UWARN: COVID-19 in WA study at the University of Washington in Seattle, WA. This study was approved by the University of Washington Human Subjects Division Institutional Review Board (STUDY00010350).

METHOD DETAILS

Plasmid construction

The SARS-CoV-2-RBD-Avi construct was synthesized by GenScript into pcDNA3.1- with an N-terminal mu-phosphatase signal peptide and a C-terminal octa-histidine tag, flexible linker, and avi tag (GHHHHHHHHGGSSGLNDIFEAQKIEWHE). The boundaries of the construct are N-$_{328}$RFPN$_{331}$ and $_{528}$KKST$_{531}$-C (Walls et al., 2020a). The GD-Pangolin (326-527), WIV1 (316-518), RaTG13 (359-562), RmYN02 (307-492), and ZXC21 (323-507) were synthesized by GenScript into vector pcDNA3.1- or CMVR with a preceding mu-phosphatase signal peptide and a C-terminal octahistidine tag. SARS-CoV-1 (306-575) was subcloned from a GenArt synthesized SARS-CoV-1 Spike ectodomain. The SARS-CoV S2P ((Pallesen et al., 2017)) was synthesized by GeneArt and placed into a modified pOPING vector with its original N-terminal mu-phosphatase signal peptide, and an engineered C-terminal extension: SG-RENLYFQG (TEV protease site), GGGSG-YIPEAPRDGQAYVRKDGEWVLLSTFL (foldon trimerization motif), G-HHHHHH (hexa-histidine tag), just upstream of the predicted transmembrane region (YIK). The SARS-CoV-2 S2P ectodomain trimer (GenBank: YP_009724390.1, BEI NR-52420) was synthesized by GenScript into pCMV with an N-terminal mu-phosphatase signal peptide and a C-terminal TEV cleavage site (GSGRENLYPQG), T4 fibritin foldon (GGGSGYIPEAPRDGQAYVRKDGEWVLLSTPL), and octa-histidine tag (GHHHHHHHH) (Walls et al., 2020a). The construct contains the 2P mutations (proline substitutions at residues 986 and 987; and an $_{682}$SGAG$_{685}$ substitution at the furin cleavage site. The SARS-CoV-2 RBD was genetically fused to the N terminus of the trimeric I53-50A nanoparticle component using 12 or 16 glycine and serine residues. RBD-12GS-I53-50A fusions were synthesized and cloned by Genscript into pCMV. The RBD-16GS-I53-50A fusion was cloned into pCMV/R using the Xba1 and AvrII restriction sites and Gibson assembly (Gibson et al., 2009). All RBD-bearing components contained an N-terminal mu-phosphatase signal peptide and a C-terminal octa-histidine tag. Human ACE2 ectodomain was genetically fused to a sequence encoding a thrombin cleavage site and a human Fc fragment at the C-terminal end. hACE2-Fc was synthesized and cloned by GenScript with a BM40 signal peptide. Genes encoding CR3022 heavy and light chains were ordered from GenScript and cloned into pCMV/R. S309 construct as previously described (Pinto et al., 2020). SARS-CoV-2 HexaPro construct is as previously described (Hsieh et al., 2020) and placed into CMVR with an octa-his tag.

Transient transfection

Proteins were produced using endotoxin free DNA in Expi293F cells grown in suspension using Expi293F expression medium (Life Technologies) at 33°C, 70% humidity, 8% CO_2 rotating at 150 rpm. The cultures were transfected using PEI-MAX (Polyscience) with cells grown to a density of 3.0 million cells per mL and cultivated for 3 days. Supernatants were clarified by centrifugation (5 min at 4000 *rcf.*), addition of PDADMAC solution to a final concentration of 0.0375% (Sigma Aldrich, #409014), and a second spin (5 min at 4000 *rcf.*).

Microbial protein expression and purification

The I53-50A and I53-50B.4.PT1 proteins (Bale et al., 2016) were expressed in Lemo21(DE3) (NEB) in LB (10 g Tryptone, 5 g Yeast Extract, 10 g NaCl) grown in 2 L baffled shake flasks or a 10 L BioFlo 320 Fermenter (Eppendorf). Cells were grown at 37°C to an OD600 ~0.8, and then induced with 1 mM IPTG. Expression temperature was reduced to 18°C and the cells shaken for ~16 h. The cells were harvested and lysed by microfluidization using a Microfluidics M110P at 18,000 psi in 50 mM Tris, 500 mM NaCl, 30 mM imidazole, 1 mM PMSF, 0.75% CHAPS. Lysates were clarified by centrifugation at 24,000 g for 30 min and applied to a 2.6 × 10 cm Ni Sepharose 6 FF column (Cytiva) for purification by IMAC on an AKTA Avant150 FPLC system (Cytiva). Protein of interest was eluted over a linear gradient of 30 mM to 500 mM imidazole in a background of 50 mM Tris pH 8, 500 mM NaCl, 0.75% CHAPS buffer. Peak fractions were pooled, concentrated in 10K MWCO centrifugal filters (Millipore), sterile filtered (0.22 μm) and applied to either a Superdex 200 Increase 10/300, or HiLoad S200 pg GL SEC column (Cytiva) using 50 mM Tris pH 8, 500 mM NaCl, 0.75% CHAPS buffer. I53-50A elutes at ~0.6 column volume (CV). I53-50B.4PT1 elutes at ~0.45 CV. After sizing, bacterial-derived components were tested to confirm low levels of endotoxin before using for nanoparticle assembly.

Protein purification

Proteins containing His tags were purified from clarified supernatants via a batch bind method where each clarified supernatant was supplemented with 1 M Tris-HCl pH 8.0 to a final concentration of 45 mM and 5 M NaCl to a final concentration of ~310 mM. Talon cobalt affinity resin (Takara) was added to the treated supernatants and allowed to incubate for 15 min with gentle shaking. Resin was collected using vacuum filtration with a 0.2 μm filter and transferred to a gravity column. The resin was washed with 20 mM Tris pH 8.0, 300 mM NaCl, and the protein was eluted with 3 column volumes of 20 mM Tris pH 8.0, 300 mM NaCl, 300 mM imidazole. The batch bind process was then repeated and the first and second elutions combined. SDS-PAGE was used to assess purity. RBD-I53-50A fusion protein IMAC elutions were concentrated to > 1 mg/mL and subjected to three rounds of dialysis into 50 mM Tris pH 7.4, 185 mM NaCl, 100 mM Arginine, 4.5% glycerol, and 0.75% w/v 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) in a hydrated 10K molecular weight cutoff dialysis cassette (Thermo Scientific). S2P and HexaPro IMAC elution fractions were concentrated to ~1 mg/mL and dialyzed three times into 50 mM Tris pH 8, 150 mM NaCl, 0.25% L-Histidine in a hydrated 10K molecular weight cutoff dialysis cassette (Thermo Scientific).

Clarified supernatants of cells expressing monoclonal antibodies and human ACE2-Fc were purified using a MabSelect PrismA 2.6 × 5 cm column (Cytiva) on an AKTA Avant150 FPLC (Cytiva). Bound antibodies were washed with five column volumes of 20 mM $NaPO_4$, 150 mM NaCl pH 7.2, then five column volumes of 20 mM $NaPO_4$, 1 M NaCl pH 7.4 and eluted with three column volumes of 100 mM glycine at pH 3.0. The eluate was neutralized with 2 M Trizma base to 50 mM final concentration. SDS-PAGE was used to assess purity.

Recombinant S309 was expressed as a Fab in expiCHO cells transiently co-transfected with plasmids expressing the heavy and light chain, as described above (see Transient transfection) (Pinto et al., 2020). The protein was affinity-purified using a HiTrap Protein A Mab select Xtra column (Cytiva) followed by desalting against 20 mM $NaPO_4$, 150 mM NaCl pH 7.2 using a HiTrap Fast desalting column (Cytiva). The protein was sterilized with a 0.22 μm filter and stored at 4°C until use.

In vitro nanoparticle assembly and purification

Total protein concentration of purified individual nanoparticle components was determined by measuring absorbance at 280 nm using a UV/vis spectrophotometer (Agilent Cary 8454) and calculated extinction coefficients. The assembly steps were performed at room temperature with addition in the following order: RBD-I53-50A trimeric fusion protein, followed by additional buffer (50 mM Tris pH 7.4, 185 mM NaCl, 100 mM Arginine, 4.5% glycerol, and 0.75% w/v CHAPS) as needed to achieve desired final concentration, and finally I53-50B.4PT1 pentameric component (in 50 mM Tris pH 8, 500 mM NaCl, 0.75% w/v CHAPS), with a molar ratio of RBD-I53-50A:I53-50B.4PT1 of 1.1:1. All RBD-I53-50 *in vitro* assemblies were incubated at 2-8°C with gentle rocking for at least 30 min before subsequent purification by SEC in order to remove residual unassembled component. Different columns were utilized depending on purpose: Superose 6 Increase 10/300 GL column was used analytically for nanoparticle size estimation, a Superdex 200 Increase 10/300 GL column used for small-scale pilot assemblies, and a HiLoad 26/600 Superdex 200 pg column used for nanoparticle production. Assembled particles were purified in 50 mM Tris pH 7.4, 185 mM NaCl, 100 mM Arginine, 4.5% glycerol, and 0.75% w/v CHAPS, and elute at ~11 mL on the Superose 6 column and in the void volume of Superdex 200 columns. Assembled nanoparticles were sterile filtered (0.22 μm) immediately prior to column application and following pooling of fractions.

Endotoxin measurements

Endotoxin levels in protein samples were measured using the EndoSafe Nexgen-MCS System (Charles River). Samples were diluted 1:50 or 1:100 in Endotoxin-free LAL reagent water, and applied into wells of an EndoSafe LAL reagent cartridge. Charles River EndoScan-V software was used to analyze endotoxin content, automatically back-calculating for the dilution factor. Endotoxin values were reported as EU/mL which were then converted to EU/mg based on UV/vis measurements. Our threshold for samples suitable for immunization was < 50 EU/mg.

Pigtail macaque immunization

Two Pigtail macaques were immunized with 250 μg of RBD-12GS-I53-50 nanoparticle (88 μg RBD antigen) with AddaVax at day 0, day 28, and O/W at day 168. Blood was collected every 14 days post-prime. Blood was collected in serum collection tubes and allowed to clot at room temperature. Serum was isolated after a 15 min spin at 1455 x g for 15 min and stored at −80C until use. Prior to vaccination or blood collection, animals were sedated with an intramuscular injection (10 mg/kg) of ketamine (Ketaset®; Henry Schein). Prior to inoculation, immunogen suspensions were gently mixed 1:1 vol/vol with AddaVax adjuvant for immunizations 1 and 2 and O/W for immunization 3 (VFI) to reach a final concentration of 0.250 mg/mL antigen. The vaccine was delivered intramuscularly into both quadriceps muscles with 1 mL per injection site on days 0, 28, and 168. All injection sites were shaved prior to injection. Animals were observed daily for general health (activity and appetite, urine/feces output) and for evidence of reactogenicity at the vaccine inoculation site (swelling, erythema, and pruritus) for up to 1 week following vaccination. They also received physical exams including temperature and weight measurements at each study time point. None of the animals became severely ill during the course of the study nor required euthanasia.

Rhesus macaque immunization

Adapted from Arunachalam et al. (2021). AS03 was kindly provided by GSK Vaccines. AS03 is an oil-in-water emulsion that contains 11.86 mg a-tocopherol, 10.69 mg squalene, and 4.86 mg polysorbate 80 (Tween-80) in PBS. For each dose, RBD-NP was diluted to 50 μg/ml (RBD component) in 250 μL of Tris-buffered saline (TBS) and mixed with an equal volume of AS03.The dose of AS03 was 50% v/v (equivalent of one human dose). Soluble HexaPro was diluted to 50 μg/ml in 250 μL of Tris-buffered saline (TBS) and mixed with an equal volume of AS03. All immunizations were administered via the intramuscular route in right forelimbs. The volume of each dose was 0.5 ml.

Deep mutational scanning

All mutations that escape serum antibody binding were mapped via a deep mutational scanning approach (Greaney et al., 2020, 2021b). We used previously described yeast-display RBD mutant libraries (Greaney et al., 2020; Starr et al., 2020a). Briefly, duplicate mutant libraries were constructed in the spike receptor binding domain (RBD) from SARS-CoV-2 (isolate Wuhan-Hu-1, GenBank: MN908947, residues N331-T531) and contain 3,804 of the 3,819 possible amino-acid mutations, with > 95% present as single mutants. Each RBD variant was linked to a unique 16-nucleotide barcode sequence to facilitate downstream sequencing. As previously

described, libraries were sorted for RBD expression and ACE2 binding to eliminate RBD variants that are completely misfolded or non-functional (i.e., lacking modest ACE2 binding affinity) (Greaney et al., 2020).

Antibody escape mapping experiments were performed in biological duplicate using two independent mutant RBD libraries, with minor modifications from Greaney et al. (2020), and exactly as described in Greaney et al. (2021b). The antibody escape mapping for the vaccinated NHP serum was performed in this study; the antibody escape mapping from convalescent human plasma was performed in Greaney et al. (2021b). Briefly, mutant yeast libraries induced to express RBD were washed and incubated with plasma or serum at a range of dilutions for 1 h at room temperature with gentle agitation. For each sample, we chose a sub-saturating dilution such that the amount of fluorescent signal due to plasma antibody binding to RBD was approximately equal across samples. A 1:1000 dilution was used for the vaccinated NHP serum, and the exact dilutions of human convalescent plasma are reported in Greaney et al. (2021b). After the antibody incubations, the libraries were secondarily labeled with 1:100 FITC-conjugated anti-MYC antibody (Immunology Consultants Lab, CYMC-45F) to label for RBD expression and and 1:200 Alexa-647-conjugated goat anti-human-IgA+IgG+IgM (Jackson ImmunoResearch 109-605-064) to label for bound serum or plasma antibodies. A flow cytometric selection gate was drawn to capture approximately 5% of the RBD mutant libraries with the lowest amount of plasma binding for their degree of RBD expression. For each sample, approximately 10 million RBD+ cells were processed on the cytometer. Antibody-escaped cells were grown overnight in SD-CAA (6.7g/L Yeast Nitrogen Base, 5.0g/L Casamino acids, 1.065 g/L MES acid, and 2% w/v dextrose) to expand cells prior to plasmid extraction.

Plasmid samples were prepared from pre-selection and overnight cultures of antibody-escaped cells (Zymoprep Yeast Plasmid Miniprep II) as previously described (Greaney et al., 2020). The 16-nucleotide barcode sequences identifying each RBD variant were amplified by PCR and sequenced on an Illumina HiSeq 2500 with 50 bp single-end reads as described in Greaney et al. (2020 and Starr et al. (2020a).

Escape fractions were computed as described in Greaney et al. (2020), and exactly as described in Greaney et al. (2021b). We used the dms_variants package (https://jbloomlab.github.io/dms_variants/, version 0.8.2) to process Illumina sequences into counts of each barcoded RBD variant in each pre-sort and antibody-escape population using the barcode/RBD look-up table from Starr et al. (2021).

For each serum selection, we computed the ''escape fraction'' for each barcoded variant using the deep sequencing counts for each variant in the original and serum-escape populations and the total fraction of the library that escaped antibody binding via the formula provided in Greaney et al. (2020). These escape fractions represent the estimated fraction of cells expressing that specific variant that fall in the antibody escape bin, such that a value of 0 means the variant is always bound by serum and a value of 1 means that it always escapes serum binding. We then applied a computational filter to remove variants with low sequencing counts or highly deleterious mutations that might cause antibody escape simply by leading to poor expression of properly folded RBD on the yeast cell surface (Greaney et al., 2020; Starr et al., 2020a). Specifically, we removed variants that had (or contained mutations with) ACE2 binding scores < -2.35 or expression scores < -1, using the variant- and mutation-level deep mutational scanning scores from Starr et al. (2020a). Note that these filtering criteria are slightly more stringent than those used in Greaney et al. (2020) but are identical to those used in Greaney et al. (2021b) and Starr et al. (2020b).

We next deconvolved variant-level escape scores into escape fraction estimates for single mutations using global epistasis models (Otwinowski et al., 2018) implemented in the dms_variants package, as detailed at (https://jbloomlab.github.io/dms_variants/dms_variants.globalepistasis.html) and described in Greaney et al. (2020). The reported escape fractions throughout the paper are the average across the libraries (correlations shown in Figure S2); these scores are also in Table S1 and at https://github.com/jbloomlab/SARS-CoV-2-RBD_MAP_RBD-nano-vax-NHP1/blob/main/results/supp_data/NHP_HCS_raw_data.csv. Sites of strong escape from each antibody were determined heuristically as sites whose summed mutational escape scores were at least 10 times the median sitewise sum of selection, and within 10-fold of the sitewise sum of the most strongly selected site. Sites shown in Figures 2 and S2A are the sites of strong escape for any of the three human convalescent plasma, plus sites 417, 452, and 501 due to their prevalence in circulating SARS-CoV-2 variants. For each plasma, the y axis is scaled to be the greatest of (a) the maximum site-wise escape metric observed for that plasma, (b) 20x the median site-wise escape fraction observed across all sites for that plasma, or (c) an absolute value of 1.0 (to appropriately scale plasma that are not ''noisy'' but for which no mutation has a strong effect on plasma binding). Full documentation of the computational analysis is at https://github.com/jbloomlab/SARS-CoV-2-RBD_MAP_RBD-nano-vax-NHP1. These results are also available in a zoomable, interactive form at https://jbloomlab.github.io/SARS-CoV-2-RBD_MAP_RBD-nano-vax-NHP1/.

ELISA

For anti-S2P ELISA, 25 μL of 2 μg/mL S2P was plated onto 384-well Nunc Maxisorp (ThermoFisher) plates in PBS and sealed overnight at RT. The next day plates were washed 4 × in Tris Buffered Saline Tween (TBST) using a plate washer (BioTek) and blocked with SuperBlock (ThermoFisher) for 1 h at 37°C. Plates were washed 4 × in TBST and 1:5 serial dilutions of mouse, NHP, or human sera were made in 25 μL TBST and incubated at 37°C for 1 h. Plates were washed 4 × in TBST, then anti-mouse (Invitrogen), anti-NHP (AlphaDiagnostics), or anti-human (Invitrogen) horseradish peroxidase-conjugated antibodies were diluted 1:5,000 and 25 μL added to each well and incubated at 37°C for 1 h. Plates were washed 4 × in TBST and 25 μL of TMB (SeraCare) was added to every well for

~5 min at room temperature. The reaction was quenched with the addition of 25 μL of 1 N HCl. Plates were immediately read at 450 nm on a BioTek plate reader and data plotted and fit in Prism (GraphPad) using nonlinear regression sigmoidal, 4PL, X is log(concentration) to determine EC_{50} values from curve fits.

Competition ELISA of NHP sera with hACE2, CR3022 IgG, and S309 IgG for immobilized SARS-CoV-2 S2P

384-well Maxisorp plates (Thermo Fisher) were coated overnight at room temperature with 3 μg/mL of SARS-CoV-2 S2P (Pallesen et al., 2017) in 20mM Tris pH 8 and 150mM NaCl. Plates were slapped dry and blocked with Blocker Casein in TBS (Thermo Fisher) for one hour at 37°C. Plates were slapped dry and NHP sera was serially diluted 1:4 in TBST with an initial dilution of 1:4 for hACE2 competition or 1:2 for antibody competition. Random primary amine biotinylated (Thermo Fisher) hACE2-Fc, CR3022 (Yuan et al., 2020), or S309 (Pinto et al., 2020) were added, bringing the concentration of each well to the EC_{50} values of 0.2nM, 2nm, and 0.01nM, respectively. Plates were left for one hour at 37°C, then washed 4x with TBST using a 405 TS Microplate Washer (BioTek) followed by addition of 1:500 streptavidin-HRP (Thermo Fisher) for one hour at 37°C. Plates were washed 4x and TMB Microwell Peroxidase (Seracare) was added. The reaction was quenched after 1-2 minutes with 1 N HCl and the A450 of each well was read using a BioTek plate reader (BioTek). Data plotted and fit in Prism (GraphPad) using nonlinear regression sigmoidal, 4PL, X is log(concentration) to determine EC_{50} values from curve fits with upper and lower constraints determined by uncompeted ELISA per antigen.

Competition ELISA of mouse sera and immobilized hACE2 or mAbs with SARS-CoV-2 or SARS-CoV S2P

384-well Maxisorp plates (Thermo Fisher) were coated overnight at room temperature with 3 μg/mL of hACE2-Fc, CR3022 (Yuan et al., 2020), or S309 (Pinto et al., 2020) in 20mM Tris pH 8 and 150mM NaCl. Plates were slapped dry and blocked with Blocker Casein in TBS (Thermo Fisher) for one hour at 37°C. Plates were slapped dry and a 30-minute pre-incubated 1:5 serial dilution of mouse sera in TBST, with in initial dilution of 1:50 for hACE2-Fc competition or 1:10 for antibody competition, and a constant concentration of biotinylated (Avidity) SARS-CoV-2 S2P or SARS-CoV 2P at their EC_{50} values were added. Spike concentrations were 0.63nM, 5.98nM, and 0.22nM of SARS-CoV-2 S2P or 4.11nM, 2.89nM, and 0.19nM of SARS-CoV S2P for immobilized hACE2, CR3022, and S309, respectively. Plates were left for one hour at 37°C, then washed 4x with TBST using a 405 TS Microplate Washer (BioTek) followed by addition of 1:500 streptavidin-HRP (Thermo Fisher) for one hour at 37°C. Plates were washed 4x and TMB Microwell Peroxidase (Seracare) was added. The reaction was quenched after 1-2 minutes with 1 N HCl and the A450 of each well was read using a BioTek plate reader (BioTek). Data plotted and fit in Prism (GraphPad) using nonlinear regression sigmoidal, 4PL, X is log(concentration) to determine EC_{50} values from curve fits.

Pseudovirus production

MLV-based D614G SARS-CoV-2 S and SARS-CoV S pseudotypes were prepared as previously described (Millet and Whittaker, 2016; Walls et al., 2020a, 2020b). Briefly, HEK293T cells were co-transfected using Lipofectamine 2000 (Life Technologies) with an S-encoding plasmid, an MLV Gag-Pol packaging construct, and the MLV transfer vector encoding a luciferase reporter according to the manufacturer's instructions. Cells were washed 3 × with Opti-MEM and incubated for 5 h at 37°C with transfection medium. DMEM containing 10% FBS was added for 60 h. The supernatants were harvested by spinning at 2,500 g, filtered through a 0.45 μm filter, concentrated with a 100 kDa membrane for 10 min at 2,500 g and then aliquoted and stored at −80°C.

HIV-based pseudotypes were prepared as previously described (Crawford et al., 2020). Briefly, HEK293T cells were cotransfected using Lipofectamine 2000 (Life Technologies) with an S-encoding plasmid (D614G SARS-CoV-2 S (YP 009724390.1), Pangolin-Guangdong S (QLR06867.1), SARS-CoV S (YP 009825051.1), P1, B.1.351 S, B.1.1.7, and B.1.1.7+E484K S) an HIV Gag-Pol, Tat, Rev1B packaging construct, and the HIV transfer vector encoding a luciferase reporter according to the manufacturer's instructions. Cells were washed 3 × with Opti-MEM and incubated for 5 h at 37°C with transfection medium. DMEM containing 10% FBS was added for 60 h. The supernatants were harvested by spinning at 2,500 g, filtered through a 0.45 μm filter, concentrated with a 100 kDa membrane for 10 min at 2,500 g and then aliquoted and stored at −80°C.

D614G SARS-CoV-2 S (YP 009724390.1), D614G SARS-CoV-2 point mutants, SHC014 (AGZ48806.1), RaTG13 S (QHR63300.2), Pangolin-Guangdong S (QLR06867.1), SARS-CoV S (YP 009825051.1), WIV1 S (AGZ48831.1), B.1.351 S, P1, and B.1.1.7 S pseudotyped VSV viruses were prepared as described previously (McCallum et al., 2021a; Sauer et al., 2021). Briefly, 293T cells in DMEM supplemented with 10% FBS, 1% PenStrep seeded in 10-cm dishes were transfected with the plasmid encoding for the corresponding S glycoprotein using lipofectamine 2000 (Life Technologies) following manufacturer's indications. One day post-transfection, cells were infected with VSV(G*ΔG-luciferase) and after 2 h were washed five times with DMEM before adding medium supplemented with anti-VSV-G antibody (I1- mouse hybridoma supernatant, CRL- 2700, ATCC). Virus pseudotypes were harvested 18-24 h post-inoculation, clarified by centrifugation at 2,500 x g for 5 min, filtered through a 0.45 μm cut off membrane, concentrated 10 times with a 30 kDa cut off membrane, aliquoted and stored at −80°C.

Pseudovirus neutralization

HEK293-hACE2 cells (Crawford et al., 2020) were cultured in DMEM with 10% FBS (Hyclone) and 1% PenStrep with 8% CO_2 in a 37°C incubator (ThermoFisher). One day prior to infection, 40 μL of poly-lysine (Sigma) was placed into 96-well plates and incubated with rotation for 5 min. Poly-lysine was removed, plates were dried for 5 min then washed 1 × with water prior to plating with 40,000

cells. The following day, cells were checked to be at 80% confluence. In an empty half-area 96-well plate a 1:3 serial dilution of sera was made in DMEM and diluted pseudovirus was then added to the serial dilution and incubated at room temperature for 30-60 min. After incubation, the sera-virus mixture was added to the cells at 37°C and 2 hours post-infection, 40 μL 20% FBS-2% PenStrep DMEM was added. After 17-20 hours (VSV) or 48 hours (HIV, MLV) 40 μL/well of One-Glo-EX substrate (Promega) was added to the cells and incubated in the dark for 5-10 min prior reading on a BioTek plate reader. Measurements were done in at least duplicate. Relative luciferase units were plotted and normalized in Prism (GraphPad). Nonlinear regression of log(inhibitor) versus normalized response was used to determine IC_{50} values from curve fits. Kruskal Wallis tests were used to compare two groups to determine whether they were statistically different.

Sarbecovirus biolayer interferometry binding analysis

Purification of Fabs from NHP serum was adapted from Boyoglu-Barnum et al. (2021). Briefly, 1 mL of day 70 serum was diluted to 10 mL with PBS and incubated with 1 mL of 3 × PBS-washed protein A beads (GenScript) with agitation overnight at 37°C. The next day beads were thoroughly washed with PBS using a gravity flow column and bound Abs were eluted with 0.1 M glycine pH 3.5 into 1M Tris-HCl (pH 8.0) to a final concentration of 100 mM. Serum and early washes that flowed through were re-bound to beads overnight again for a second, repeat elution. IgGs were concentrated (Amicon 30 kDa) and buffer exchanged into PBS. 2 × digestion buffer (40 mM sodium phosphate pH 6.5, 20 mM EDTA, 40 mM cysteine) was added to concentrated and pooled IgGs. 500 μL of resuspended immobilized papain resin (ThermoFisher Scientific) freshly washed in 1 × digestion buffer (20 mM sodium phosphate, 10 mM EDTA, 20 mM cysteine, pH 6.5) was added to purified IgGs in 2 × digestion buffer and samples were agitated for 5 h at 37°C. The supernatant was separated from resin and resin washes were collected and pooled with the resin flow through. Pooled supernatants were sterile-filtered at 0.22 μm and applied 6 × to PBS-washed protein A beads in a gravity flow column. The column was eluted as described above and the papain procedure repeated overnight with undigested IgGs to increase yield. The protein A flow throughs were pooled, concentrated (using an Amicon 10 kDa), and buffer exchanged into PBS. Purity was checked by SDS-PAGE.

Assays were performed and analyzed using biolayer interferometry on an Octet Red 96 System (Pall Forte Bio/Sartorius) at ambient temperature with shaking at 1000 rpm. Different Sarbeco RBDs were purified like in Walls et al., 2020 and were diluted with different acetate buffers and applied to a black 96-well Greiner Bio-one microplate at 200ul per well. GD-Pangolin RBD was diluted in pH 6 buffer to 5 μg/mL, RmNY02 were diluted in pH 5 to 25 μg/mL, WIV16 was diluted in pH 5 to 10 μg/mL, SARS-CoV-2 was diluted in pH 6 to 5 μg/mL, RaTG13 was diluted in pH 6 to 10 μg/mL, RaTG13 was diluted in pH 6 to 10 μg/mL, SARS-CoV was diluted in pH 6 to 50 μg/mL, and finally ZXC21 was diluted in pH 6 to 10μg/mL. AR2G biosensors (ForteBio/Sartorius) following 600 s hydration were normalized in water for 180 s. Then tips were NHS-EDC activated for 300 s and the different sarbecovirus RBDs were loaded up to a 1.50 nm threshold for up to 600 s. Immobilized RBDs on the tips were quenched for 300 s in ethanolamine and dipped into kinetics buffer for a 60 s baseline. The association step was performed by dipping the mobilized RBDs into diluted purified polyclonal pigtail macaque IgGs for 600 s. Dissociation was measured by inserting the biosensors in kinetics buffer for 600 s. The data were baseline subtracted and the plots fitted using the Pall ForteBio/Sartorius analysis software (version 12.0).

Cocktail and mosaic bio-layer interferometry (antigenicity)

Binding of hACE2-Fc to monovalent RBD-I53-50 nanoparticles, mosaic-RBD-I53-50 nanoparticles, and cocktail of RBD-nanoparticles was analyzed for antigenicity experiments and real-time stability studies using an Octet Red 96 System (Pall FortéBio/Sartorius) at ambient temperature with shaking at 1000 rpm. Protein samples were diluted to 100 nM in Kinetics buffer (1 × HEPES-EP+ (Pall Forté Bio), 0.05% nonfat milk, and 0.02% sodium azide). Buffer, receptor, and analyte were then applied to a black 96-well Greiner Bio-one microplate at 200 μL per well. Protein A biosensors (FortéBio/Sartorius) were first hydrated for 10 minutes in Kinetics buffer, then dipped into hACE2-Fc diluted to 10 μg/mL in Kinetics buffer in the immobilization step. After 150 s, the tips were transferred to kinetics buffer for 60 s to reach a baseline. The association step was performed by dipping the loaded biosensors into the immunogens for 300 s, and subsequent dissociation was performed by dipping the biosensors back into Kinetics buffer for an additional 300 s. The data were baseline subtracted prior for plotting using the FortéBio analysis software (version 12.0). Plots in Figure S3 show the 600 s of association and dissociation.

Sandwich bio-layer interferometry (mosaic display antigenicity)

Binding of hACE2-Fc or S2H14 mAb and S230 Fab to WIV1-RBD-I53-50, RaTG13-RBD-I53-50, SARS-CoV-SARS-CoV2-RBD-I53-50, SARS-CoV2-I53-50, and mosaic-RBD-I53-50 nanoparticles were analyzed for co-display of RBDs using an Octet Red 96 System (Pall FortéBio/Sartorius) at ambient temperature with shaking at 1000 rpm. Nanoparticles were diluted to 100 nM in Kinetics buffer. Kinetics buffer, mAb, nanoparticles and Fab were then applied to a black 96-well Greiner Bio-one microplate at 200 μL per well. Protein A biosensors (FortéBio/Sartorius) were first hydrated for 10 minutes in Kinetics buffer, then dipped into hACE2-Fc or S2H14 mAb diluted to 10 μg/mL in Kinetics buffer in the immobilization step. After 150 s, the tips were transferred to Kinetics buffer for 60 s to reach a baseline. The receptor or mAb was then loaded with nanoparticle by dipping the loaded biosensors into the immunogens for 300 s, and subsequent baseline was performed by dipping the biosensors back into the Kinetics buffer for an additional 60 s. Association of S230 Fab diluted to 100 nM in Kinetics buffer was then measured for 300 s and subsequent dissociation in Kinetics buffer of S230 Fab for 300 s. The data were baseline subtracted prior for plotting using the FortéBio analysis software (version 12.0). Plots in Figure S3 exclude the initial mAb loading and the first baseline.

Cocktail and mosaic negative stain electron microscopy

Monovalent RBD-I53-50 nanoparticles, mosaic-RBD-I53-50 nanoparticles, and cocktail of RBD-nanoparticles were first diluted to 75 μg/mL in 50 mM Tris pH 7.4, 185 mM NaCl, 100 mM Arginine, 4.5% v/v Glycerol, 0.75% w/v CHAPS prior to application of 3 μL of sample onto freshly glow-discharged 300-mesh copper grids. Sample was incubated on the grid for 1 minute before the grid was dipped in a 50 μL droplet of water and excess liquid blotted away with filter paper (Whatman). The grids were then dipped into 6 μL of 0.75% w/v uranyl formate stain. Stain was blotted off with filter paper, then the grids were dipped into another 6 μL of stain and incubated for ~70 s. Finally, the stain was blotted away and the grids were allowed to dry for 1 minute. Prepared grids were imaged in a Talos model L120C electron microscope at 57,000 × (nanoparticles).

Cocktail and mosaic dynamic light scattering

Dynamic Light Scattering (DLS) was used to measure hydrodynamic diameter (Dh) and % Polydispersity (%Pd) of monovalent RBD-I53-50 nanoparticles, mosaic-RBD-I53-50 nanoparticles, and cocktail of RBD-nanoparticles on an UNcle Nano-DSF (UNchained Laboratories). Sample was applied to a 8.8 μL quartz capillary cassette (UNi, UNchained Laboratories) and measured with 10 acquisitions of 5 s each, using auto-attenuation of the laser. Increased viscosity due to 4.5% v/v glycerol in the RBD nanoparticle buffer was accounted for by the UNcle Client software in Dh measurements.

Mouse immunizations and challenge

At six weeks of age, 8 female BALB/c mice per dosing group were vaccinated with a prime immunization, and three weeks later mice were boosted with a second vaccination (IACUC protocol 4470.01). Prior to inoculation, immunogen suspensions were gently mixed 1:1 vol/vol with AddaVax adjuvant (Invivogen, San Diego, CA) to reach a final concentration of 0.01 mg/mL antigen. Mice were injected intramuscularly into the gastrocnemius muscle of each hind leg using a 27-gauge needle (BD, San Diego, CA) with 50 μL per injection site (100 μL total) of immunogen under isoflurane anesthesia. To obtain sera all mice were bled two weeks after prime and boost immunizations. Blood was collected via submental venous puncture and rested in 1.5 mL plastic Eppendorf tubes at room temperature for 30 min to allow for coagulation. Serum was separated from red blood cells via centrifugation at 2,000 g for 10 min. Complement factors and pathogens in isolated serum were heat-inactivated via incubation at 56°C for 60 min. Serum was stored at 4°C or −80°C until use. The study was repeated twice. Five weeks post-boost, mice (aged 14 weeks) were exported from Comparative Medicine Facility at the University of Washington, Seattle, WA to an AAALAC accredited Animal Biosafety Level 3 (ABSL3) Laboratory at the University of North Carolina, Chapel Hill. After a 7-day acclimation time, mice were anesthetized with a mixture of ketamine/xylazine and challenged intranasally with 10^5 plaque-forming units (pfu) of mouse-adapted SARS-CoV-2 MA10 or SARS-CoV MA15 strain for the evaluation of vaccine efficacy (IACUC protocol 20-114.0). After infection, body weight and congestion score were monitored daily until the termination of the study two days post-infection, when lung and nasal turbinate tissues were harvested to evaluate viral load by plaque assay.

Histopathology of post challenge mouse lungs

For each mouse the left lung was incubated in formalin at 4°C for at least 7 days to fix tissue and inactivate virus. The fixed tissue was processed and embedded in paraffin. 5 μm sections were cut and stained with either Congo red or hematoxylin and eosin (H&E). Eosinophils were enumerated by counting the Congo red positive leukocytes in 10 high power fields (400X final magnification) per mouse lung. Representative images were minimally and similarly adjusted with Adobe Photoshop 2020 to enhance contrast. Airway pathology was assessed in H&E stained sections to assess bronchial epithelial cell death (score: 0 = no dead cells, 1 = 1-5 dead cells, 2 = 6-10 dead cells, 3 = 11-20 dead cells and 4 = > 20 dead cells; scored for 10 400X fields per mouse lung), peribronchial inflammation (score 0 = none, 1 = 0%–25% circumference with > 1 leukocyte cell layer, 2 = 26%–50% circumference with > 1 leukocyte cell layer, and 3 = 50%–100% circumference with > 1 leukocyte cell layer; scored for 10 400X fields per mouse lung), and interstitial pneumonitis (score = percentage of pulmonary alveolar parenchyma with septae expanded by leukocyte; scored for 10 100X fields).

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical details of experiments can be found in the figure legends. For NHP experiments, 2-6 sera samples were used and experiments were done in at least duplicate unless mentioned. For mouse ELISAs, neutralization, and challenge experiments, sera from 6, 8, or 10 BALB/c animals were used and experiments were completed in at least duplicate unless mentioned. Geometric mean titers were calculated. Kruskal Wallis tests were performed to compare two groups to determine whether they were statistically different for ELISA and neutralization experiments. Significance is indicated with stars: *, $p < 0.05$; **** $p < 0.0001$ and non significant groups are not shown.

Supplemental figures

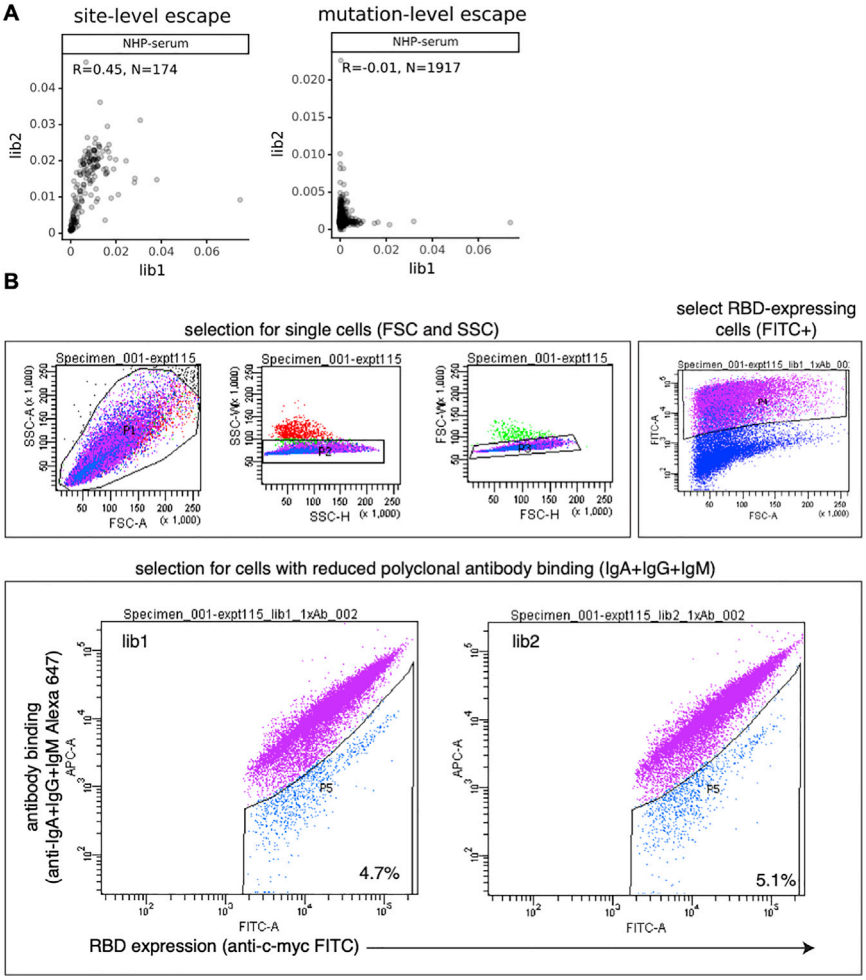

Figure S1. Effects of mutations on binding of HCP Abs to RBD and FACS gating strategy, related to Figure 2

(A) Correlation plots of site- and mutation-level escape for each of the two independent RBD mutant libraries for the Ab-escape map shown in Figure 2B. Site-level escape is the sum of the escape fractions for each mutation at a site. (B) Hierarchical FACS gating strategy used for selecting yeast cells expressing Ab-escape RBD variants. First, gates are selected to enrich for single cells (SSC-A versus FSC-A, and FSC-W versus FSC-H) that also express RBD (FITC-A versus FSC-A, cells in pink). Second, cells expressing RBD mutants with reduced polyclonal Ab binding, detected with an anti-IgA+IgG+IgM secondary Ab, were selected with a gate that captured the ~5% of cells with the lowest Ab binding (cells in blue).

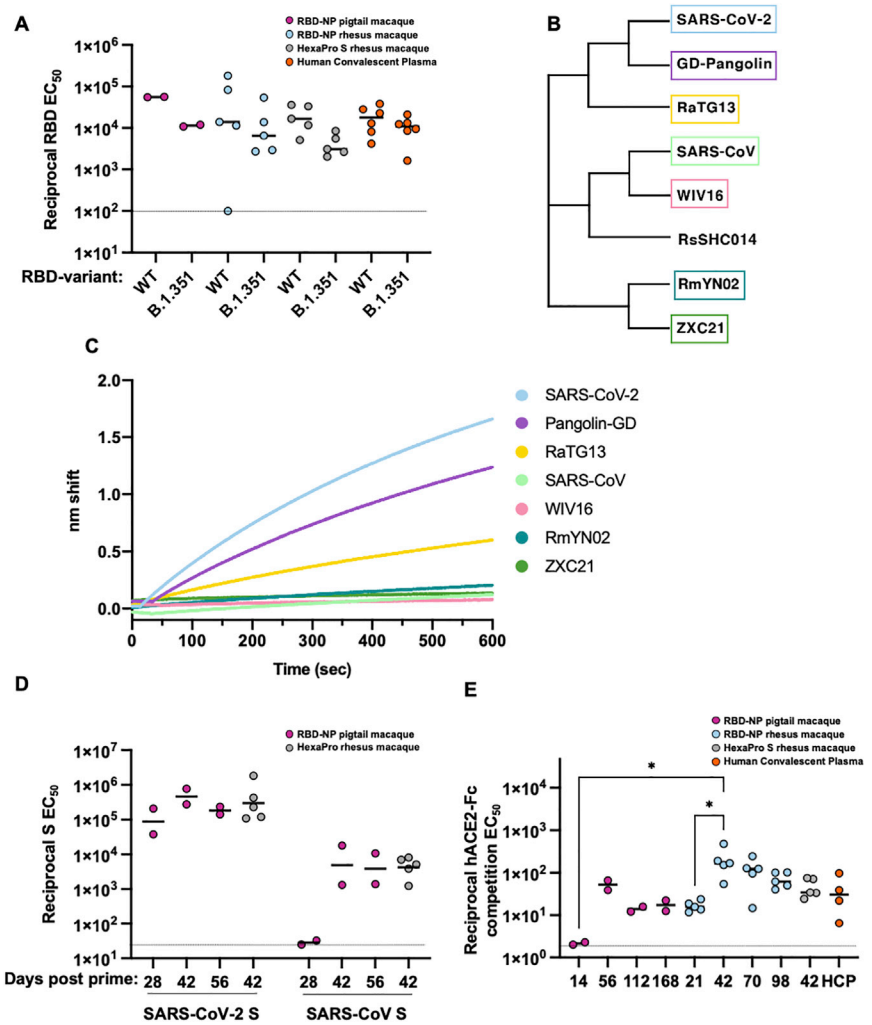

Figure S2. Evaluation of vaccine-elicited binding and neutralizing Ab titers against SARS-CoV-2 variants and sarbecoviruses, related to Figure 3

(A) Wild-type (Wuhan-Hu-1) and B.1.351 SARS-CoV-2 RBD-specific Ab binding titers of RBD-NP-elicited sera in pigtail macaques (magenta, n = 2) and rhesus macaques (blue, n = 5), HexaPro S-elicited sera in rhesus macaques (gray, n = 5), or HCP (orange, n = 6, Table S1) analyzed by ELISA with an LOD of $1x10^2$. (B) Cladogram based on sarbecovirus RBD amino acid sequences. (C) Biolayer interferometry analysis of binding of 1 μM purified polyclonal pigtail macaque IgGs (obtained 70 days post prime) to sarbecovirus RBDs immobilized at the surface of biosensors. (D) SARS-CoV-2 S2P (left) or SARS-CoV S2P (right) Ab binding titers of RBD-NP-elicited sera in pigtail macaques (magenta) or HexaPro S-elicited sera in rhesus macaques (gray) analyzed by ELISA with an LOD of 2.5×10^1. (E) Competition ELISA between 0.13 nM human ACE2-Fc and RBD-NP-elicited sera in pigtail macaques (magenta) and rhesus macaques (blue), or HexaPro S-elicited sera in rhesus macaques (gray) at various time points following vaccination, benchmarked against COVID-19 HCP with an LOD of $4 \times 10^{\circ}$, showing the magnitude of inhibition of ACE2 binding to immobilized SARS-CoV S2P expressed as reciprocal serum dilution blocking 50% of the maximum binding response. Statistical significance was determined by Kruskal-Wallis test and shown when significant. **, $p < 0.01$. All data repeated twice. LODs are shown as gray horizontal dotted lines. Raw data curves shown in Data S1.

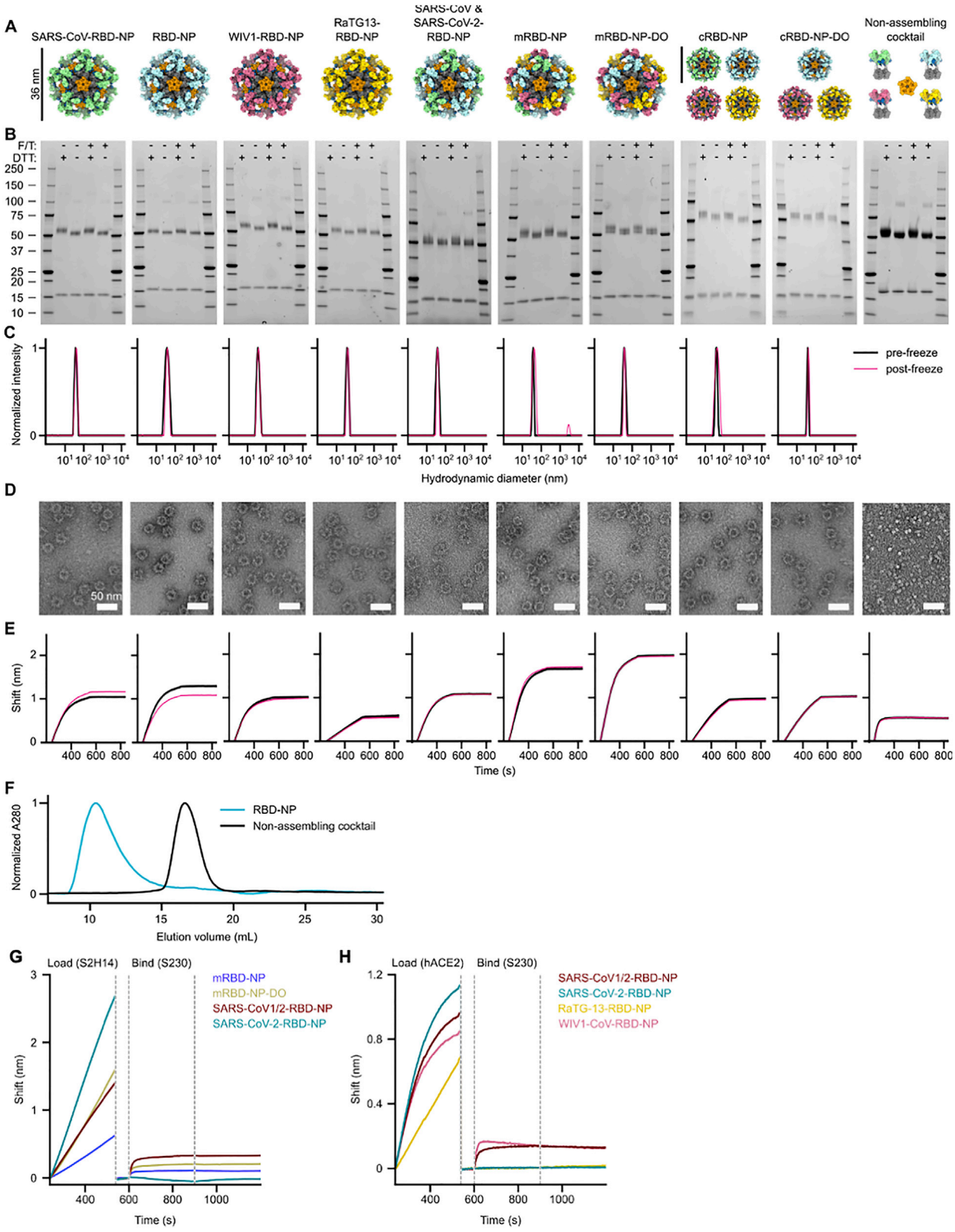

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Figure S3. *In vitro* characterization and confirmation of co-display of sarbecovirus RBD-NP immunogens, related to Figure 4

(A) Design models of the various vaccine candidates evaluated. Scale bars, 36 nm. (B) SDS-PAGE analysis of purified nanoparticles. DTT, dithiothreitol; F/T, freeze/thaw. (C) Dynamic light scattering. (D) Electron micrographs of negatively stained samples. Scale bars, 50 nm. (E) Binding of 100 nM SEC-purified nanoparticle immunogens and the non-assembling cocktail immunogen (which was not purified with SEC) to immobilized hACE2-Fc. (F) SEC chromatogram overlay of purified RBD-NP and non-assembling cocktail. (G-H) Sandwich biolayer interferometry. The SARS-CoV-2 S-specific mAb S2H14 immobilized on protein A biosensors was used to capture various nanoparticle immunogens from 300-480 s. The captured nanoparticles were subsequently exposed to a Fab derived from the SARS-CoV S-specific mAb S230 from 600-900 s (G). hACE2-Fc immobilized on protein A biosensors was used to capture various nanoparticle immunogens from 300-480 s. The captured nanoparticles were subsequently exposed to a Fab derived from the SARS-CoV S-specific mAb S230 from 600-900 s (H).

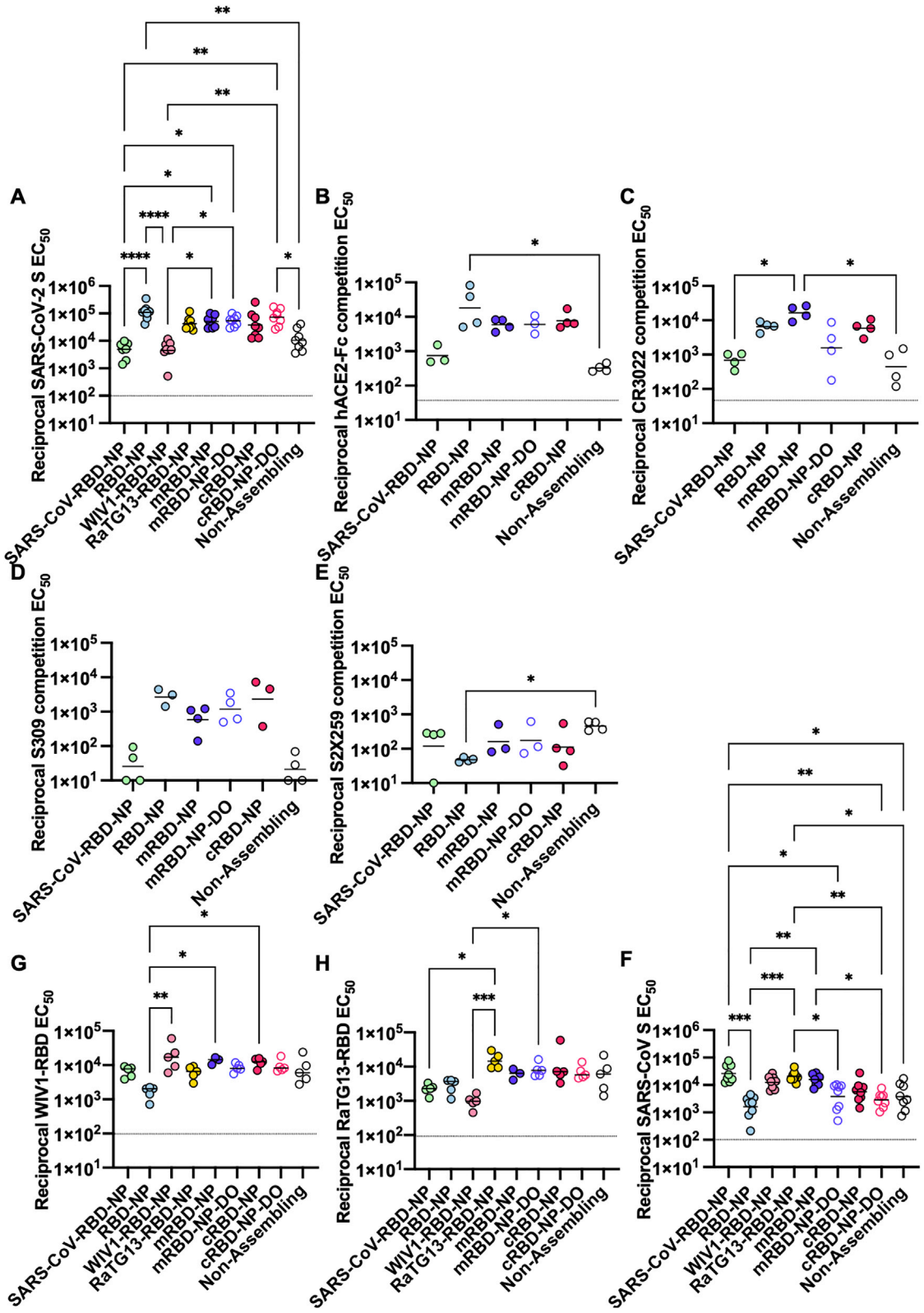

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Figure S4. Serum Ab binding titers elicited by mosaic and cocktail RBD-NPs, related to Figure 5

(A) Ab binding titers to SARS-CoV-2 S2P at five weeks post prime analyzed by ELISA with an LOD of $1x10^2$. (B–E) Titers of SARS-CoV-2 S-specific Abs competing with ACE2-Fc with an LOD of $5x10^1$ (B), CR3022 with an LOD of $5x10^1$ (C), S309 with an LOD of $1x10^1$ (D), and S2X259 with an LOD of $1x10^1$ (E) in immunized mouse sera analyzed by competition ELISA. (F) Ab binding titers to SARS-CoV S2P at week 5 analyzed by ELISA. (G–H) Ab binding titers to the WIV1 (G), and RaTG13 (H) RBDs analyzed by ELISA with an LOD of $1x10^2$. Statistical significance was determined by Kruskal Wallis test and shown when significant. $^{**}p < 0.01$. LODs are shown as gray horizontal dotted lines. Raw data curves shown in Data S1.

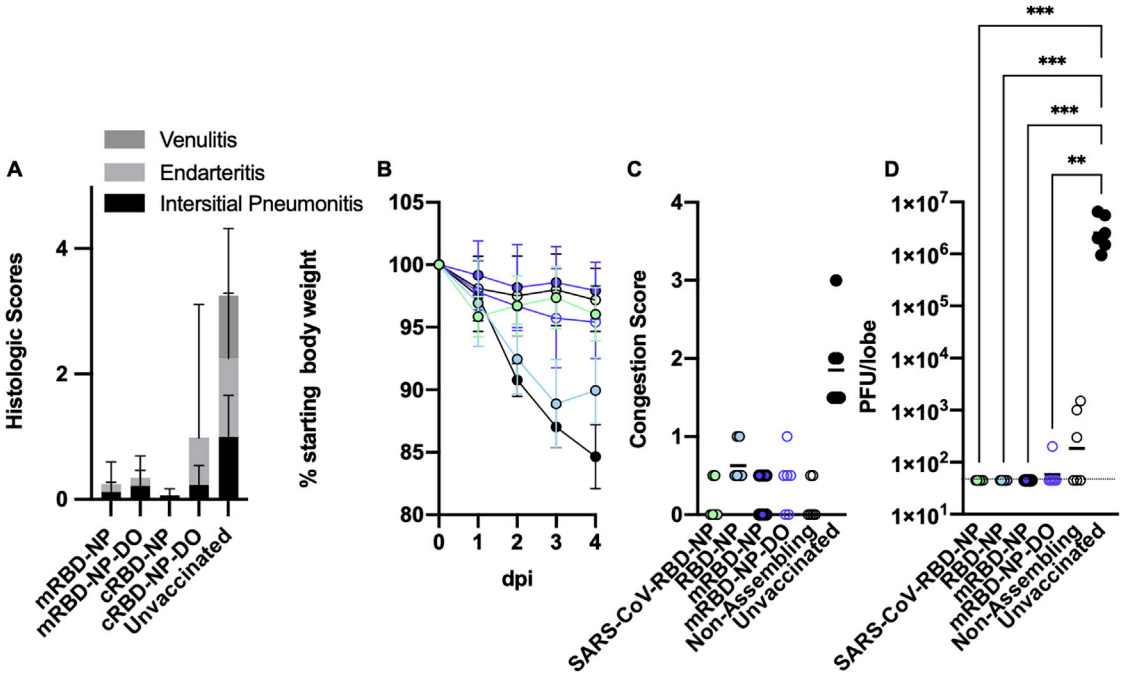

Figure S5. Monovalent, mosaic, and cocktail RBD-NPs protect against heterotypic SARS-CoV-MA15 challenge in 15-week-old BALB/c cByJ mice, related to Figure 6

(A) Normalized active inflammation following SARS-CoV MA 15 challenge shown in Figure 6 with venulitis, endarteritis, and interstitial pneumonitis shown as stacked bar graphs in dark gray, light gray, and black respectively. (B) Weight loss following SARS-CoV MA15 challenge (N = 6). Unvaccinated animals are shown as black circles. (C) Congestion score following SARS-CoV MA15 challenge with a score of 0 indicating unchanged lung color and 4 indicating a darkened and diseased lung (N = 6). (D) Viral titers in mice lungs (expressed in plaque forming units per lobe) following challenge (N = 8) with an LOD of $9x10^1$. Statistical significance was determined by Kruskal Wallis test and shown when significant and $^{**}p < 0.01$. LOD is shown as a gray horizontal dotted line.

From: Broder, Christopher (b) (6)
To: (b) (6)
Subject: NIAID funding new RFA
Date: Thursday, March 23, 2023 4:08:46 PM
Attachments: RFA-AI-23-020_RandD Vacs-mabs for Pandemic Prep-Bunya, Paramyxo and Picorna-U19.pdf
Veesler-SARS-nanoparticle-2021.pdf

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hi all,

thanks for taking the time for the call today on the sG Nipah/Hendra patch/PCEP vaccine project.
It is looking really terrific.

Attached is the RFA from NIAID on Pandemic Preparedness that our patch vaccine approaches can be integrated into.

I envision, continuation with NiV/HeV patch,
but the new area is doing Langya virus sG and/or Mojiang virus (both are the other henipaviruses
with different features. Perhaps also Ghana bat virus. All three have human infections, and/or evidence of human infections.
In parallel, we can propose reverse genetics rescue of these viruses for experimental pathogenesis studies.

The other vaccine method to continue, would be with nanoparticles which can also be explored with the patch. (attached)
(b) (6) would potentially also be a structural bio Core for the submission. We are now testing
a pilot exp with the nanoparticle method now with (b) (6) s group.

Bunyavirus (focus on Hantaan virus) can be integrated with either vaccine approach. We have a
pending submission for nanobody generation against these related viruses in collaboration with (b) (6) at UTMB
with recombinant proteins produced by Genovac. (b) (6) at Genovac, has been able to make Langya Henipavirus soluble
F and G viral glycoproteins which we just received to take a look at. Genovac is also state of the art for
producing human and animal mAbs.

Looking forward to keep our collaborations going.

(b) (6)

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From: (b) (6)
To: Broder, Christopher; (b) (6)
Subject: FW: PAD henipavirus RFP
Date: Monday, July 25, 2022 6:10:13 PM
Attachments: INV-048917_UIC_Investment Document_proposal-07-25-22.docx
Copy of FINAL_ INV-048917_UIC_budget_proposalJuly2022 (003).xlsx

---

Hi all,

Fyi, we just submitted the full proposal to Gates Foundation. I assume they will work with us to make it fit their requirements.
Hopefully we will be funded (cautiously optimistic).

Best,
(b) (6)

[redacted]

From: (b) (6)
Sent: Monday, July 25, 2022 1:51 PM
To: (b) (6) [redacted]
Cc: (b) (6) [redacted]
Subject: RE: PAD henipavirus RFP

Dear (b) (6) [redacted],

Attached are the full proposal from us. Please acknowledge when you receive it. We look forward to working with you to make this “perfect”. Please kindly let us know if we miss anything. Thank you so much for your guidance!

Best,
(b) (6)

[redacted]

(b) (6)

From: (b) (6)
Sent: Monday, July 25, 2022 1:46 PM
To: (b) (6)
Cc: (b) (6)
Subject: RE: PAD henipavirus RFP

Hi (b) (6),
Submitting through email is preferred. If the files are too big to send via email, please send us a Dropbox link or I'm happy to set up a MS Teams site for file sharing.
Thanks!

(b) (6)

From: (b) (6)
Sent: Monday, July 25, 2022 2:41 PM
To: (b) (6)
Cc: (b) (6)
Subject: RE: PAD henipavirus RFP

Dear (b) (6),

Could you please let us know the link we need to submit the proposal? Thank you so much!

Best,
(b) (6)

(b) (6)

From: (b) (6)
Sent: Wednesday, July 13, 2022 1:11 PM
To: (b) (6)
Cc: (b) (6)
Subject: RE: PAD henipavirus RFP

Hi (b) (6),
We don't require any signature at this point as we shape the proposal documents. Once both parties have agreed on the final proposal documents (we typically go back and forth on a few iterations of the Investment Document and budget), we will then create the Grant Agreement that references both proposal documents. It is the Grant Agreement that will need a signature from UIC.
Hope that helps!

(b) (6)

From: (b) (6)
Sent: Monday, July 11, 2022 11:25 AM
To: (b) (6)
Cc: (b) (6)
Subject: RE: PAD henipavirus RFP

Thank you (b) (6) for your prompt response!
(b) (6), please advise us how to proceed.

Best,
(b) (6)

From: (b) (6)
Sent: Monday, July 11, 2022 9:19 AM

To: (b) (6)
Cc: (b) (6)
Subject: RE: PAD henipavirus RFP

Hi (b) (6),
I believe and email approval will be sufficient but I will let (b) (6) provide the final word here (b) (6) is out of the office until Wednesday this week.
Best regards,
(b) (6)

From: (b) (6)
Sent: Monday, July 11, 2022 9:47 AM
To: (b) (6)
Cc: (b) (6)
Subject: RE: PAD henipavirus RFP

Hi (b) (6),

(b) (6) will help us to get the proposal and budget together here at UIC. We need some clarification on the institutional approval.
What do you need from UIC, a letter or email from UIC? Thank you so much for the information.

Best regards,
(b) (6)

From: (b) (6)
Sent: Wednesday, July 6, 2022 11:52 AM
To: (b) (6)
Cc: (b) (6)
Subject: RE: PAD henipavirus RFP

Hi (b) (6) and all,

We look forward to talking with you tmmr.

Best,
(b) (6)

[redacted]

From: (b) (6)
Sent: Wednesday, July 6, 2022 10:41 AM
To: (b) (6)
Cc: (b) (6)
[redacted]
Subject: RE: PAD henipavirus RFP

Hi (b) (6),
Thursday July 7 at 1p seems like a good time to meet. Please invite others who will participate in the proposal. (b) (6) (cc'd) will provide the grant template, budget template and instructions for the proposal. We would like to receive a full proposal for review by July 22. Two areas that we would like to discuss are your plans for medicinal chemistry and overlap with AViDD programs. I will send the meeting invitation for 1p PST July 7.
We look forward to speaking with you soon.
Best regards,
(b) (6)

From: (b) (6)
Sent: Friday, July 1, 2022 9:24 AM
To: (b) (6)
Cc: (b) (6)
Subject: RE: PAD henipavirus RFP

Dear (b) (6),

We have very pleased and honored to be accepted to submit a full proposal from the PAD program, and we are eager to learn about the next step, and seek your guidance on it.

I am available next week:

Thursday July 7, 3 pm-5 pm (Central time) or Pacific time: 1-3 pm
Friday July 8th 3-5 pm (CT), or PT: 1-3 pm

Does that work out for you?

Also is it OK if I include Dr. (b) (6) to this meeting since she will work with me on the full proposal?

We look forward to talking with you!

Best,
(b) (6)

[redacted]

From: (b) (6) [redacted]
Sent: Friday, July 1, 2022 6:49 AM
To: (b) (6) [redacted]
Cc: (b) (6) [redacted]
Subject: PAD henipavirus RFP

Dear (b) (6),

Congratulations on being accepted to submit a full proposal for the PAD program. It would be good for us to have a quick call to answer questions and make sure we're on the same page with the process. We would like to have the full proposal submitted by July 22 if possible so that we can go through the internal review process and make any adjustments as needed. I will be the senior program officer (SPO) managing this proposal and my colleague (b) (6) will be helping as the secondary program officer.

Please let me know a few dates and times next week (after July 5) that you might be available for a call.

We look forward to working with you on this interesting project.
Best regards,
(b) (6)

(b) (6)

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From: (b) (6)
To: (b) (6)
Cc: (b) (6) Broder, Chris (b) (6)
Subject: Re: R01 reviews
Date: Sunday, June 26, 2022 9:33:32 PM
Attachments: 1R01AI168287-01A1.pdf

Great reviews, (b) (6). It is interesting to see the panel is almost all human infectious disease people. So it's great that you were able to get a One Health proposal through and with such strong reviews.

Sent from my iPhone

> On Jun 26, 2022, at 7:13 AM, (b) (6) wrote:
>
> ****External Sender****
>
> Attached are the reviews, FYI.
>
> (b) (6)
>
> ---
>
> **From:** (b) (6)
> **Sent:** Wednesday, June 22, 2022 11:12 AM
> **To:** (b) (6)
> **Cc:** Broder, Chris (b) (6)
> **Subject:** Re: plans for R01 submission
>
> This makes me so happy. Well done (b) (6) and team on leading this. 5th percentile! Looking forward to working with everyone.
> (b) (6)
>
> [redacted]

(b) (6)

From: (b) (6)
Date: Wednesday, 22 June 2022 at 15:59
To: (b) (6)
Cc: Broder, Chris (b) (6)
Subject: Re: plans for R01 submission

Yesss!!! This is terrific news!

On Wed, Jun 22, 2022 at 5:42 PM (b) (6) wrote:

> Congrats!!!
>
> (b) (6)
>
> ---
>
> **From:** (b) (6)
> **Sent:** Wednesday, June 22, 2022 8:30 AM
> **To:** (b) (6) Laing, Eric (b) (6) ; Broder, Chris (b) (6)
> **Cc:** (b) (6)
> **Subject:** [EXTERNAL] RE: plans for R01 submission
>
> Dear colleagues,

We've received scores back on our resubmission. Impact score is (b) (6) and we're at the (b) (5) percentile. With these numbers, we should expect to be funded. Congratulations.

I've heard from our Program Officer about timelines. Funding will start in fiscal year 2023, which begins on Oct 1, 2022. Her best guess is that we'll be funded to begin before the end of 2022, but exact timing will depend on when the US government approves their annual budget.

I look forward to working with you all on this exciting study.

(b) (6)

From: (b) (6)
Sent: Monday, January 10, 2022 4:13 PM
To: (b) (6) ; Laing, Eric (b) (6) ; christopher.broder (b) (6) ; (b) (6)
Subject: plans for R01 submission

Dear colleagues,

Happy New Year to you all!

I'm writing to follow up and move us forward with plans to resubmit the R01. The date for submission is Feb 7th, but it's likely that our admin team will require documents well before that (b) (6) is working to put together our timeline now – updates shortly.

Based on my understanding, these are the updates that need to happen to respond to our last round of reviews:

1. Add in reference to preprint from (b) (6) group on the sero assay
2. Add in appendix with biosafety protocols from icddr,b, including staff safety, and perhaps letter from biosafety committee at icddr,b
3. Updating research plan to mention these updates, and address question of collecting some additional samples from sick animals (per reviewer comment)
4. Drafting one page response to reviewers to include in resubmission

I will draft the response to reviewers for your review and comment. (b) (6), grateful for updates and reference for your preprint. (b) (6), look forward to receiving the biosafety SOPs (and thoughts on letter) as soon as you're able.

I assume that there are no changes required to budget. If this assumption is

incorrect for you, please get in touch with me ASAP with the changes you'd like to include. However, there are new updates to biosketches that could be time consuming – (b) (6) will send out guidance on this so please be on the lookout.

Look forward to getting this back in!

(b) (6)

[redacted]

Expert Panel Discussion for Vaccine Library virus selection #2

1. Introduction/overview of next steps.

2. Presentation and discussion of Spillover program- Dr. (b) (6)

3. Questions for panel discussion.

What below factors in selecting viruses for a potential Disease X emergence do you believe are important in selecting a virus for a Disease X emergence?

1. RNA vs DNA virus with RNA>DNA
2. Virus characteristics: mutation rate of the virus, ability to reassort and/or recombine, fitness, tropism, number of recognized generic lineages ie genetic diversity?
3. Mode of human to human transmission: respiratory > oral-fecal > vector-borne (mosquito >tick>midge>sandflies) > sexual transmission > water-borne > fomite > others? A virus that uses several modes of transmission ie vector plus sexual would this increase its importance?
4. Zoonotic transmission: NHP>wild birds > bats>domestic animal ie (cattle>pigs>horses>birds>camels>fish) > market food (bush meat, civets, snakes, snails)> others?
5. Is the type of zoonotic transmission important and can it be ranked: wild-birds > domestic birds > cattle > pigs > sheep> horse > domestic dogs/cats > goats > camels > NHP > guinea pigs > others?
6. Should we consider viruses with a successful vaccine ie measles, chickenpox, polio or should we anticipate escape mutants that aren't protected by a current vaccine and develop vaccines against these pathogens that are more broadly protective?
7. R_0 if known of the virus or within a family?
8. Reported human outbreaks of viruses in a family and if they are: sporadic outbreaks, cyclical, annual, endemic, pandemic pathogens.
9. Number of spillover events from zoonotic to humans in total, per year; spillover to human with human-to-human transmission, spillover to vector to human?
10. If vector-borne, characteristics of the vector ie stable distribution, expanding due to environmental changes, number of viable vectors, phylogenetic distance ie diversity in the vectors?
11. Susceptible population: for example, restricted regional occurrence leaving a large population susceptible.
12. Potential disease impact/burden, mortality, and/or morbidity?

Do you think the human outbreak characteristics of one virus in a family could be a potential characteristic of other viral pathogens within a family ie do they share potentially common virulence factors? For example Lassa fever and Junin viruses would they elevate specific viruses in the viral family of arenaviruses that may share common characteristic but are not known human pathogens yet? Knowing the potential of SARS-Cov and MERS before Covid-19 would that have elevated the concerns for other coronaviruses

In the selection of viruses within a family, do you believe there is short-term heterotypic protection from one virus within or beyond a genera and this should be considered in selecting viruses for the vaccine library? For example, acute DENV-1 may provide short-term protection against other serotypes,

JEV vaccination may provide short-term protection against DENV infection. If yes, is this specific for a virus family and not generic across many families?

4. Summary

(b) (5)

(b) (5)

(b) (5)

From: (b) (6)
To: (b) (6) ; Broder, Christopher; (b) (6)
Cc: (b) (6)
Subject: RE: Vaccine library virus selection available times for meetings #2
Date: Monday, April 4, 2022 1:55:21 PM
Attachments: 2022 0405 Apr - Experts Agenda meeting #2 questions for discussion.docx
2022 0405 Virus panel selection.xlsx

Dear all,

Please see attached the agenda for tomorrow's Expert panel meeting #2.
Included also is the spreadsheet for the virus rankings for the paramyxo and arenaviruses.

Looking forward to a good discussion tomorrow.
Thank you. Best regards,

(b) (6) on behalf of (b) (6)

-----Original Appointment-----
From: (b) (6)
Sent: Thursday, March 17, 2022 1:00 PM
To: (b) (6) ; Broder, Christopher; (b) (6)
Cc: (b) (6)
Subject: Vaccine library virus selection available times for meetings #2
When: Tuesday, April 5, 2022 6:00 PM-8:00 PM (UTC+00:00) Dublin, Edinburgh, Lisbon, London.
Where: https://cepi-net.zoom.us/j/85749182660

Vaccine library virus selection available times for meetings #2

Join Zoom Meeting
https://cepi-net.zoom.us/j/85749182660

Meeting ID: 857 4918 2660
Passcode: 412457
One tap mobile
+16699006833,,85749182660#,,,,*412457# US (San Jose)
+19292056099,,85749182660#,,,,*412457# US (New York)

Dial by your location
+1 669 900 6833 US (San Jose)

+1 929 205 6099 US (New York)
+1 253 215 8782 US (Tacoma)
+1 301 715 8592 US (Washington DC)
+1 312 626 6799 US (Chicago)
+1 346 248 7799 US (Houston)
833 548 0276 US Toll-free
833 548 0282 US Toll-free
877 853 5257 US Toll-free
888 475 4499 US Toll-free
+47 2400 4735 Norway
+47 2400 4736 Norway
800 248 88 Norway Toll-free
+44 330 088 5830 United Kingdom
+44 131 460 1196 United Kingdom
+44 203 481 5237 United Kingdom
+44 203 481 5240 United Kingdom
+44 203 901 7895 United Kingdom
+44 208 080 6591 United Kingdom
+44 208 080 6592 United Kingdom
0 800 031 5717 United Kingdom Toll-free
0 800 260 5801 United Kingdom Toll-free
0 800 358 2817 United Kingdom Toll-free
0 800 456 1369 United Kingdom Toll-free

Meeting ID: 857 4918 2660
Passcode: 412457
Find your local number: https://cepi-net.zoom.us/u/kekfEjM11E

Sensitivity: CEPI Internal

Arenaviridae

Sheli R. Radoshitzky, Michael J. Buchmeier, Rémi N. Charrel, J. Christopher S. Clegg, Jean-Paul J. Gonzalez, Stephan Günther, Jussi Hepojoki, Jens H. Kuhn, Igor S. Lukashevich, Víctor Romanowski, Maria S. Salvato, Manuela Sironi, Mark D. Stenglein and Juan Carlos de la Torre

The citation for this ICTV Report chapter is the summary published as Radoshitzky et al., (2019): ICTV Virus Taxonomy Profile: *Arenaviridae*, Journal of General Virology, 100, 1200–1201.

Corresponding author: Juan Carlos de la Torre (juanct@scripps.edu)
Edited by: Jens H. Kuhn, Stuart G. Siddell and Peter J. Walker
Posted: May 2019, updated September 2020
PDF: ICTV_*Arenaviridae*.pdf

Summary

Members of the family *Arenaviridae* produce enveloped virions containing genomes consisting of 2 to 3 single-stranded RNA segments totaling about 10.5 kb (Table 1.*Arenaviridae*). Arenaviruses are currently classified into four genera (*Antennavirus*, *Hartmanivirus, Mammarenavirus*, and *Reptarenavirus*). These viruses infect fish (antennaviruses), snakes (hartmaniviruses and reptarenaviruses) and mammals (mammarenaviruses). Some reptarenaviruses cause boid inclusion body disease in captive snakes, whereas some mammarenaviruses can infect humans and other primates, causing mild, severe, and sometimes fatal diseases.

Table 1.*Arenaviridae.* Characteristics of members of the family *Arenaviridae.*

Characteristic	Description*
Typical member	lymphocytic choriomeningitis virus Armstrong 53b [S segment: AY847350; L segment: AY847351], species *Lymphocytic choriomeningitis mammarenavirus*, genus *Mammarenavirus.*
Virion	Enveloped, pleomorphic virions 40–200 nm in diameter with trimeric surface spikes
Genome	Two or three single-stranded, usually ambisense coding arrangement, RNA molecules called small (S), medium (M), and large (L)
Replication	Ribonucleoprotein (RNP) complexes are generated that contain anti-genomic

	RNA serving as coding templates for synthesis of genomic RNA
Translation	Proteins are produced from capped and non-polyadenylated mRNAs. The 5′-cap structure is derived by polymerase slippage or cap-snatching from cellular mRNAs
Host range	Fish (antennaviruses), predominantly small mammals (mammarenaviruses), and reptiles (hartmaniviruses and reptarenaviruses), but potentially also bats and ticks
Taxonomy	Realm *Riboviria*, phylum *Negarnaviricota*, subphylum *Polyploviricotina*, class *Ellioviricetes*, order *Bunyavirales*. The family includes 4 genera and 50 species

* mostly based on experiments with mammalian arenaviruses

Viruses assigned to each of the 4 genera form a monophyletic clade based on phylogenetic analysis of large protein/RNA-directed RNA polymerase (L/RdRP) and nucleoprotein (NP) sequences. Viruses from all four genera share one or more of the following characteristics: (i) enveloped spherical or pleomorphic virions; (ii) segmented single-stranded, ambisense RNA genome without polyadenylated tracts at the 3′-termini; (iii) genomic 5′- and 3′-end sequence complementarity; (iv) nucleotide sequences that could form one or more hairpin configurations within non-coding intergenic regions (IGRs) of genomic segments; (v) capped but not polyadenylated virus mRNAs; and (vi) induction of a persistent and frequently asymptomatic infection in reservoir hosts, in which chronic viremia and/or viruria occur (Radoshitzky et al., 2015).

Piscine host

Genus *Antennavirus*. This recently established genus currently includes 2 species for 2 viruses discovered in actinopterygian fish. Antennaviruses are notable for having genomes consisting of 3, rather than 2, genomic segments and likely not encoding the zinc -binding matrix (Z) protein, which is encoded by mammarenaviruses and reptarenaviruses.

Reptilian host

Genus *Hartmanivirus*. This recently established genus currently includes 4 species for 6 viruses discovered in captive snakes with boid inclusion body disease (BIBD). Hartmaniviruses are notable for genomes lacking a gene encoding the Z protein, which is encoded by mammarenaviruses and reptarenaviruses.

Genus *Reptarenavirus*. This genus currently includes 5 species for 8 viruses discovered in captive snakes, some of which were suffering from BIBD. Reptarenaviruses are notable for their transmembrane surface GP2 glycoproteins, which are more closely related to those of ebolaviruses (order *Mononegavirales*, family *Filoviridae*) than to those of antennaviruses, hartmaniviruses,

mammarenaviruses or other bunyaviruses. Reptarenaviruses are also unusual in that they are prone to cause co-infections, with multiple distinct S and L segments, not necessarily in a 1:1 ratio, being detectable in snakes.

Mammalian host

Genus *Mammarenavirus*. The genus currently includes 39 species for 46 viruses. These viruses have been detected in rodent hosts, apart from Tacaribe virus (TCRV) which has been found only in phyllostomid bats and ixodid lone star ticks. Mammarenavirus infections of their natural rodent hosts are generally asymptomatic. In humans, some mammarenaviruses, such as Western African Lassa virus (LASV) or several viruses of South American origin, can cause severe and often fatal diseases with hemorrhagic manifestations. Lymphocytic choriomeningitis virus (LCMV), the typical mammalian arenavirus, can also cause disease in humans and poses a serious threat to immunocompromised individuals.

Virion

Morphology

Virions are spherical or pleomorphic in shape, 40–200 nm in diameter, with dense lipid envelopes (Figure 1.*Arenaviridae*). The virion surface layer is covered with club-shaped projections with distinctive stalk and head regions. These projections are made of trimeric spike structures of two virus-encoded membrane glycoprotein (GP) subunits (GP1 and GP2) and in case of some arenaviruses, a third component (stable signal peptide [SSP]). Isolated RNP complexes are organized into “beads-on-a-string”-like structures (Hetzel et al., 2013, Li et al., 2016, Neuman et al., 2005, Buchmeier 2002, Charrel and de Lamballerie 2003, Jay et al., 2005, Meyer et al., 2002, Hepojoki et al., 2018).

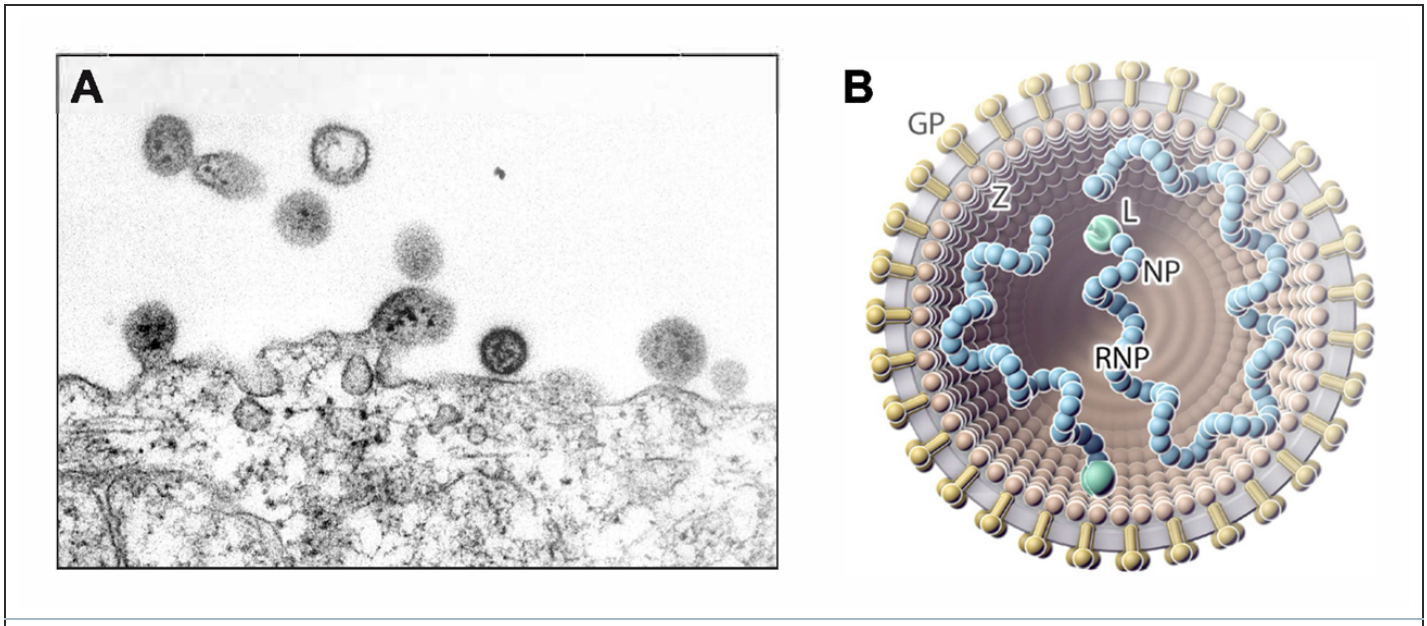


Figure 1.*Arenaviridae.* A) Electron micrograph of (mammalian) arenavirus particles, showing dark internal inclusion bodies (Latin: *arena*, sand), budding from an infected cell. **B)** Schematic illustration of an arenavirus particle. Shown is the spherical and enveloped (grey) particle that is spiked with glycoproteins (GP, gold) around a layer of zinc-binding matrix proteins (Z, brown; missing in hartmaniviruses). The small (S) and large (L) ribonucleoprotein (RNP) complexes inside the particle consist of nucleoprotein (NP, blue) and RNA-directed RNA polymerase (L, green).

Physicochemical and physical properties

Mainly known for members of the genus *Mammarenavirus* (see section on genus page).

Nucleic acid

Arenavirions typically contain 2 or 3 linear, ambisense or negative-sense single-stranded RNA segments that are encapsidated independently. These RNAs are uncapped (Leung et al., 1977) and contain a single non-templated G at each of the 5′-ends (Garcin and Kolakofsky 1990, Raju et al., 1990, Shi et al., 2018). No poly(A) tracts are present at the 3′-termini. The termini of the RNAs ends have inverted complementary sequences encoding transcription and replication initiation signals (Hepojoki et al., 2018, Salvato et al., 1989, Harnish et al., 1993, Young and Howard 1983).

Proteins

Arenaviruses express 3 (hartmaniviruses) or 4 (antennaviruses, mammarenaviruses, reptarenaviruses) structural proteins. The most abundant structural protein in virions is the nucleoprotein (NP), which encapsidates the virus genomic segments. The least abundant protein is the RNA-directed RNA polymerase (L), which mediates virus genome replication and transcription. The zinc-binding matrix (Z) protein, which is absent in antennaviruses and hartmaniviruses, is a matrix protein. Glycoproteins (GP1 or G1, GP2 or G2) are derived by post-translational cleavage of an intracellular GP precursor, the “glycoprotein-cell-associated” preprotein (GPC) by the cellular S1P/SKI protease. A third GPC cleavage product, the signal peptide, stays attached to the GP complex in hartmaniviruses and mammarenaviruses (stable signal peptide [SSP]), but not in reptarenaviruses (signal peptide [SP]). The GP structure of antennaviruses is unknown (Hepojoki et al., 2018, Shi et al., 2018, Buchmeier et al., 1987, Kunz et al., 2003, Lenz et al., 2001, Koellhoffer et al., 2014, Bederka et al., 2014, Eichler et al., 2003, York et al., 2004).

Lipids

Only known for members of the genus *Mammarenavirus* (see section on genus page).

Carbohydrates

Only known for members of the genus *Mammarenavirus* (see section on genus page).

Genome organization and replication

The arenavirus genome typically consists of two or three single-stranded, typically ambisense RNA molecules, termed S, (M), and L. Some of these RNAs encode two proteins in non-overlapping open reading frames (ORF) of opposite polarities (ambisense coding arrangement) that are separated by non-coding intergenic regions (IGRs) (Figure 2.*Arenaviridae*). The S RNA encodes NP in the virus genome-complementary sequence, and, in many cases, the GPC in the virus genome-sense sequence. The L RNA encodes L in the virus genome-complementary sequence, and, in some case, Z in the virus genome-sense sequence. Antennaviruses and hartmaniviruses lack the Z ORF, and antennaviruses encode at least one protein of unknown function. The IGRs form one or more energetically stable stem-loop (hairpin) structures and which function in structure-dependent transcription termination and in virion assembly and budding.

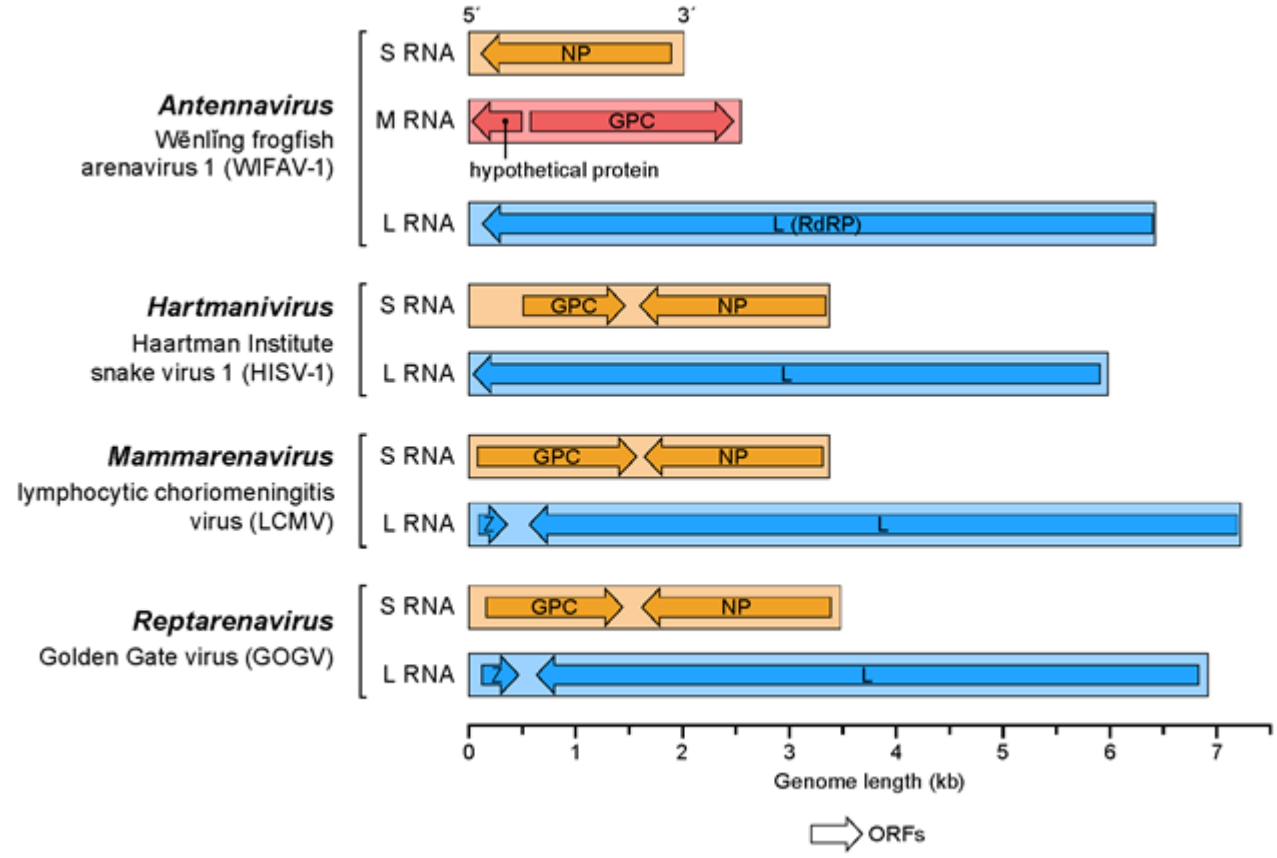


Figure 2.*Arenaviridae*. Schematic representation of the bi- or tri-segmented arenavirus genome organization. The 5′- and 3′-ends of all segments (S, [M], and L) are complementary at their termini, likely promoting the formation of circular ribonucleoprotein complexes within the virion. GPC, glycoprotein precursor; L, RNA-directed RNA polymerase; NP, nucleoprotein; Z, zinc-binding matrix

protein. Open reading frames are separated by non-coding intergenic regions (IGRs), with predicted hairpin structures (not shown).

Arenavirus infection starts with attachment to cell-surface receptors and entry via the endosomal route (Martinez et al., 2007, Vela et al., 2007, Borrow and Oldstone 1994, Radoshitzky et al., 2007, Cao et al., 1998, Raaben et al., 2017, Glushakova and Lukashevich 1989) (Figure 3.*Arenaviridae*). pH-dependent fusion with late endosomes releases the virion RNP complex into the cytoplasm. In the case of some mammalian arenaviruses (LASV), this fusion event involves a pH-dependent switch to an intracellular receptor, lysosomal associated membrane protein 1 (LAMP1) (Jae et al., 2014). The virus RNP directs both RNA genome replication and gene transcription (Meyer et al., 2002). During replication, L reads through the IGR transcription-termination signal and generates uncapped antigenomic and genomic RNAs (Leung et al., 1977). Because these RNAs contain a single non-templated G at the 5′-ends (Garcin and Kolakofsky 1990, Raju et al., 1990), replication initiation might involve a slippage mechanism of L on the nascent RNA (Garcin and Kolakofsky 1992). In case of ambisense coding arrangements, only mRNAs encoding NP or L can be synthesized from genomic RNAs. Transcription of mRNAs encoding GPC or Z occurs only after the first round of virus replication, during which S and L antigenomes are produced.

Virus proteins are synthesized from subgenomic capped mRNAs that lack terminal poly(A) (Meyer and Southern 1993, Singh et al., 1987, Southern et al., 1987). The 5′-ends of virus mRNAs contain several non-templated bases, suggesting that arenaviruses use either polymerase slippage or a cap-snatching mechanism similar to that used by other members of the subphylum *Polyploviricotina* (Garcin and Kolakofsky 1990, Raju et al., 1990, Meyer and Southern 1993). Cap-snatching would require an endonuclease presumed to be present in the N-terminal part of L, which cleaves cellular mRNAs to generate a cap leader that is subsequently used to prime arenavirus transcription. The 3′-termini of the mRNAs have been mapped to locations in the IGRs.

Virion budding occurs from the cellular plasma membrane, thereby providing the virion envelope (Dalton et al., 1968, Eichler et al., 2004, Perez et al., 2003, Strecker et al., 2003).

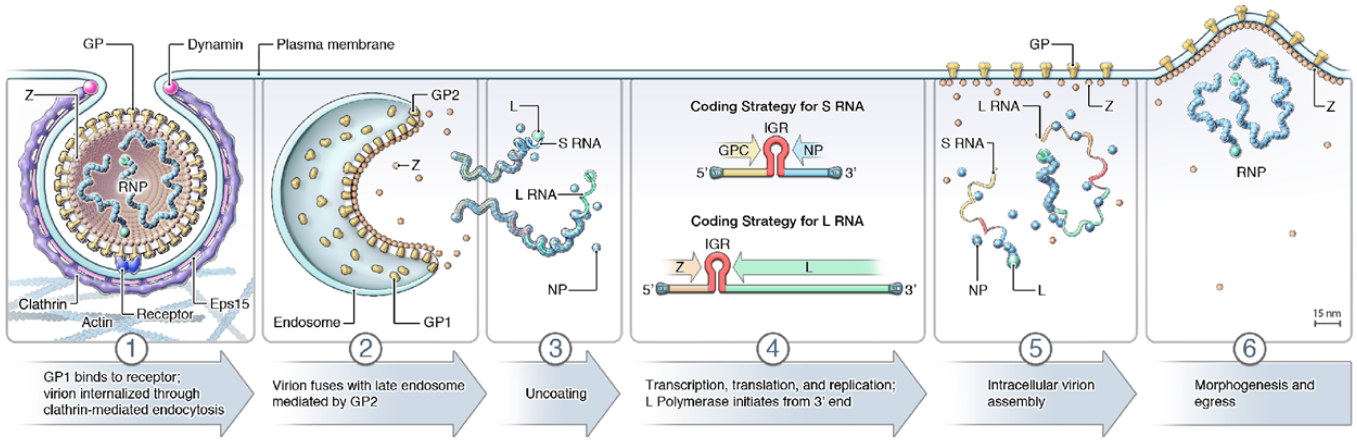


Figure 3.*Arenaviridae*. Lifecycle of arenaviruses. (1) Virion uptake; (2) virus-cell membrane fusion;

(3) uncoating; (4) transcription, translation, and replication; (5) virion assembly; and (6) virion budding. GP, glycoprotein; IGR, intergenic region; L, RNA-directed RNA polymerase; NP, nucleoprotein; RNP, ribonucleoprotein; Z, zinc-binding matrix protein. Note that antennaviruses and hartmaniviruses do not encode Z.

Biology

Arenaviruses are ecologically diverse: they have been isolated from fish (antennaviruses) (Shi et al., 2018), rodents, bats, and ticks (mammarenaviruses) (Downs et al., 1963, Sayler et al., 2014), and snakes (reptarenaviruses, hartmaniviruses) (Hetzel et al., 2013, Hepojoki et al., 2018, Hepojoki et al., 2015, Stenglein et al., 2012). The geographic distribution of arenaviruses overlaps with the distribution of their hosts. Most mammalian arenaviruses infect rodents of preferentially one or a few species and are, therefore, geographically constrained to their hosts, but LCMV, which infects the ubiquitous house mouse (*Mus musculus* Linnaeus, 1758) appears distributed globally (Childs 1993). The natural distribution of reptilian arenaviruses is unknown as they have only been detected in captive snakes thus far (Hetzel et al., 2013, Hepojoki et al., 2018, Hepojoki et al., 2015, Stenglein et al., 2012). A diverse range of vertebrate cell lines are permissive to mammalian arenavirus infection *in vitro*; certain reptilian cell lines support replication of reptilian arenaviruses (Hepojoki et al., 2018, Stenglein et al., 2012, Lukashevich et al., 1983).

Antigenicity

Systematic antigenicity studies have only been reported for mammarenavirions (see section on *Mammarenavirus* genus page).

Genus demarcation criteria

Classification of arenaviruses is currently based on pairwise sequence comparisons (PASC) of coding-complete genomes. Based on the most current sequence dataset, S segment and L segment nucleotide sequence identities for viruses within the same genus need to be higher than 40% and 35%, respectively (Radoshitzky et al., 2015). Four genera have been established to date. Viruses assigned to a genus form a monophyletic clade in well-supported maximum likelihood trees using complete L and NP nucleotide sequences and/or core L palm domain sequences. Use of L and NP for taxonomic purposes is justified by the presence of broadly conserved domains and the rarity of reassortment between genetic segments, at least in mammarenaviruses. Hence, the availability of at least coding-complete sequences of all genome segments may be sufficient for arenavirus classification in the absence of a cultured isolate. Classification is also possible when at least a coding-complete genomic S segment sequence is available together with a cultured isolate (Radoshitzky et al., 2015). However, at the present time, classification also includes the

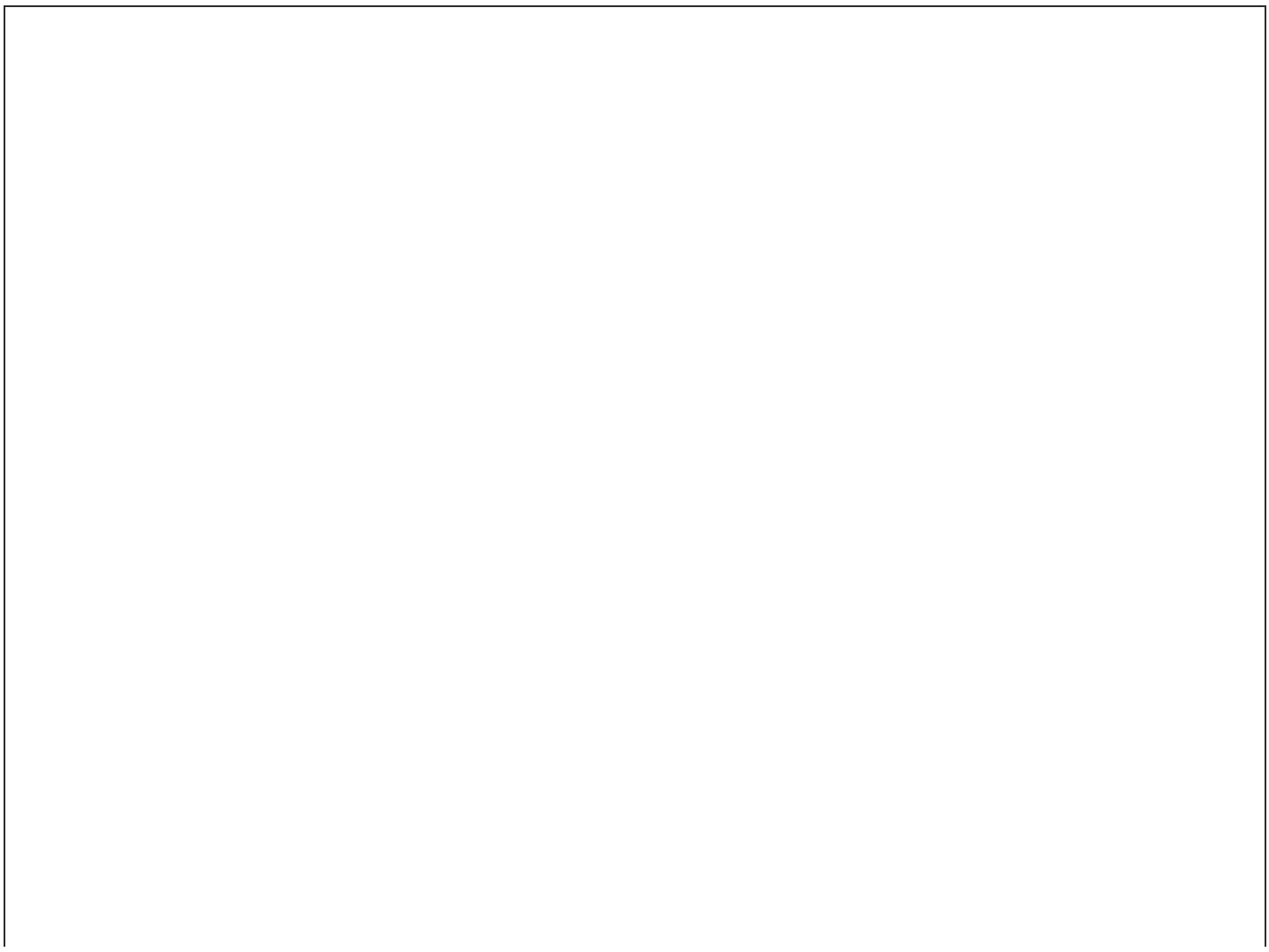
consideration of phenotypic characters such as significant differences in member virus genome architecture, virion antigenicity, and virus ecology (e.g., host range, pathobiology, and transmission patterns).

Derivation of names

Arenaviridae: from the Latin *arenosus* meaning “sandy” and *arena* meaning “sand,” in recognition of the “sandy” appearance of mammarenavirus particles observed in electron-microscopic thin sections (Rowe et al., 1970a).

Relationships within the family

Phylogenetic relationships across the family have been established from maximum likelihood trees generated using complete L amino acid sequences (Figure 4.*Arenaviridae*). Phylogenetic relationships between viruses assigned to more closely related genera and within genera can also be established using other structural protein genes, notably NP.



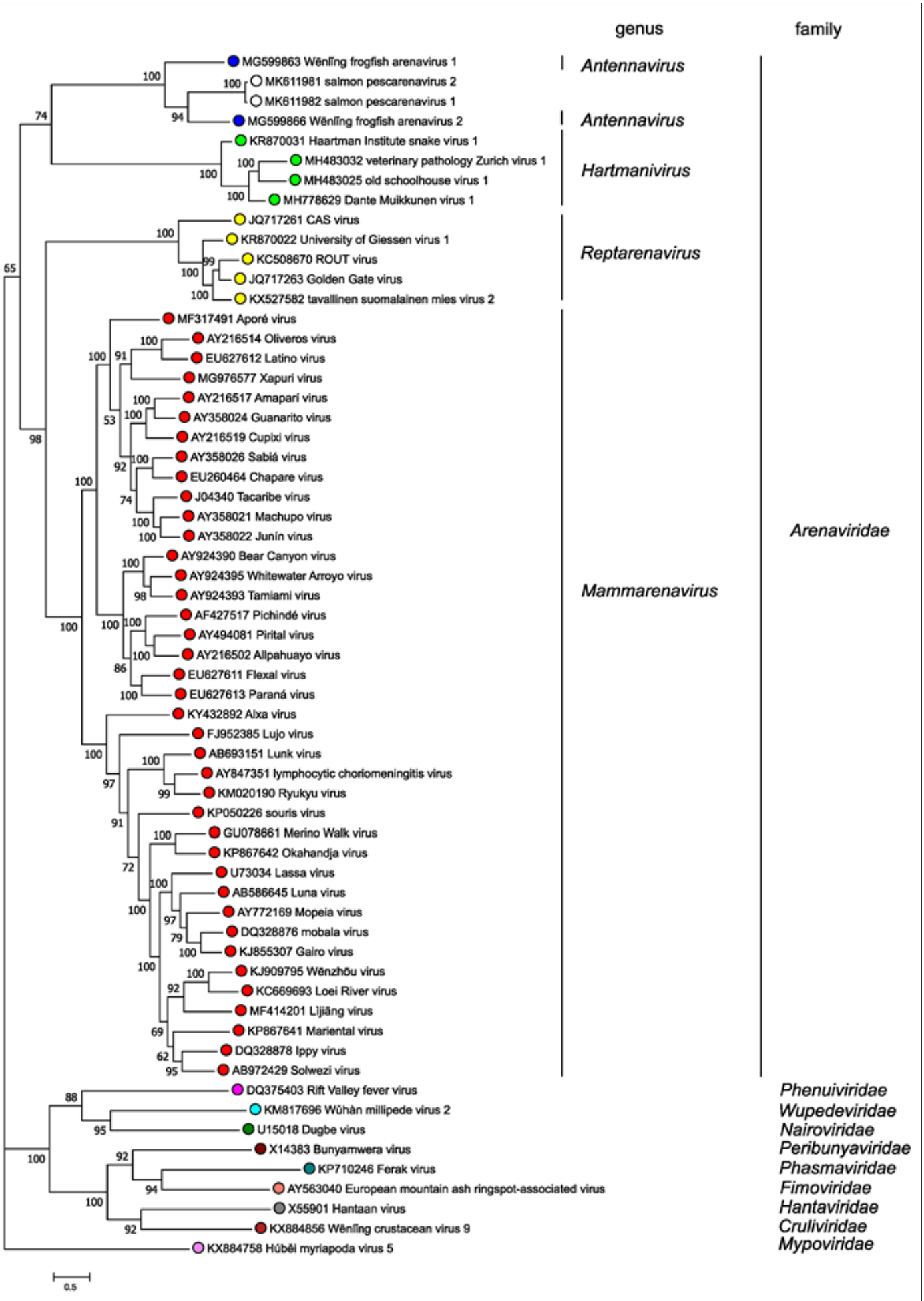

genus
family
MG599863 Wēnlǐng frogfish arenavirus 1
MK611981 salmon pescarenavirus 2
MK611982 salmon pescarenavirus 1
MG599866 Wēnlǐng frogfish arenavirus 2
KR870031 Haartman Institute snake virus 1
MH483032 veterinary pathology Zurich virus 1
MH483025 old schoolhouse virus 1
MH778629 Dante Muikkunen virus 1
JQ717261 CAS virus
KR870022 University of Giessen virus 1
KC508670 ROUT virus
JQ717263 Golden Gate virus
KX527582 tavallinen suomalainen mies virus 2
MF317491 Aporé virus
AY216514 Oliveros virus
EU627612 Latino virus
MG976577 Xapuri virus
AY216517 Amapari virus
AY358024 Guanarito virus
AY216519 Cupixi virus
AY358026 Sabiá virus
EU260464 Chapare virus
J04340 Tacaribe virus
AY358021 Machupo virus
AY358022 Junín virus
AY924390 Bear Canyon virus
AY924395 Whitewater Arroyo virus
AY924393 Tamiami virus
AF427517 Pichindé virus
AY494081 Pirital virus
AY216502 Allpahuayo virus
EU627611 Flexal virus
EU627613 Paraná virus
KY432892 Alxa virus
FJ952385 Lujo virus
AB693151 Lunk virus
AY847351 lymphocytic choriomeningitis virus
KM020190 Ryukyu virus
KP050226 souris virus
GU078661 Merino Walk virus
KP867642 Okahandja virus
U73034 Lassa virus
AB586645 Luna virus
AY772169 Mopeia virus
DQ328876 mobala virus
KJ855307 Gairo virus
KJ909795 Wēnzhōu virus
KC669693 Loei River virus
MF414201 Lijiāng virus
KP867641 Mariental virus
DQ328878 Ippy virus
AB972429 Solwezi virus
DQ375403 Rift Valley fever virus
KM817696 Wǔhàn millipede virus 2
U15018 Dugbe virus
X14383 Bunyamwera virus
KP710246 Ferak virus
AY563040 European mountain ash ringspot-associated virus
X55901 Hantaan virus
KX884856 Wēnlǐng crustacean virus 9
KX884758 Húběi myriapoda virus 5
Antennavirus
Antennavirus
Hartmanivirus
Reptarenavirus
Mammarenavirus
Arenaviridae
Phenuiviridae
Wupedeviridae
Nairoviridae
Peribunyaviridae
Phasmaviridae
Fimoviridae
Hantaviridae
Cruliviridae
Mypoviridae
0.5

Figure 4.*Arenaviridae*. Maximum likelihood phylogenetic tree inferred from PRANK alignment (Löytynoja and Goldman 2008) of the complete L amino acid sequences of 50 arenaviruses assigned to the genera *Antennavirus* (blue dots, blue rings for unclassified viruses the genus), *Hartmanivirus*(green dots), *Mammarenavirus* (red dots) and *Reptarenavirus* (yellow dots), along with representative viruses of other bunyavirus families (other colors of dots). The best-fit model of protein evolution (LG+G) was selected using ProtTest 3 (v. 3.4.2) (Darriba et al., 2011). The maximum likelihood tree with 1,000 bootstrap replicates was produced using RAxML (v. 8) (Stamatakis 2014). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap is shown next to branch nodes (when ≥ 70%). The tree was visualized using FigTree (http://tree.bio.ed.ac.uk) and is mid-point rooted. This phylogenetic tree and corresponding sequence alignment are available to download from the Resources page.

Relationships with other taxa

Arenaviruses are closely related to Húběi myriapoda virus 5 (*Bunyavirales*: *Mypoviridae*) (Shi et al., 2016).

Related, unclassified viruses

Additional unclassified arenaviruses that are probable members of existing genera are listed under individual genus descriptions.

Virus name	Accession number	Virus abbreviation
DF 20/00 virus (Granzow et al., 2014)	Not available	-
DF 26/02 virus (Granzow et al., 2014)	Not available	-
Hyriopsis cumingii Lea plague virus (Carella et al., 2016, Zhong et al., 2011)	Not available	HcPV

Virus names and abbreviations are not official ICTV designations.

Member taxa

- *Antennavirus*
- *Hartmanivirus*
- *Mammarenavirus*
- *Reptarenavirus*

Paramyxoviridae

Bert Rima, Anne Balkema-Buschmann, William G. Dundon, Paul Duprex, Andrew Easton, Ron Fouchier, Gael Kurath, Robert Lamb, Benhur Lee, Paul Rota and Linfa Wang

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Corresponding author: Bert Rima (b.rima@qub.ac.uk)
Edited by: Jens H. Kuhn and Stuart G. Siddell
Posted: September 2019, updated August 2020
PDF: ICTV_*Paramyxoviridae*.pdf

Summary

The family *Paramyxoviridae* consists of large enveloped RNA viruses infecting mammals and birds, or in some cases reptiles and fish (Table 1.*Paramyxoviridae*). Many paramyxoviruses are host-specific and several such as measles virus, mumps virus, Nipah virus, Hendra virus and several parainfluenza viruses are pathogenic for humans. Virus transmission is horizontal, mainly through direct contact and airborne routes; no vectors are known.

Table 1.*Paramyxoviridae*. Characteristics of members of the family *Paramyxoviridae*

Characteristic	Description
Typical member	measles virus, Ichinose-B95a (AB016162), species *Measles morbillivirus*, genus *Morbillivirus*
Virion	Enveloped, pleomorphic (mostly spherical) virions with a diameter of 300–500 nm enclosing a ribonucleoprotein
Genome	Negative-sense, non-segmented RNA genomes of 14.6–20.1 kb
Replication	Cytoplasmic, by the virus ribonucleoprotein complex, involves replication of antigenome and transcription of 6–8 positive-sense mRNAs
Translation	Cytoplasmic, by cellular machinery from capped and poly-adenylated mRNAs
Host range	Mammals, birds, fish and reptiles

Taxonomy	Realm Riboviria, phylum *Negarnaviricota*, class *Monjiviricetes*, order *Mononegavirales*. Currently 4 subfamilies, 17 genera and 78 species

Virion

Morphology

Virions are 150 nm or more (up to 500nm) in diameter, pleomorphic, but usually spherical in shape in vitreous ice. Virions consist of a lipid envelope surrounding a nucleocapsid. The envelope is derived directly from the host cell plasma membrane by budding and contains two transmembrane glycoproteins (Figure 1.*Paramyxoviridae*). These are present as homo-oligomers and form spike-like projections, 8–12 nm in length, spaced 7–10 nm apart (depending on virus genus affiliation). Also, depending on the genus, one or two additional transmembrane proteins may be present. One non-glycosylated membrane or matrix protein is associated with the inner face of the envelope. The virus nucleocapsid consists of negative-sense virus genome RNA and the nucleocapsid protein (N). The nucleocapsid has helical symmetry and is approximately 18 nm in diameter with a 7 nm pitch; its length can be up to 1,000 nm in viruses of some genera. The ribonucleoprotein (RNP) complex in the virion consists of the nucleocapsid together with the polymerase-associated or phosphoprotein (P) and the L protein (L, including RNA-directed RNA polymerase, capping and cap methylation activities) (Lamb and Parks 2007). Multiploid virions are found, although the vast majority of virions contain a single functional genome.

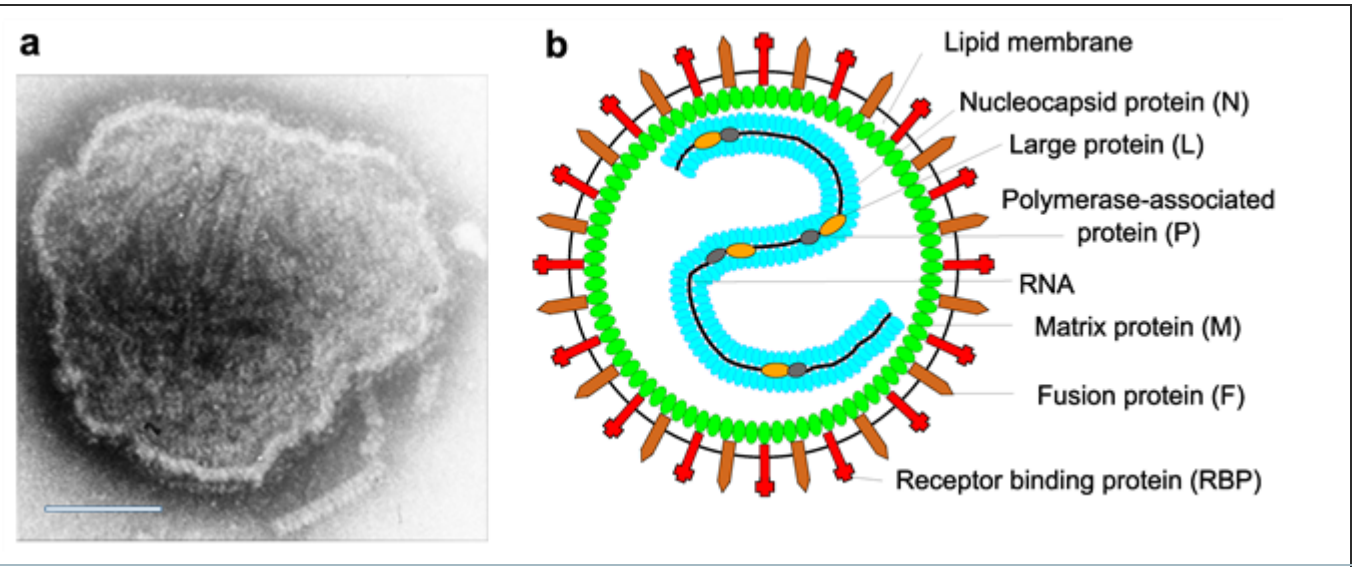


Figure 1.*Paramyxoviridae.* Paramyxovirus virion structure. (A) Negative-contrast electron micrograph of intact measles virus particle (genus *Morbillivirus*). Scale bar = 100 nm. (B) Schematic diagram of paramyxovirus particle in cross-section.

Physicochemical and physical properties

Virion Mr is about 500 ×10^6, and much greater for multiploid virions. Virion buoyant density in sucrose is 1.18–1.20 g cm^{-3}. Virion $S_{20,w}$ is at least 1000S. Virions are very sensitive to heat, lipid solvents, ionic and non-ionic detergents, formaldehyde and oxidizing agents.

Nucleic acid

Virions contain a single molecule of linear, negative-sense, single stranded RNA that is not infectious alone but is infectious if the RNP complex is introduced into the cytoplasm. The RNA genome varies from 14,296 nucleotides for Antarctic penguin virus B to 20,148 nt for Pohorje Myodes paramyxovirus 1. Genomes of all viruses in the family *Paramyxoviridae* are multiples of 6 nt, which is a requirement for efficient replication (Calain and Roux 1993). Some virions may contain positive-sense RNA and so partial self-annealing of extracted RNA may occur. Intracellularly, or in virions, genome-length RNA is found exclusively encapsidated in ribonucleocapsids (RNPs). The genome RNA does not contain a 5′-cap, nor a covalently linked protein. The genome 3′-end is not polyadenylated.

Proteins

Members of the family *Paramyxoviridae* encode 6–10 proteins (5–250 kDa) of which several can be derived either from gene editing events in the P locus and an overlapping ORF in the P gene itself (Figure 2.*Paramyxoviridae*). Virion proteins common to all genera include: three nucleocapsid-associated proteins, i.e., an RNA-binding nucleocapsid protein (N), a polymerase-associated phosphoprotein (P) and a large protein (L, including an RNA-directed RNA polymerase (RdRP), mRNA guanylyl- and methyltransferases, and methylation functions required for the capping of mRNAs), and three membrane-associated proteins, i.e., an unglycosylated inner membrane or matrix protein (M) and two glycosylated envelope proteins, comprising a fusion protein (F) and an attachment or receptor-binding protein (RBP, designated variably as HN, haemagglutinin-neuraminidase protein, H, haemagglutinin or G, glycoprotein). The F protein is synthesized within infected cells as a precursor (F_0) that is activated following cleavage by cellular protease(s) to produce the virion disulfide-linked F_1 and F_2 subunits (order: N-F_2-S-S-F_1-C). Some viruses also encode putative non-structural proteins (C), a cysteine-rich protein that binds Zn^{2+} (V) that can be structural or non-structural depending on the virus, a small integral membrane protein (SH) and transmembrane proteins (tM). Some virus genomes, such as that of the fer-de-lance virus, contain transcription units encoding proteins with unidentified functions. Virion enzyme activities include the RNA-directed RNA polymerase and mRNA guanylyl- and methyltransferases functionally encoded in the L protein. Variously represented among the genera are neuraminidases associated with the RBP.

Lipids

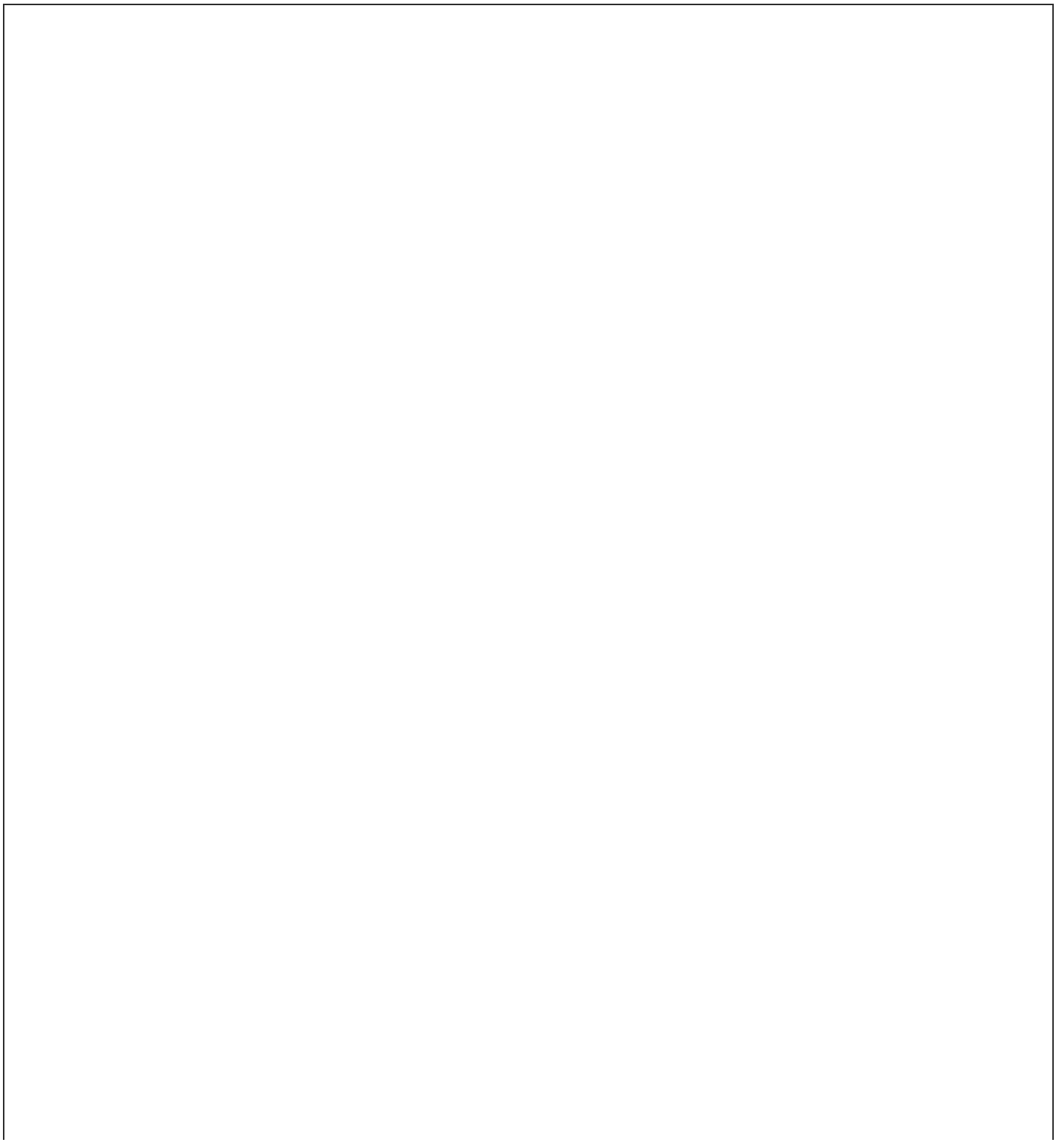
Lipids in the virus envelope are derived from host cell plasma membrane.

Carbohydrates

Virions are composed of approximately 6% carbohydrate by weight; composition is dependent on the host cell. Fusion and RBP proteins are glycosylated by *N*-linked carbohydrate side chains.

Genome organization and replication

The genome organization is illustrated in Figure 2.*Paramyxoviridae* for viruses representing 14 of the genera in the family.



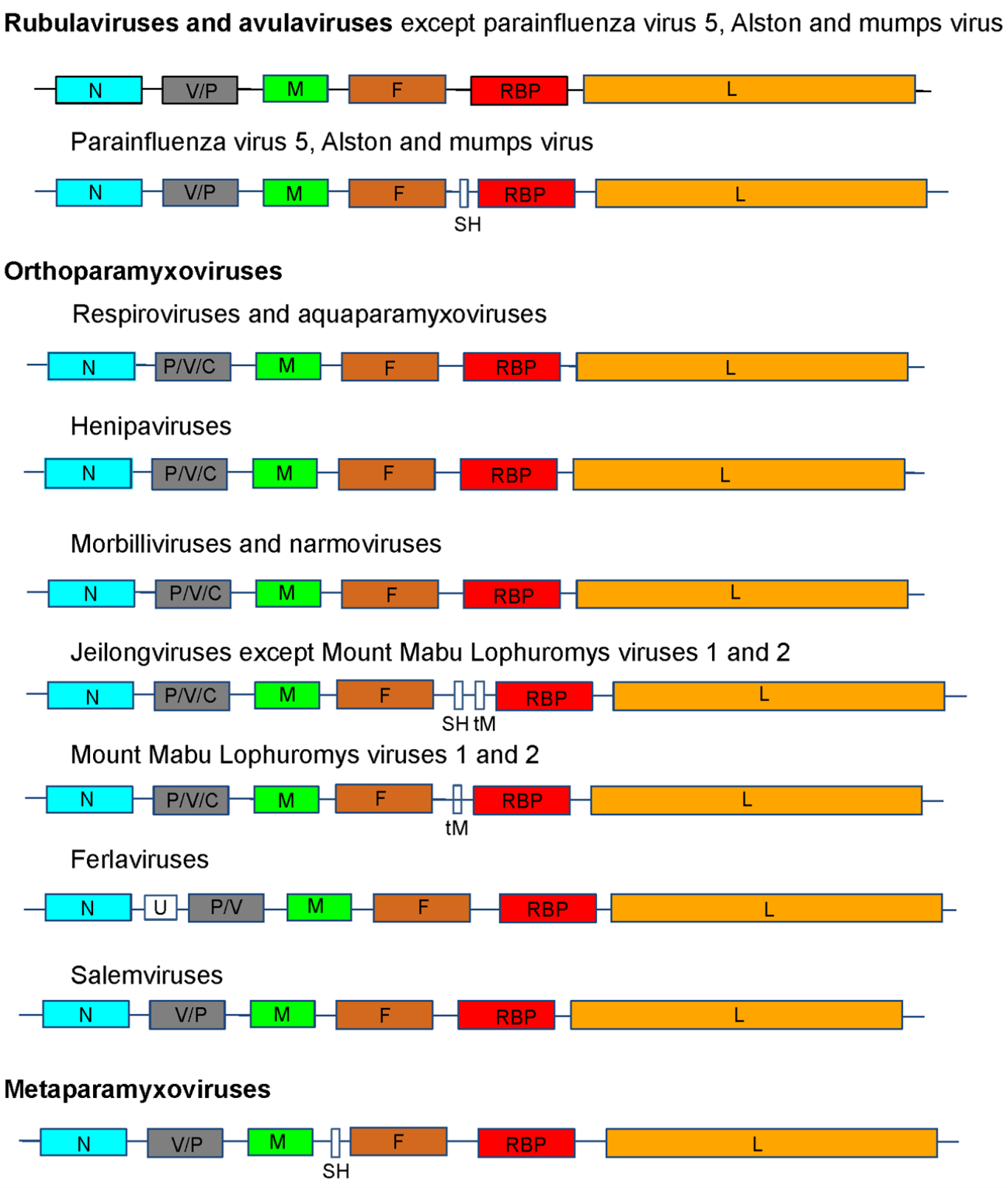


Figure 2.*Paramyxoviridae.* Genome organization (3′-to-5′) of viruses in the family *Paramyxoviridae*. Each box represents a separately encoded coding sequence; slashes indicate where multiple distinct ORFs are present within mRNA transcripts. Co-transcriptional editing leads to expression of the V or the P protein: the first shown is derived from the unedited sequence. The lengths of the boxes are approximately to scale although the non-coding sequences (NCS) are not to scale. Certain viruses express additional C proteins by the using multiple secondary translational start sites within the P

gene. In human parainfluenza virus 1 and human parainfluenza virus 3 of the genus *Respirovirus*, the V ORF may be a non-expressed relic, the function of which may be partially compensated by an edited D protein. U is an additional transcription unit between the N and P genes in ferlavirus genomes.

After attachment to cell receptors, virion entry is achieved by fusion of the virion envelope with the cell surface membrane. This can occur at neutral pH. Virus replication occurs in the cell cytoplasm and is thought to be independent of host nuclear functions. The genome is transcribed processively from the 3′-end by the virion-associated RdRP into 6–8 separate positive-sense mRNAs. Transcription is guided by short (10–13 nt) conserved gene start (GS) and gene end (GE) signals flanking the intergenic sequence. The mRNAs are capped by the guanylyl- and methyltransferase activities of the L protein and possess 3′-poly(A) tracts synthesized by reiterative copying of U tracts in GE sequence. Intergenic regions are highly conserved in length (3 nt) and sequence (CUU with few exceptions see Table 2.*Paramyxoviridae* for details) in the orthoparamyxoviruses and metaparamyxoviruses. Neither, the length or sequence of the intergenic sequences is conserved in avulavirus or rubulavirus genomes. RNA replication occurs through an intermediate, the antigenome, which is an exact positive-sense copy of the genome.

RNP assembly occurs in the cytoplasm and is tightly linked to RNA synthesis. RNPs are enveloped by budding at the cell surface plasma membrane at sites containing virion envelope proteins. Orthoparamyxovirus genomes contain 6–8 transcriptional elements that encode 7–11 proteins. Each element encodes a single mRNA with the exception of the P/V element. This element is transcribed into an exact copy mRNA (P or V mRNA, depending on genus) and into alternative versions in which the RNA transcriptase ‘stutters’ on the template at an editing motif midway down the element. This stuttering results in the insertion of one or more pseudo-templated G nucleotides (“RNA editing”) and shifts the reading frame to access alternative ORFs. The exact copy and edited mRNAs synthesize two alternative proteins, P and V, which have identical amino-terminal domains but due to the insertions of G residues have different carboxy-terminal domains. Other truncated, or chimeric, proteins (called I, W, or D, depending on the virus) can be produced by shifting into the third reading frame. The C ORF present in henipavirus, morbillivirus, narmovirus, jeilongvirus, aquaparamyxovirus and respirovirus genomes overlaps the P ORF and can initiate synthesis at an AUG codon that is accessed by ribosomal choice or at alternative start codons in the same ORF.

Biology

Paramyxoviruses have been conclusively identified only in vertebrates and mostly in mammals and birds, although they have recently also been detected in reptiles and fish, including boneless fish. Most viruses have a narrow host range in nature but can infect a broader range of cultured cells. Infection of cultured cells is generally lytic, but temperate or persistent infections are common in this family *in vitro*and *in vivo*. Other features of infection include the formation of inclusion bodies and syncytia. Host cell surface

molecules reported to serve as receptors for the attachment for members of the family vary (Thibault et al., 2017). Respiroviruses, some rubulaviruses and all avulaviruses use sialoglycoproteins and glycolipids as receptors. The cell surface proteins signaling lymphocytic activation molecule family member 1 (SLAMF1, aka CD150) and nectin cell adhesion molecule 4 (nectin 4) are major receptors for measles virus and other morbilliviruses. Henipaviruses use ephrin B2 (EFNB2) and B3 (EFNB3) proteins as cellular entry receptors (Table 2.*Paramyxoviridae*).

Table 2.*Paramyxoviridae.* Receptor and receptor binding protein properties of paramyxoviruses

Orthoparamyxovirinae

Genus	**Virus**	**RBP name / amino acid residues#**	**Sequence at start of RBP propeller blade 2***	**Cell receptor**	**Intergenic trinucleotides**
Aquaparamyxovirus	Atlantic salmon paramyxovirus	HN 576	**NRKSCS**	? probably neuraminic acid	CUU + CAU (F-HN)
Aquaparamyxovirus	Pacific salmon paramyxovirus	HN 578	**NRKSCS**	? probably neuraminic acid	CUU
Ferlavirus	fer-de-lance virus	HN 564	**NRKSCS**	? probably neuraminic acid	CCU(3x)+ CUU(4x) alternating
Jeilongvirus	Beilong virus	“G” 734	NRRSCT	?	CUU
Jeilongvirus	Tailam virus	“G” 1052	NRRSCT	?	CUU
Jeilongvirus	J-virus	“G” 709	NRRSCS	?	CUU
Jeilongvirus	Pohorje Myodes paramyxovirus 1	“G” 1589	NRRSCT	?	CUU
Jeilongvirus	Mount Mabu Lophuromys virus 1	“G” 854	NRKSCT	?	CUU
Jeilongvirus	Mount Mabu Lophuromys virus 2	“G” 810	**NRKSCS**	? probably neuraminic acid	CUU
Jeilongvirus	Shaan virus	HN 588	**NRKSCS**	? probably neuraminic acid	CUU + CGU (F-SH)
Henipavirus	Hendra virus	G 604	TIHHCS	EFNB2/3	CUU

Henipavirus	Nipah virus	G 420	TVYHCS	EFNB2/3	CUU
Henipavirus	Cedar virus	G 622	QVINCV	EFNB2	CUU
Henipavirus	Mòjiāng virus	G 625	IINSCA	protein?	CUU
Henipavirus	Ghana virus	G 632	NYHSCT	EFNB2	CUU + CUG (F-G)
Morbillivirus	measles virus wt	H 617	DFSNCM	SLAMF1/NECTIN4	CUU + CGU (H-L)
Morbillivirus	measles Ed-Zag vac	H 617	DLSNCM	SLAMF1/NECTIN4/CD46	CUU + CGU (H-L)
Morbillivirus	canine distemper virus	H 607	KTKVCT	SLAMF1/NECTIN4	CUU + CUA (H-L)
Morbillivirus	canine distemper virus vaccine	H 607	KAKVCT	SLAMF1/NECTIN4/?	CUU + CUA (H-L)
Morbillivirus	phocine distemper virus	H 607	NTKICT	SLAMF1/NECTIN4t	CUU + CUA (H-L)
Morbillivirus	rinderpest virus	H 609	ELETCM	SLAMF1/NECTIN4	CUU + CGU (H-L)
Morbillivirus	peste des petits ruminants virus	H 609	DYRSCL	SLAMF1/NECTIN4	CUU
Morbillivirus	dolphin morbillivirus	H 604	GLNFCL	SLAMF1/ NECTIN4	CUU
Morbillivirus	feline morbillivirus	H 595	GMESCT	SLAMF1/ NECTIN4	CUU + CUA (M-F)
Narmovirus	Nariva virus	“H” 657	AYDGCA	protein?	CUU
Narmovirus	Mossman virus	“G” 632	VFDGCS	protein?	CUU + CGU (F-H)
Narmovirus	bank vole virus 1	“G” 625	LRDSCT	protein?	CUU + CUA (P-M and F-H and L-t); CAU (M-F)
Narmovirus	Tupaia paramyxovirus	“H” 665	NLRDCS	protein?	CUU
Respirovirus	human parainfluenza virus 1	HN 575	**NRKSCS**	Neuraminic acid	CUU + CGU (P-M)
Respirovirus	Sendai virus	HN 575	**NRKSCS**	Neuraminic acid	CUU + CCC (HN-L)

Respirovirus	giant squirrel virus	HN 574	**NRKSCS**	? probably neuraminic acid	CUU +CAU (HN-L)
Respirovirus	human parainfluenza virus 3	HN 572	**NRKSCS**	Neuraminic acid	CUU
Respirovirus	bovine parainfluenza virus 3	HN 572	**NRKSCS**	Neuraminic acid	CUU
Respirovirus	porcine parainfluenza virus 1	HN 576	**NRKSCS**	? probably neuraminic acid	CUU
Respirovirus	caprine parainfluenza virus 3	HN 574	**NRKSCS**	? probably neuraminic acid	CUU
Salemvirus	Salem virus	“G” 620	LSGKCT	protein?	CUU + CCU(P-M) + CGU (F-G)

Metaparaymyxovirinae

Genus	**Virus**	**RBP name / amino acid residues#**	**Sequence at start of RBP propeller blade 2***	**Cell receptor**	**Intergenic trinucleotides**
Synodonvirus	Wēnlǐng triplecross lizardfish paramyxovirus	“HN”621	PAPSCP	protein?	CUU + CAUCUU (F-HN)

Rubulavirinae

Genus	**Virus**	**RBP name/ amino acid residues#**	**Sequence at start of RBP propeller blade 2***	**Cell receptor**
Orthorubulavirus	mumps virus	HN 582	**NRKSCS**	Neuraminic acid
Orthorubulavirus	La Piedad Michoacán Mexico virus	HN 576	**NRKSCS**	? probably neuraminic.acid
Orthorubulavirus	Mapuera virus	HN 582	**NRKSCS**	? probably neuraminic acid
Orthorubulavirus	simian virus 41	HN 568	**NRKSCS**	? probably neuraminic acid
Orthorubulavirus	human parainfluenza virus 2	HN 571	**NRKSCS**	? probably neuraminic acid

Orthorubulavirus	human parainfluenza virus 4	HN 579	**NRKSCS**	? probably neuraminic acid
Orthorubulavirus	parainfluenza virus 5	HN 532	**NRKSCS**	Neuraminic acid
Orthorubulavirus	Alston virus	HN 565	**NRKSCS**	? probably neuraminic acid
Pararubulavirus	Menangle virus	“HN” 595	PVRTCS	protein?
Pararubulavirus	Tioman virus	“HN” 593	QARGCS	protein?
Pararubulavirus	Teviot virus	“HN” 595	QTRGCS	protein?
Pararubulavirus	Achimota virus 1	“HN” 595	VTYQCS	protein?
Pararubulavirus	Achimota virus 2	“HN” 583	FRRGCS	protein?
Pararubulavirus	Hervey virus	“HN” 543	PKRSCS	protein?
Pararubulavirus	Tuhoko virus 1	“HN” 580	WLRSCS	protein?
Pararubulavirus	Tuhoko virus 2	“HN” 588	VSRQCS	protein?
Pararubulavirus	Tuhoko virus 3	“HN” 582	RLYHCS	protein?
Pararubulavirus	Sosuga virus	“HN” 582	RLYHCS	protein?

Avulavirinae

Genus	**Virus**	**RBP name/ amino acid residues**	**Sequence at start of RBP propeller blade 2***	**Cell receptor**
Metaavulavirus	avian paramyxovirus 2	HN 580	**NRKSCS**	? probably neuraminic acid
Metaavulavirus	avian paramyxovirus 5	HN 574	**NRKSCS**	? probably neuraminic acid
Metaavulavirus	avian paramyxovirus 6	HN 613	**NRKSCS**	? probably neuraminic acid
Metaavulavirus	avian paramyxovirus 7	HN 569	**NRKSCS**	? probably neuraminic acid
Metaavulavirus	avian paramyxovirus 8	HN 577	**NRKSCS**	? probably neuraminic acid

Metaavulavirus	avian paramyxovirus 10	HN 575	**NRKSCS**	? probably neuraminic acid
Metaavulavirus	avian paramyxovirus 11	HN 583	**NRKSCS**	? probably neuraminic acid
Metaavulavirus	avian paramyxovirus 14	HN 580	**NRKSCS**	? probably neuraminic acid
Metaavulavirus	avian paramyxovirus 15	HN 579	**NRKSCS**	? probably neuraminic acid
Metaavulavirus	avian paramyxovirus 20	HN 574	**NRKSCS**	? probably neuraminic acid
Orthoavulavirus	avian paramyxovirus 1 (NDV)	HN 571	**NRKSCS**	Neuraminic acid
Orthoavulavirus	avian paramyxovirus 9	HN 579	**NRKSCS**	? probably neuraminic acid
Orthoavulavirus	avian paramyxovirus 12	HN 614	**NRKSCS**	? probably neuraminic acid
Orthoavulavirus	avian paramyxovirus 13	HN 579	**NRKSCS**	? probably neuraminic acid
Orthoavulavirus	avian paramyxovirus 16	HN 618	**NRKSCS**	? probably neuraminic acid
Orthoavulavirus	Antarctic penguin virus A	HN 599	**NRKSCS**	? probably neuraminic acid
Orthoavulavirus	Antarctic penguin virus B	HN 591	**NRKSCS**	? probably neuraminic acid
Orthoavulavirus	Antarctic penguin virus C	HN 587	**NRKSCS**	? probably neuraminic acid
Orthoavulavirus	avian paramyxovirus 21	HN 567	**NRKSCS**	? probably neuraminic acid
Paraavulavirus	avian paramyxovirus 3	HN 577	**NRKSCS**	? probably neuraminic acid
Paraavulavirus	avian paramyxovirus 4	HN 569	**NRKSCS**	? probably neuraminic acid

[#] The nomenclature for RBP (G, H or HN) used in the accessions in the data bank submissions is shown in quotation marks.

[*] The canonical NRKSCS sequence at the start of propeller blade 2 (Langedijk et al., 1997) is shown in bold lettering; in the *Avulavirinae* and *Rubulavirinae* intergenic sequences vary widely in length and sequence and hence are not recorded in the Table.

Nucleocapsids associate with virus membrane proteins at the plasma membrane and are enveloped by budding out at the membrane.

Transmission of paramyxoviruses is horizontal, mainly through airborne and direct contact routes; no vectors are known. Paramyxovirus infection typically begins in the respiratory tract and may remain at that site (e.g., human parainfluenza virus 1 [HPIV-1]) or spread to secondary sites (e.g., lymphoid and endothelial tissues for measles virus (MV) (Griffin 2007), the parotid gland, CNS and endothelial tissues for mumps virus (MuV) (Carbone and Rubin 2007) or lung and CNS for Hendra virus (HeV) and Nipah virus (NiV) (Eaton et al., 2007). In general, paramyxovirus infections are limited, and eliminated, by host immunity. However, virus can sometimes be shed for periods of weeks or months in healthy and, especially, in immunocompromised individuals. Latent infection is unknown. However, long-term persistent infection is known for several morbilliviruses such as MV in subacute sclerosing panencephalitis, a rare complication that involves persistence of a defective measles virus in the CNS for periods of, on average, 8 years. Old dog distemper can involve persistence of defective or fully infectious canine distemper virus for weeks or months in healthy and, especially, immunocompromised animals. Feline morbillivirus has been shown to be shed for long periods from the kidneys of cats. The recurrence of neurological manifestations has also been noted in NiV patients more than 4 years after recovery from acute encephalitis (Eaton et al., 2007).

Antigenicity

The RBP and F proteins are of primary importance in inducing virus-neutralizing antibodies and immunity against reinfection. Antibodies to N and, variably, to other virus proteins also are induced by infection. Following processing into small peptides the virus proteins also stimulate cell-mediated immune responses.

Derivation of names

Avula: from avian and rubula

Cynoglossusvirus: from the genus *Cynoglossus* of the fish from which the virus sequence was obtained

Henipa: from Hendra and Nipah viruses, the first isolates assigned to this genus

Hoplichthysvirus: from the genus *Hoplichthys* of the fish from which the virus sequence was obtained

Meta: from Greek *meta*, meaning “after, beyond”.

Morbilli: from Latin *morbillus*, diminutive of *morbus*, “disease”.

Ortho: from Greek *orthos*, “straight”.

Paramyxo: from Greek *para*, “by the side of”, and *myxa*, “mucus”.

Pneumo: from Greek *pneuma*, “breath”.

Respiro: from Latin *respirare*, “respire, breathe”.

Rubula: from *Rubula inflans* – old name for the disease mumps from Latin *Rubula,* red; *inflans,* swelling or puffing up.

Scoliodonvirus: from binomial name *Scoliodon macrorhynchos* (Bleeker 1852) of Pacific spadenose shark from which the virus sequence was obtained

Subfamily, genus and species demarcation criteria

The current paramyxovirus taxonomic structure is based on a comparison of complete L protein amino acid sequences. The *Paramyxoviridae* Study Group decided to use this as a sole criterion on the basis of the likely monophyly of this large and complex virus protein (Wolf et al., 2018, Dolja and Koonin 2018)consequential upon the ICTV decision to classify viruses even if only known from their genome sequences (Simmonds et al., 2017) . The genetic-based classification reflected previous classifications based on biological characteristics, which are unlikely to be known for all the new paramyxovirus sequences that have become available (Rima et al., 2018). Four subfamilies have been established on the basis of their genetic distance from the node distinguishing the family *Paramyxoviridae* from the *Sunviridae*, which is the closest related outlier family. These distances (substitutions per site) are respectively 0.64 for the *Metaparamyxovirinae*, 0.80 for the *Avulavirinae*, 0.82 for the *Orthoparamyxovirinae* and 0.90 for the *Rubulavirinae*.

Relationships within the family

Phylogenetic analysis of complete L protein amino acid sequences (Figure 3.*Paramyxoviridae*) supports the classification of paramyxoviruses into four subfamilies and fourteen genera based on genetic distances; in addition, three viruses are members of species that are not assigned to a genus or subfamily

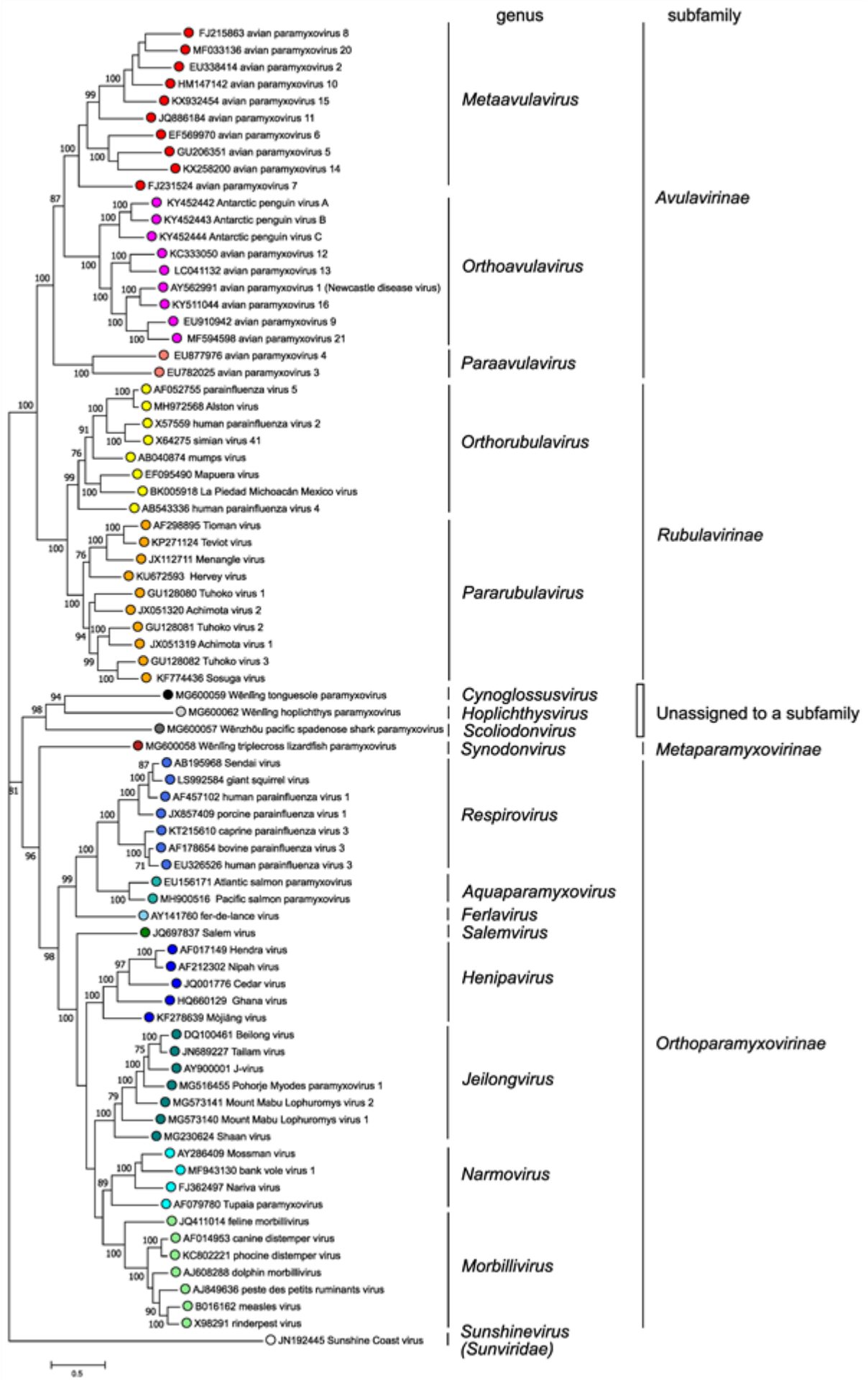

genus
subfamily
FJ215863 avian paramyxovirus 8
MF033136 avian paramyxovirus 20
EU338414 avian paramyxovirus 2
HM147142 avian paramyxovirus 10
KX932454 avian paramyxovirus 15
JQ886184 avian paramyxovirus 11
EF569970 avian paramyxovirus 6
GU206351 avian paramyxovirus 5
KX258200 avian paramyxovirus 14
Metaavulavirus
FJ231524 avian paramyxovirus 7
Avulavirinae
KY452442 Antarctic penguin virus A
KY452443 Antarctic penguin virus B
KY452444 Antarctic penguin virus C
KC333050 avian paramyxovirus 12
LC041132 avian paramyxovirus 13
AY562991 avian paramyxovirus 1 (Newcastle disease virus)
KY511044 avian paramyxovirus 16
EU910942 avian paramyxovirus 9
MF594598 avian paramyxovirus 21
Orthoavulavirus
EU877976 avian paramyxovirus 4
EU782025 avian paramyxovirus 3
Paraavulavirus
AF052755 parainfluenza virus 5
MH972568 Alston virus
X57559 human parainfluenza virus 2
X64275 simian virus 41
AB040874 mumps virus
EF095490 Mapuera virus
BK005918 La Piedad Michoacán Mexico virus
AB543336 human parainfluenza virus 4
Orthorubulavirus
AF298895 Tioman virus
KP271124 Teviot virus
JX112711 Menangle virus
KU672593 Hervey virus
GU128080 Tuhoko virus 1
JX051320 Achimota virus 2
GU128081 Tuhoko virus 2
JX051319 Achimota virus 1
GU128082 Tuhoko virus 3
KF774436 Sosuga virus
Pararubulavirus
Rubulavirinae
MG600059 Wēnlíng tonguesole paramyxovirus
MG600062 Wēnlíng hoplichthys paramyxovirus
MG600057 Wēnzhōu pacific spadenose shark paramyxovirus
MG600058 Wēnlíng triplecross lizardfish paramyxovirus
Cynoglossusvirus
Hoplichthysvirus
Scoliodonvirus
Synodonvirus
Unassigned to a subfamily
Metaparamyxovirinae
AB195968 Sendai virus
LS992584 giant squirrel virus
AF457102 human parainfluenza virus 1
JX857409 porcine parainfluenza virus 1
KT215610 caprine parainfluenza virus 3
AF178654 bovine parainfluenza virus 3
EU326526 human parainfluenza virus 3
Respirovirus
EU156171 Atlantic salmon paramyxovirus
MH900516 Pacific salmon paramyxovirus
Aquaparamyxovirus
AY141760 fer-de-lance virus
Ferlavirus
JQ697837 Salem virus
Salemvirus
AF017149 Hendra virus
AF212302 Nipah virus
JQ001776 Cedar virus
HQ660129 Ghana virus
KF278639 Mòjiāng virus
Henipavirus
DQ100461 Beilong virus
JN689227 Tailam virus
AY900001 J-virus
MG516455 Pohorje Myodes paramyxovirus 1
MG573141 Mount Mabu Lophuromys virus 2
MG573140 Mount Mabu Lophuromys virus 1
MG230624 Shaan virus
Jeilongvirus
Orthoparamyxovirinae
AY286409 Mossman virus
MF943130 bank vole virus 1
FJ362497 Nariva virus
AF079780 Tupaia paramyxovirus
Narmovirus
JQ411014 feline morbillivirus
AF014953 canine distemper virus
KC802221 phocine distemper virus
AJ608288 dolphin morbillivirus
AJ849636 peste des petits ruminants virus
B016162 measles virus
X98291 rinderpest virus
Morbillivirus
JN192445 Sunshine Coast virus
Sunshinevirus
(Sunviridae)
0.5

Figure 3.*Paramyxoviridae.* Phylogenetic analysis of complete L protein amino acid sequences of members of the family *Paramyxoviridae*. Complete L protein amino acid sequences were aligned by Clustal W with gap generation penalties of 5 and extension penalties of 1 in both multi and pairwise alignments. The evolutionary history was inferred by using the Maximum Likelihood method and JTT matrix-based model. The tree with the highest log likelihood (-258124.74) is shown. The percentage of 500 trees in which the associated taxa clustered together is shown next to the branches where this was > 70%. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 78 amino acid sequences. There were a total of 2745 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018). This phylogenetic tree and corresponding sequence alignment are available to download from the Resources page.

Relationships with other taxa

The member viruses of the family *Paramyxoviridae* have a similar strategy of gene expression and replication and gene order to those of other mononegaviruses, specifically filoviruses and rhabdoviruses.

Member taxa

- *Avulavirinae*
  - *Metaavulavirus*
  - *Orthoavulavirus*
  - *Paraavulavirus*
- *Metaparamyxovirinae*
  - *Synodonvirus*
- *Orthoparamyxovirinae*
  - *Aquaparamyxovirus*
  - *Ferlavirus*
  - *Henipavirus*
  - *Jeilongvirus*
  - *Morbillivirus*
  - *Narmovirus*
  - *Respirovirus*
  - *Salemvirus*
- *Rubulavirinae*
  - *Orthorubulavirus*

    - *Pararubulavirus*
- Genera unassigned to a subfamily
    - *Cynoglossusvirus*
    - *Hoplichthysvirus*
    - *Scoliodonvirus*

(b) (5)

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(b) (5)

Non-Disclosure Agreement

Agreement Summary

[COUNTERPARTY] INFORMATION	
Name:	[●]
Mailing Address:	[●]
Project Lead:	[●]
Management Contact:	[●]

CEPI INFORMATION	
Name:	Coalition for Epidemic Preparedness Innovations ("**CEPI**")
Mailing Address:	(b) (6)
Project Lead:	[●] (b) (6)
Management Contact:	[●] (b) (6)

AGREEMENT INFORMATION	
Project Name:	[●] Virus Expert Panel for Disease X Vaccine Libraries
Effective Date:	January 17, 2022
Expiration Date:	[●] January 17, 2023
This Agreement includes and incorporates by reference:	The agreement (referred to as the "**Agreement**") means this Agreement Summary together with the following: - Terms and Conditions ("T&Cs") (Annex A)

THIS AGREEMENT is between the Counterparty and CEPI (each as defined above) and comes into force as of the Effective Date. Each party to this Agreement may be referred to individually as a "**Party**" and together as the "**Parties**".

This Agreement sets out the terms and conditions governing the exchange of information between the Parties and their representatives in the context of the selection of viruses for the Disease X vaccine l brary (the "**Purpose**"). As a pre-condition of this information exchange, the Parties enter into this Agreement by having their authorized representatives sign below.

Signed for and on behalf of [**COUNTERPARTY**] by:

Signature:……

Name:……..

Title:……

Date:……

Signed for and on behalf of **COALITION FOR EPIDEMIC PREPAREDNESS INNOVATIONS** by:

Signature:……

Name:……..

Title:…….

Date:…….

Non-Disclosure Agreement - Terms and Conditions

1. Definitions

1.1 In this Agreement, terms shall have the following meaning:

1.2 "**Applicable Laws**" means all national and supranational laws and regulations and other mandatory professional regulations applicable to a Party or any activities carried out or to be carried in order to achieve the Purpose or a Party's activities or obligations described under or pursuant to this Agreement;

1.3 "**Business Day**" means a day which is not a Saturday or Sunday, or a bank or public holiday in England, Norway, and/or the country in which a Party's mailing address (as set out in the Agreement Summary) is located;

1.4 "**Confidential Information**" means any and all non-public information which by its nature or the manner of its disclosure is reasonably identifiable as Confidential Information, including but not limited to data, results, know-how, software (further including non-open source code), plans, details of research work, discoveries, inventions, intended publications, intended or pending patent applications, designs, technical information, business plans, budgets and strategies, business or financial information or other information in any medium, and any physical items, prototypes, compounds, samples, components, non-public regulatory filings, non-public submissions to regulatory authorities or other articles or materials disclosed on or after the Effective Date of this Agreement by one Party to the other Party whether orally or in writing or in any other form. Confidential information will not include:

a. information that is or was already known to the receiving Party at the time of disclosure, as shown by written records, without any obligation to keep it confidential;

b. information that is independently developed by employees of the receiving Party who have not had access to the Confidential Information of the disclosing Party as evidenced by contemporaneous written records;

c. information that at the time of being disclosed or obtained by the receiving Party or at any time thereafter, is published or otherwise generally available to the public other than due to default by the receiving Party of its obligations hereunder;

d. information properly obtained by the receiving Party from a source which, to the best knowledge of the receiving Party, is not known to be bound by a confidentiality agreement, fiduciary obligation or other legal or contractual restriction that may prohibit the disclosure of such Confidential Information;

e. information necessarily disclosed by the receiving Party pursuant to a statutory obligation and the Party required to make that disclosure has informed the other, within a reasonable time after being required to make such disclosure, of the requirement to disclose and the information required to be disclosed; and

f. information approved for release in writing by an authorised representative of the disclosing Party.

1.5 "**CEPI Group"** means the nodes of CEPI established in Norway, England, India, the United States of America and any other node of CEPI which may be established from time to time.

1.6 "**Group**" means in relation to a company (other than CEPI), that company, any subsidiary or any holding company from time to time of that company, and any subsidiary from time to time of a holding company of that company. Each company in a Group is a member of the Group;

1.7 all terms defined in the Agreement Summary shall have the same meaning throughout the Agreement.

2. Confidentiality; Restricted Use

2.1 All Confidential Information shall be used by the receiving Party ("**Receiving Party"**) exclusively for the Purpose, unless otherwise expressly agreed to in writing by the disclosing Party ("**Disclosing Party"**).

2.2 Each Party undertakes that during the term of this Agreement and for a period of five (5) years after its termination or expiry, it shall keep confidential and not disclose any Confidential Information of the other Party disclosed to or obtained by it in connection with this Agreement or the Purpose other than representatives, employees, agents, consultants, professional advisers, sub-contractors, regulatory

authorities of itself or members of its Group and, in the case of CEPI, its funders or members of the CEPI Group, in each case who have a need to know Confidential Information of the other Party disclosed to or obtained by it in connection with the Purpose.

2.3 Each Party shall ensure that all representatives, employees, agents, consultants, professional advisers, sub-contractors (to the extent it can legally do so) and third parties to which Confidential Information of the other Party is disclosed are:

a. informed of the confidentiality provisions of this Agreement and

b. bound by obligations of confidentiality and non-use at least as protective as the provisions contained herein, it being understood that where such person is a natural person (i.e. individual), such person shall not be allowed to disclose the Confidential Information to any other person.

2.4 Each Party shall take reasonable security precautions to protect against unauthorized disclosure of Confidential Information.

2.5 **Required Disclosure.** The disclosure of information that is required to be disclosed by a competent Court or regulatory authority or otherwise by Applicable Law may be disclosed as required, provided that where it is free to do so, the Receiving Party shall give notice of such disclosure to the Disclosing Party as soon as reasonably practicable and all such information shall be marked as “Confidential”.

2.6 **Permitted Disclosures.** Notwithstanding the above, nothing in this Agreement shall restrict a Party’s (or any of its representatives, employees, agents, consultants, professional advisers, sub-contractors) right to

a. disclose the existence of a relationship between the Parties for the purpose of declaring a potential conflict of interest;

b. disclose Confidential Information to any committee or regulatory body in furtherance of Party’s statutory or regulatory duties.

3. Refusal

3.1 Each Party shall have the right to refuse to accept any information under this Agreement prior to any disclosure; information disclosed despite such a refusal is not covered by the confidentiality obligation under this Agreement. Nothing herein shall require either Party to disclose any particular information.

4. No Licence

4.1 Each Party reserves all rights in its Confidential Information. The disclosure of Confidential Information by one Party does not give the other Party or any other person any licence or other right in respect of any Confidential Information beyond the rights expressly set out in this Agreement.

5. No Warranty

5.1 Except as expressly stated in this Agreement, neither Party makes any express or implied warranty or representation concerning its Confidential Information, including but not limited to the accuracy or completeness of the Confidential Information.

5.2 The disclosure of Confidential Information by the parties shall not form any offer by, or representation or warranty on the part of, that party to enter into any further agreement with the other party in relation to the Purpose.

6. Termination

6.1 This Agreement shall come into force on the Effective Date and shall automatically expire five (5) years later. It may be prematurely terminated by mutual agreement of the Parties or by one Party upon thirty (30) days' prior written notice to the other Party of its intention to terminate. The rights and obligations of the Parties which have accrued prior to termination shall, however, survive the termination of this Agreement for a period of five (5) years.

7. Return

7.1 Within sixty (60) days after termination or expiry of this Agreement the disclosing Party may request in writing from the Receiving Party that the Receiving Party at the Disclosing Party's discretion either return or destroy all Confidential Information received from the Disclosing Party and stored electronically and/or on record-bearing media as well as any copies thereof. The Receiving Party shall confirm in writing such destruction or return the Confidential Information as well as any copies thereof to the Disclosing Party within fourteen (14) days after receipt of the Disclosing Party's request.

7.2 The Parties acknowledge that return and/or destruction of Confidential Information constitutes an administrative burden, and agree to keep requests for return and/or destruction of Confidential Information to the minimum required.

7.3 The provisions of Clause **Error! Reference source not found.** above shall not apply to copies of electronically exchanged Confidential Information made as a matter of routine information technology backup and to Confidential Information or copies thereof which must be stored by the receiving Party according to provisions of mandatory law or to the receiving Party's internal compliance guidelines, provided that such Confidential Information or copies thereof shall be subject to an indefinite confidentiality obligation according to the terms and conditions set forth herein until returned and/or destroyed, as the case may be.

8. Resolving Differences

8.1 **Escalation process**. Any question, difference or dispute which may arise concerning the construction, meaning or effect of this Agreement, or concerning the rights or liabilities of the Parties hereunder, or any other matter arising out of or in connection with this Agreement shall first be submitted to the Chief Executive Officer of CEPI and to the Chief Executive Officer of the other Party (the "**Senior Officers**") for resolution (each of whom may call on others to advise them as they see fit). The Senior Officers shall discuss the matter arising in good faith and in a timely manner and endeavour to reach a mutually agreeable solution. If the Parties are unable to resolve such dispute through such negotiations within sixty (60) days of such dispute being escalated to the Senior Officers, then in respect of any dispute, controversy or claim the Parties irrevocably submit to arbitration in accordance with Clause **Error! Reference source not found.**.

8.2 **Arbitration**. All disputes arising out of or in connection with this Agreement, including disputes on its conclusion, binding effect, amendment and termination, shall be resolved by arbitration, in accordance with the arbitration rules of the United Nations Commission of International Trade Law (UNCITRAL Arbitration rules) then-current in force. The appointing authority shall be the President of the Swiss Arbitration Association. The number of arbitrators shall be one, unless the parties agree otherwise. The arbitration proceedings shall take place in Geneva. The language of the arbitration shall be in English. The Parties agree to be bound by any award made by the arbitrator(s). An award shall be final and not be subject to any setting aside proceedings before any court absent fraud or misconduct. This clause shall be governed by and construed in accordance with the law of England and Wales without giving effect to any choice of law or conflict of law provisions or rules that would cause the application of the laws of any other jurisdiction.

8.3 **Good faith cooperation in resolving differences**: The Parties will cooperate in good faith to resolve differences and disputes pursuant to this Clause **Error! Reference source not found.**.

9. Limitation and exclusion of liability

9.1 **Liability cap**. Unless otherwise agreed by the Parties in writing, each Party's maximum liability in aggregate to the other Party arising out of this Agreement shall not exceed the higher of:

(a) US Dollars $10,000; and

(b) the amount (if higher) that such Party can recover from its insurer for the liability in question.

9.2 **Exclusions**. Neither Party shall be liable to the other Party for any loss of an indirect or consequential nature, nor for any loss of turnover, profits, business or goodwill, whether in contract, warranty, negligence, tort, strict liability or otherwise, arising out of any breach of or failure to perform any of the provisions of this Agreement.

9.3 **Exclusions from Liability Cap**. Notwithstanding the foregoing, nothing in this Agreement shall limit the liability of either Party in respect of: personal injury or death arising out of that Party's negligence or wilful

misconduct; or fraud or fraudulent misrepresentation or wilful misconduct; or for any other liability which cannot be limited or excluded as a matter of Applicable Laws.

10. General Provisions

10.1 **Choice of law**. This Agreement and any dispute or claim (including non-contractual disputes or claims) arising out of or in connection with it or its subject matter or formation shall be governed by and construed in accordance with the law of England and Wales without giving effect to any choice of law or conflict of law provisions or rules that would cause the application of the laws of any other jurisdiction.

10.2 **No Rights for Third Parties**. A person who is not a Party to this Agreement has no right under the Contracts (Rights of Third Parties) Act 1999 or otherwise to enforce or to enjoy the benefit of any term of this Agreement.

10.3 **Notices**. Any notice to be given pursuant to this Agreement shall be in writing in the English language and shall be delivered by overnight courier, by registered, recorded delivery or certified mail (postage prepaid) to the address of the recipient Party provided in the Agreement Summary or such other address as a Party may from time to time designate by written notice. Any notice given pursuant to this clause shall be deemed to have been received on the day of receipt, provided receipt occurs on a Business Day of the recipient Party or otherwise on the next following Business Day of the recipient. The Parties agree that email and fax are not valid methods of giving notice under this Agreement.

10.4 **No Waiver**. Neither Party shall be deemed to have waived any of its rights or remedies under this Agreement unless the waiver is expressly made in writing and signed by a duly authorized representative of that Party.

10.5 **Assignment.** Neither this Agreement nor any rights and obligations under this Agreement may be assigned or delegated by either Party without the prior written consent of the other Party, such consent not to be unreasonably withheld.

10.6 **Entire Agreement**. This Agreement, including its Agreement Summary and Annex(es), constitutes the entire agreement and understanding between the Parties relating to its subject matter and together they supersede and replace all prior arrangements, whether written or oral, between the Parties relating to the subject matter of this Agreement.

10.7 **Export regulations.** The Parties shall abide by the applicable export license regulations of the respective country(ies) and, if required, the disclosing Party shall apply for an export license grant prior to any transmission of Confidential Information and to inform the receiving Party sufficiently of any existing limitation.

10.8 **Variation**. No variation, amendment, modification or supplement to this Agreement will be valid unless and until it is made in writing and signed by a duly authorised representative of each Party.

Expert Panel

*committed

Paramyxoviruses

PL (b) (6)
PM (b) (6)

CEPI:
(b) (6)

CEPI SAC
(b) (6)

External Experts
(b) (6)
Chris Broder PhD * (USUHS) (b) (6)

Arenaviruses

PL (b) (6)
PM (b) (6)

CEPI
(b) (6)

CEPI SAC

(b) (6) [redacted]

External Experts

(b) (6) [redacted]

From: (b) (6)
To: (b) (6) ; Broder, Christopher; (b) (6)
Cc: (b) (6)
Subject: FW: Vaccine library virus selection
Date: Sunday, January 16, 2022 4:39:25 PM
Attachments: Arenaviridae - Arenaviridae - Negative-sense RNA Viruses - ICTV.pdf
Paramyxoviridae - Paramyxoviridae - Negative-sense RNA Viruses - ICTV.pdf
Viruses.xlsx
Non-disclosure-Agreement-CEPI-01172022.docx
Expert Panel 12062021.docx
image001.png
image002.png
image003.png
image004.png
image005.png
image006.png

Dear Expert Panel, from all of us at CEPI we are grateful for your taking the time and agreeing to serve on our expert panel to select viruses for our vaccine library efforts to meet the 100-day mission of having a vaccine available for the next Disease X outbreak. The task at hand in which we need your expertise is to help us in selecting 10-15 viruses from each of our two pilot vaccine projects to develop vaccine libraries for the paramyxoviruses and the arenaviruses.

Organization of the Expert Panel: Attached is a listing of the participants in the expert panels for the paramyxoviruses and the arenaviruses. Some of the experts will be serving on both panels because of your broad expertise. Others will be serving on one due to time constraints or a particular interest in one family. However, if those serving on one panel would like to be involved in both please let me know as you will be welcome to participate. If you see an essential expert I should have invited but failed to please let me know.

Housekeeping: Per CEPI procedure we request that you complete with your information and sign the attached NDA. For members of our Scientific Advisory Committee your current NDA with us will suffice. For those of you who can accept an honorarium we would like to provide a $1,000 honorarium for your participation on each panel. This of course doesn't cover your time commitment to this project but hope you will accept this as our appreciation for your efforts.

Background Information: Attached you will find some background information on the viruses from each family as listed in the ICTV by subfamilies and genera. I also highlighted those that are human pathogens and those that are zoonotic. In the excel file I merged in information from the UC Davis Spillover program on the spillover likelihood of some specific viruses. These are combined and also in a separate file. The spillover program lists viruses recently identified that are not yet listed in the ICTV or assigned a genera. Dr (b) (6) and Dr (b) (6) who are on the expert panel are part of the DARPA sponsored Spillover program and will provide more information on this dataset in our general discussion meeting. Also attached are some general information and the latest phylogenetic trees from ICTV. As we progress we at CEPI are available to do the leg work on any additional information you will need ie research papers etc and please send me requests and I'll make sure they are available prior to the meetings.

Organization of Meetings: Due to travel constraints all meetings will be held by zoom meetings. I will

be sending out a doodle poll for availability for our first Zoom meeting. Our participants cross 5 different time zones so will try for early morning meetings US time which will be late afternoon for our UK members and early evening for those in SE Asia. For the first meeting I would like both expert panels for each virus family to participate as I'll give details in a presentation on our Disease X vaccine planning process as well as regulatory approach to meet the 100-day mission and the objectives for this panel to select 10-15 viruses with a likelihood for Disease X emergence for each virus family. I am planning a 1.5 hour first meeting leaving time for discussion and feedback. I envision that for each virus family we will need an additional two 1.5 hour meetings consisting of a general discussion on factors to be considered and a final meeting for virus selection. We can schedule additional meetings if needed. After the general discussion I'll send out a listing of the viruses and for each of you to select 10-15 viruses you think should go into the library. I'll then consolidate the information for our final virus selection meeting as I suspect we will have consensus on a portion of the viruses and can focus on the other viruses for discussion and a final consensus selection. First up will be the paramyxoviruses then the arenaviruses.

Please let me know if you would like additional information or recommendations on ways to improve this selection process.

Again thank you for your participation and very excited to have you involved in this.

Best,

(b) (6)

[redacted]

CEPI New vaccines for a safer world

[redacted]

[redacted]

[redacted]

www.cepi.net

This e-mail and any attachments may contain confidential and/or privileged information.

Sensitivity: CEPI Internal

The Research Discovery of a variant genotype of Hendra virus (HeVg2) affording prospective spillover detection and revision of geographical spillover risk.

(b) (6)

University of Sydney | Sydney School of Veterinary Science and Sydney Institute of Infectious Diseases, Sydney, NSW.
CSIRO | Health and Biosecurity, Black Mountain, ACT.
EquiEpiVet | Equine Veterinary and One Health Epidemiology, Aireys Inlet, Vic.

Horses as Sentinels for Emerging Infectious Disease

with BAT ONE HEALTH

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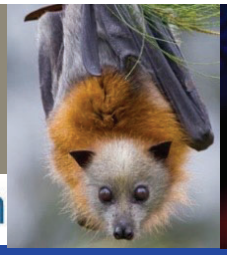

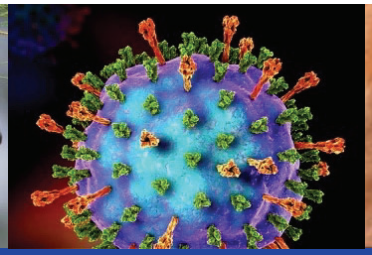

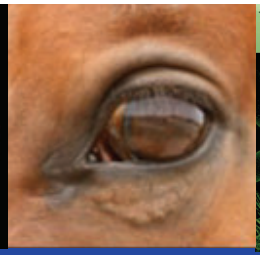

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HeV spillover to horses and humans as a low sporadic incidence but HIGH CONSEQUENCE disease

Any single missed detection of an infected case (false negatives) place humans and animals at risk of fatal illness

And yet **missed detections are inevitable** due to LOW and SPORADIC INCIDENCE of disease

YEAR/S	QLD	NSW	TOTAL	HORSE FATALITIES	Human	
					Exposures / illnesses requiring intensive treatment	Fatalities
1994-2010	13	1	14	48	10 (7+3)	4 (3+1)
2011	10	8	18	24	0	0
2012	8	0	8	10	1	0
2013	4	4	8	8	(1*)	0
2014	3	1	4	4	6	0
2015	2	2	4	4	(1*)	0
2016	0	1	1	1	0	0
2017	1	3	4	4	3	0
2018	0	1	1	1	0	0
2019	0	1	1	1	0	0
2020	0	1	1	1	0	0
2021	0	1	1	1	0	0
TOTALS	41	24	65	107	22 (13)+2*	4

Horse cases listed in blue include recently identified HeVg2 disease events. Human cases listed in blue indicate received monoclonal antibody m102.4
Cases marked with an * represent human laboratory exposures (2013 US NiV 2015 CSIRO HeV) and thus are not included in the spillover tally.

HeV Outbreaks in Horses 1994 - 2021

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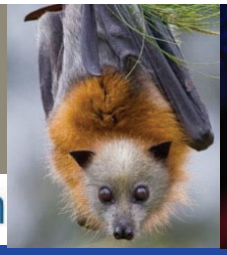
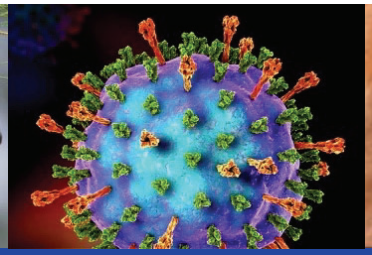
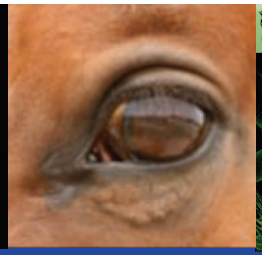

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Veterinary Testing for unvaccinated horses

- Samples tested for Hendra virus exclusion
- Negative samples are not thoroughly investigated for other pathogens
- Significance realised with detection of two fatal cases of ABLV in 2013

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Australian VETERINARY JOURNAL
THE JOURNAL OF THE AUSTRALIAN VETERINARY ASSOCIATION LTD
EQUINE

CASE SERIES AND CLINICAL REVIEW

Clinical review of two fatal equine cases of infection with the insectivorous bat strain of Australian bat lyssavirus

EJ Annand[a]* and PA Reid[b]

Veterinary Microbiology 173 (2014) 224–231

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

ELSEVIER

Australian bat lyssavirus infection in two horses

Mustaghfira Wafa Shinwari[a], Edward J. Annand[b], Luke Driver[a], David Warrilow[c], Bruce Harrower[c], Richard J.N. Allcock[d,e], Dennis Pukallus[c], Jennifer Harper[f], John Bingham[f], Nina Kung[g], Ibrahim S. Diallo[a,*]

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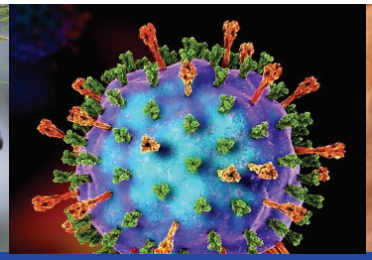

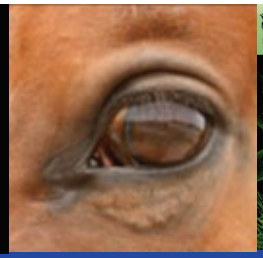

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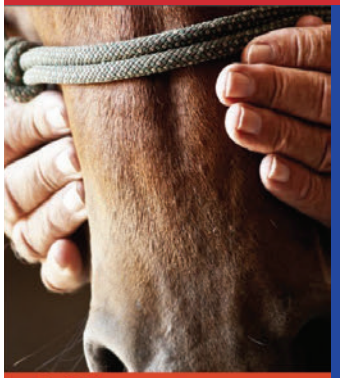


Horses as Sentinels of Emerging Infectious Disease

Which pathogens are causing neurological and respiratory symptoms in Hendra negative horses?

Testing Hendra negative horses to Discover Emerging Pathogens causing Hendra like illnesses.


THE LAND

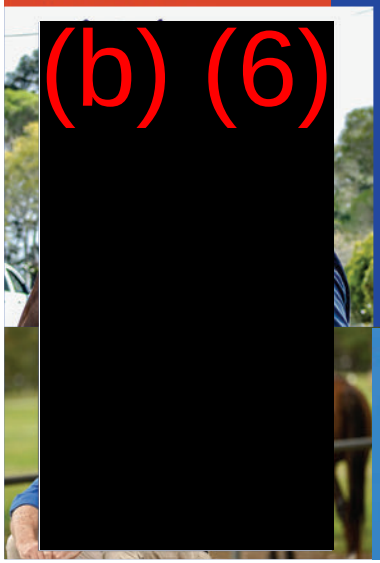
NEWS SPECIAL FEATURES CATTLE SHEEP CROPPING WEATHER
NEWS EVENTS PROPERTY PHOTOS LIFE & STYLE OPINION MORE

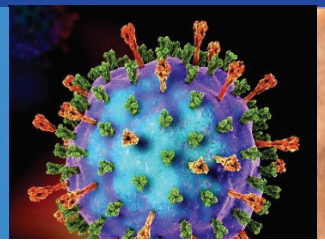
Researchers target new wave of emerging infectious diseases

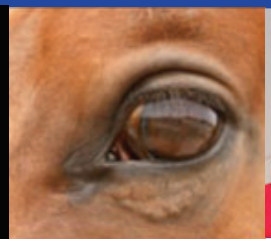
Penelope Arthur

Driven by the hypothesis that:

Some cases of severe Hendra virus-like disease in Australian horses is due to spillover infection by related but divergent bat borne paramyxoviruses such as those in the Rubulavirus and Henipavirus families of which our awareness is emerging.







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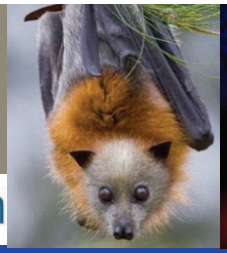

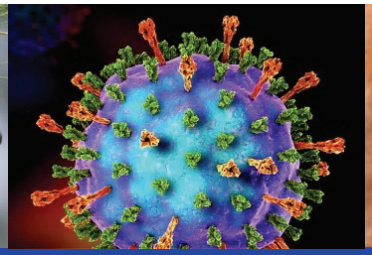

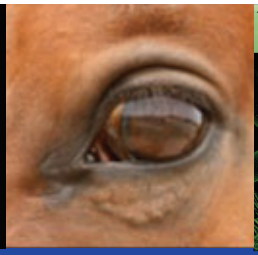

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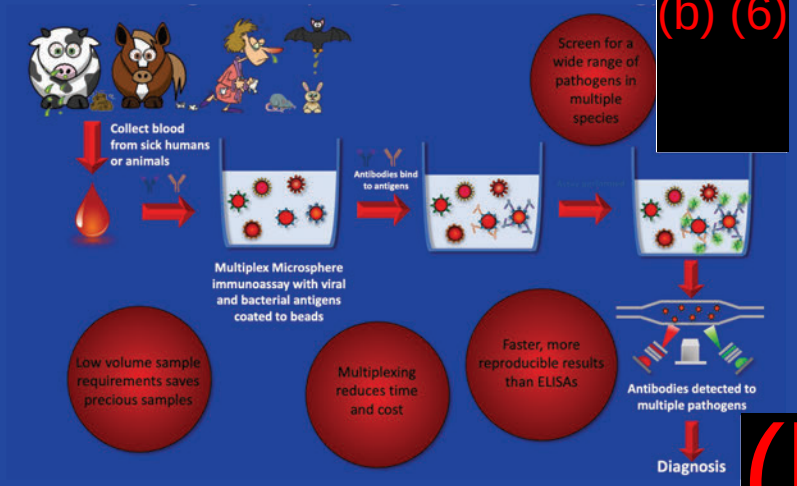
33 plex Microbead Fluorescent Immunoassay Targeting EIDs of Putative EID Relevance to Australian Geography and Horses



(b) (6)

Antigens

Brucella suis repA	Paramyxoviruses	Filovirus	Coronavirus
Brucella OMP	Hendra	EBOV (G)	SARS1
Leptospira	Nipah	Bundibugyo	SARS2 (S and N)
Chikungunya virus E1 protein	Cedar	Reston	MERS
Sindbis virus E1 protein	Mojang	Marburg	Rs4874
Hepatitis E virus Capsid protein	Ghana	Ravn	
Hervey pteropid gammaretrovirus	Menangle (N and HN)	Lloviu	
La Crosse virus N protein	Grove	Mengla	
West Nile virus E protein	Yeppoon	Zaire N	

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Sources: Commercial, collaborators-CSIRO, Burnett Institute, Vilnius University, Lithuania, Uniformed Services University

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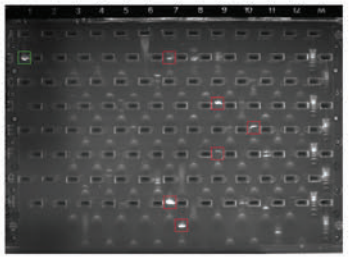
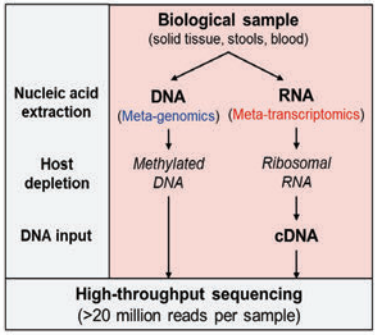
Nested Conventional RT-PCR and Total RNA NGS

Viral family pan-RT-PCR



Paramyxo, Corona, Filo, Alpha & Flaviviruses

Deep RNA sequencing



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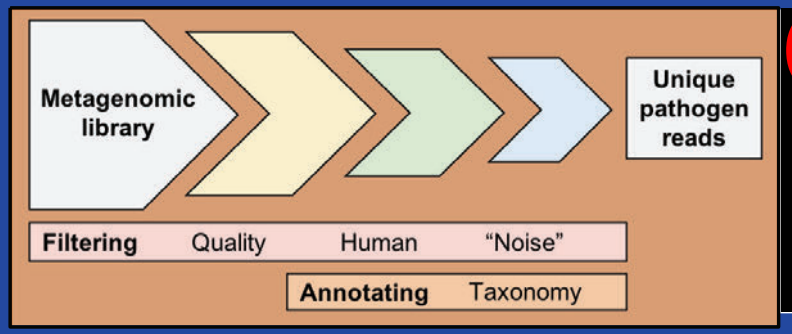
Sensitive Bioinformatical Pipeline



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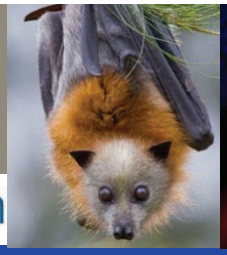

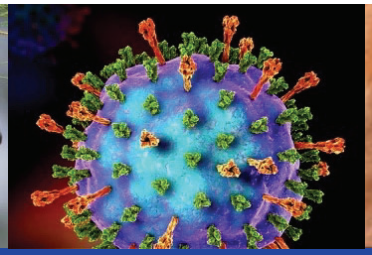

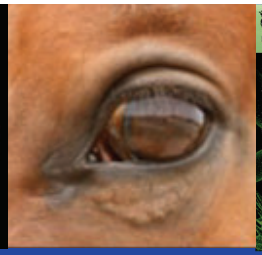

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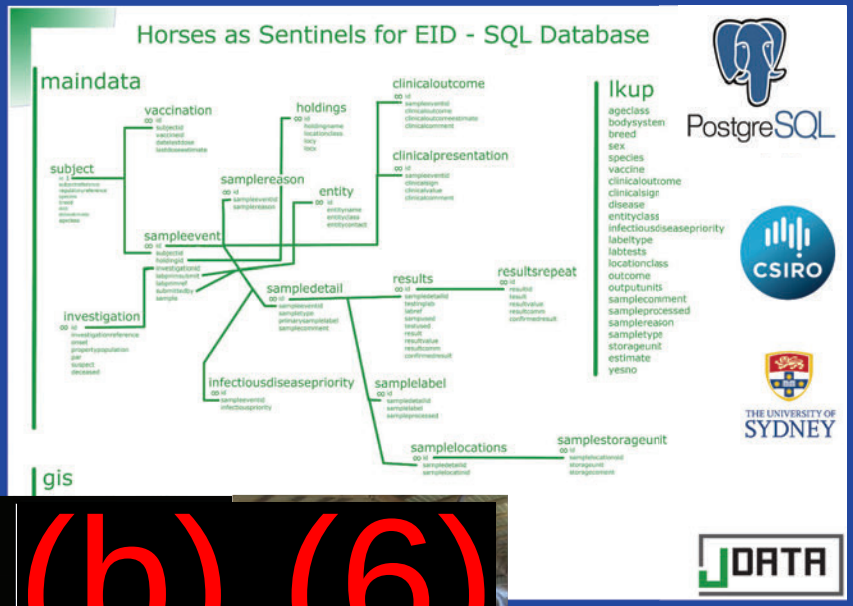
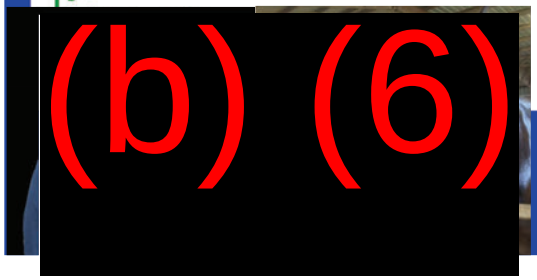
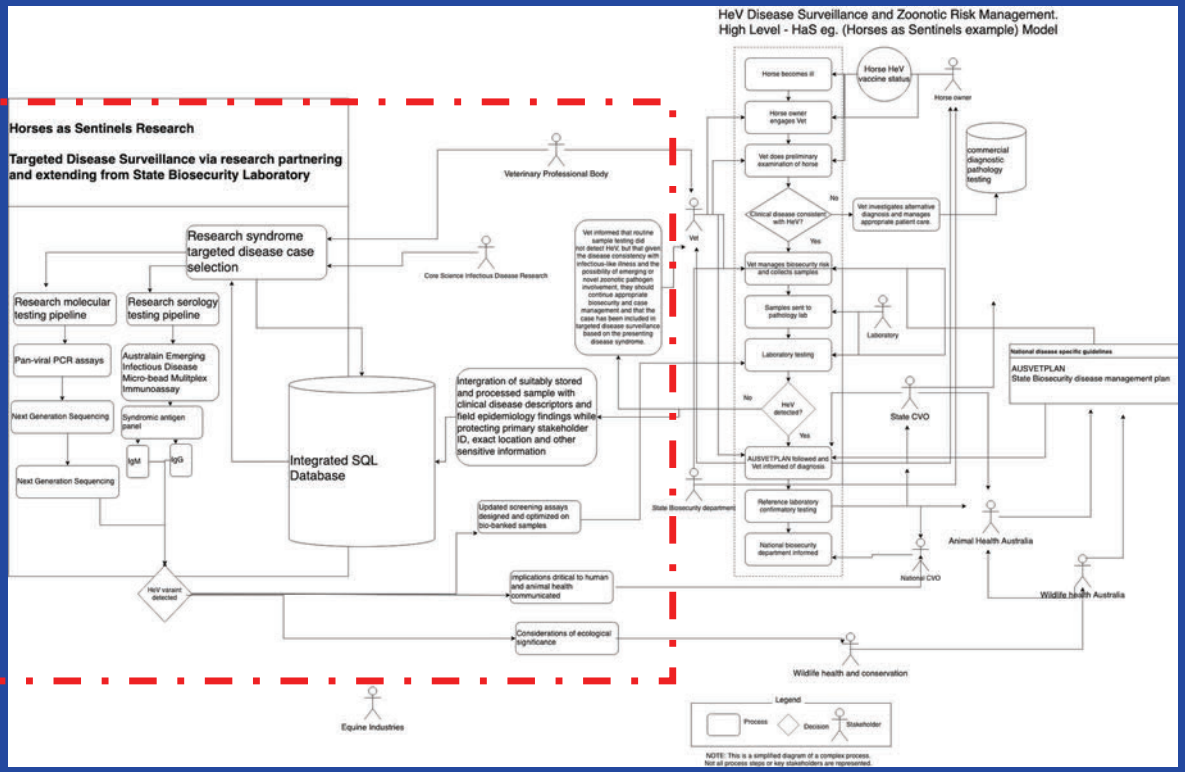
Horses as Sentinels – Targeted Disease Surveillance via research partnering with and extending routine Biosecurity

SQL database allowing syndromic classification, sample processing and multiple testing pipelines.







Additional pathway extended from and supporting routine surveillance

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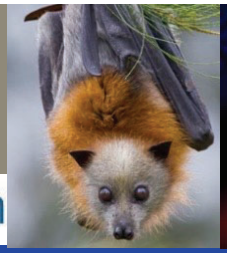

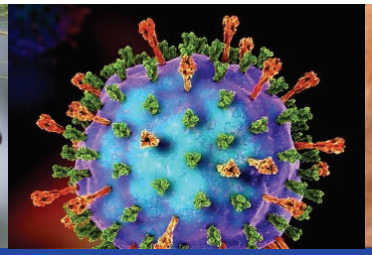

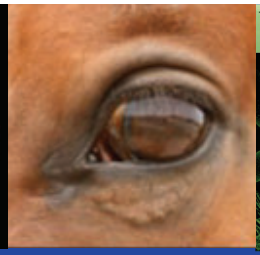

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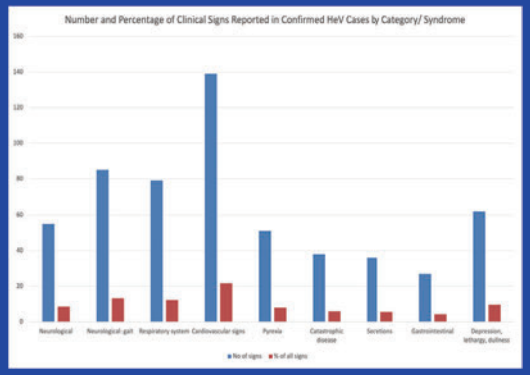
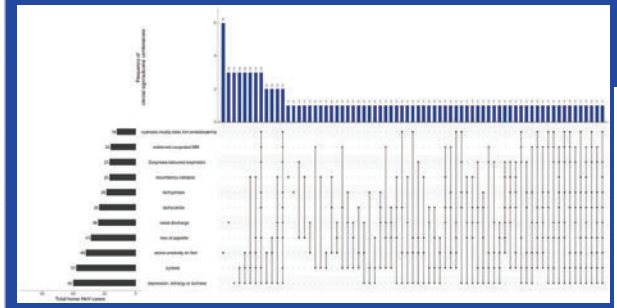
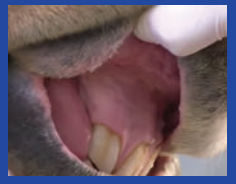
EquiEpiVet

Understanding HeV disease in horses in its pathogenic basis and the syndromic presentation





Upset plot reported HeV cases



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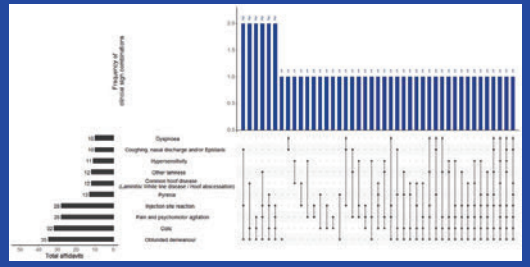

Upset plot AEFI

Table 1. Infectious disease prioritization categories (with examples) used in this study to identify Hendra-negative equine disease cases with highest likelihood of similar undiagnosed viral cause from larger cohort for further investigation

Infectious disease priority	Description	Example
Category 1 Highest infectious disease suspect	Case features 'pyrexia' or 'abnormal mucous membranes AND one or more other clinical signs related to infectious disease OR the presence of either change AND 'epidemiological observation indicative of infectious cause' based on temporal and/or spatial relationship to similar disease cases	Pyrexia with tachycardia and acute onset respiratory consolidation and/or secretions. Pyrexia and neurological symptoms. Pyrexia and 'injected/congested' mucous membranes. 'Congested/injected mucous membranes' with acute severe respiratory dysfunction. Clustering of similar cases on same or neighboring properties
Category 2 High infectious disease suspect	Pyrexia OR other clinical signs associated with infectious disease of interest	Acute onset abnormal respiratory secretions. Fever of unknown origin. Colic with the presence of neurological symptoms
Category 3 Moderate infectious disease suspect	Clinical signs may be associated equally with infectious and non-infectious causes	Colic with the presence of dehydration and mucous membrane changes. Ataxia with the absence of pyrexia or known trauma
Category 4 Low infectious disease suspect	Non-infectious etiologies more common or most likely on differential diagnosis list, but infectious cause still possible	Ataxia following known traumatic event. Traumatic wounds following unusual behavioral event. Acute lethargy following chronic non-infectious disease condition
Category 5 No infectious disease suspect	No clinical signs of illness or no infectious cause considered likely	Traumatic wounds in the absence of underlying disease. Screening in unvaccinated horses to manage biosecurity risk prior to invasive procedures addressing non-infectious disease such as is a common requirement for dentistry or admission to equine hospitals in Australia
Category 6 Confirmed infectious disease	Other infectious disease confirmed via diagnostic testing	A case submitted for HeV testing, found negative and then testing positive for alternative known infectious disease such as ABLV, WNV, EHV or RRV*

*ABLV, Australian bat lyssavirus; EHV, Equine herpes virus; HeV, Hendra virus; RRV, Ross River Virus; WNV, West Nile virus

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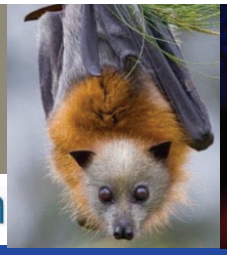

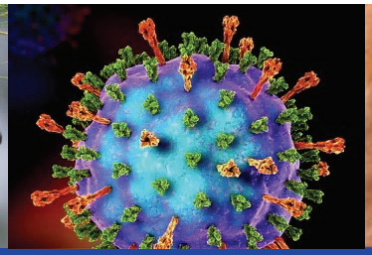

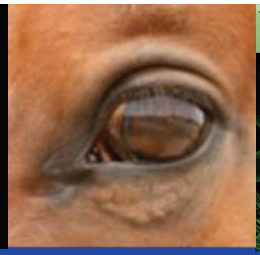

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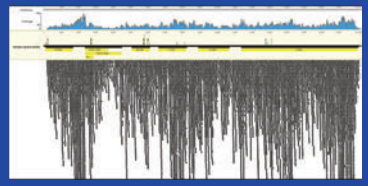
Important update for HeV surveillance in humans and animals.
Hendra-virus-variant - Horse South-East Qld 2015



(b) (6)

A Hendra virus variant (HeV-var) detected:

- 2015 South-east Queensland case of fatal equine disease;
- Previously found negative by routine state PCR testing for HeV at the time;
- Not detected by the molecular methods routinely relied upon due to sufficient nucleotide mismatches in genomic sequence (approx. 15%).
- Equine HeV disease caused by this variant was clinically indistinguishable from the most severe reported form of acute HeV disease.
- This 2015 horse HeV-variant shares near-identical genomic sequence to that detected in a grey-headed flying fox (*Pteropus poliocephalus*) from Adelaide in 2013.

2013 viral sequence was shared with us in good-will prior to publication will by relevant CSIRO scientists.

Hendra virus variant primers shared with Human and Animal Health laboratory and biosecurity network 24th February 2021 courtesy of *'Horses as Sentinels Research' group in recommendation for urgent prepublication use on compassionate grounds. Primer design by JS Eden WIMR and I Smith CSIRO.*

(b) (6)

Initial action:

- Timely communication with state biosecurity research partners and CVO allowing for immediate actions relevant to local stakeholders.
- Redesigned PCR assays suitable for routine biosecurity screening and shared these with state and national human and animal health laboratory network.
- Undertook genomic and phenotypic analysis of the HeV-variant to support the understanding that immune-protection should be afforded as for the prototypic HeV by both the Equivac HeV® vaccine in horses and the mono-clonal antibody m102.4 post-exposure therapy in humans.

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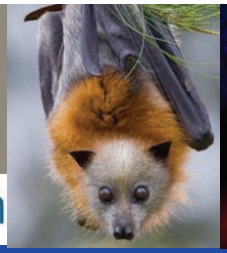

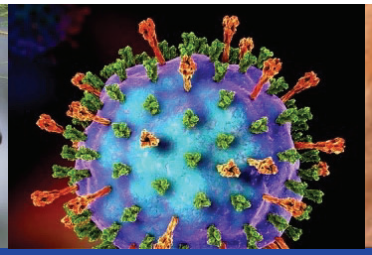

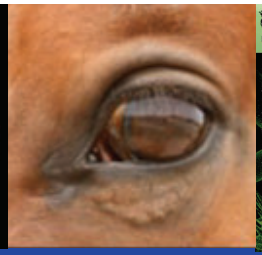

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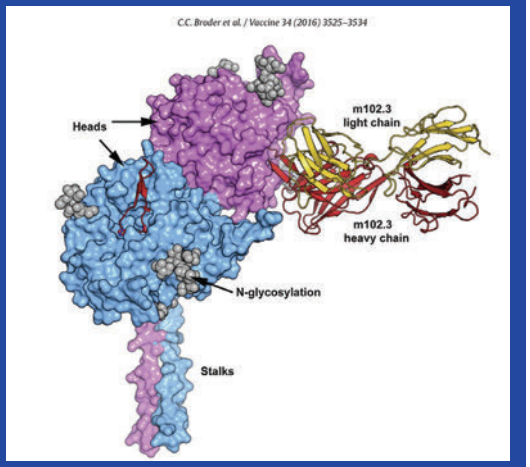
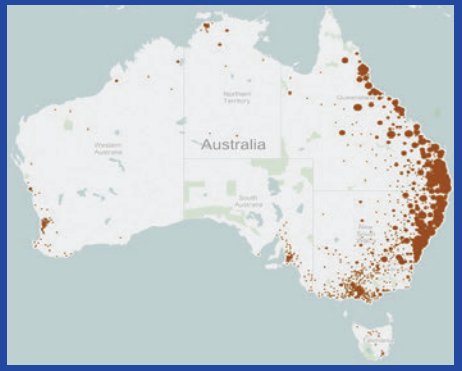
It should be emphasized, that the HeV RBP shares only 79% amino acid identity to NiV RBP, yet the HeV-sG subunit vaccine provides 100% protection against lethal challenge with both HeV and NiV in animal models (11).

Both the higher similarity between the HeV-var and HeV RBP (92.5% amino acid identity) and structural consistency of critical epitopes mentioned above, suggest that current vaccination utilizing the HEV RBP will elicit similarly protective antibodies against this HeV-var. Current serological assays based on the HeV RBP are not expected to distinguish between exposure to the variants.



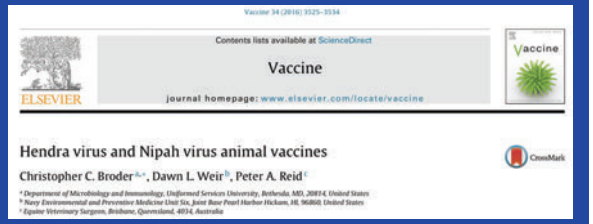
Neutralisation Titres on Vaccinated Horses

Serum	HeV	HeV-var/g2
1	64	64
2	256	256
3	128	128
4	128	128
5	64	64
6	2048	1024
7	16	16
8	1024	1024
9	2048	2048
10	64	64
11	512	1024
12	2048	2048
13	512	512
14	512	512



>150,000 horses vaccinated from 2012 to 2019

>650,000 doses administered.


Contents lists available at ScienceDirect
Vaccine
journal homepage: www.elsevier.com/locate/vaccine
Hendra virus and Nipah virus animal vaccines
Christopher C. Broder, Dawn L. Weir, Peter A. Reid

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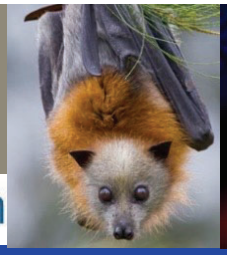

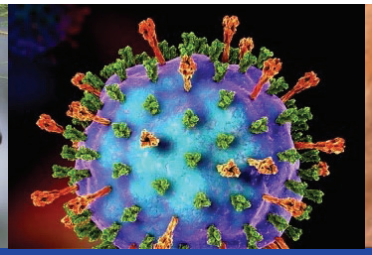

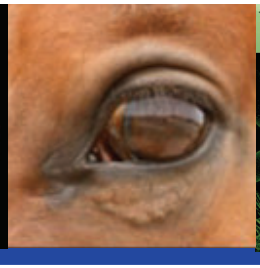

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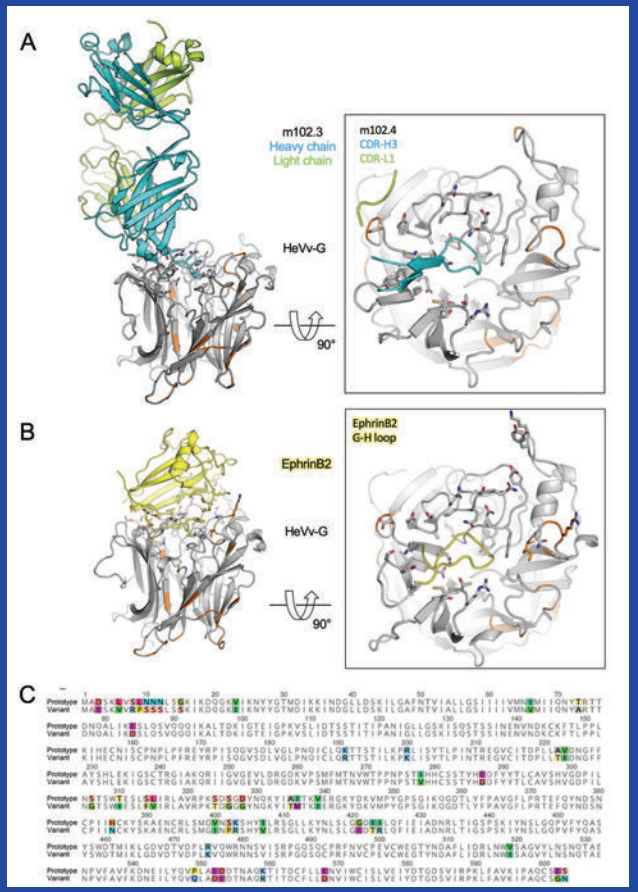
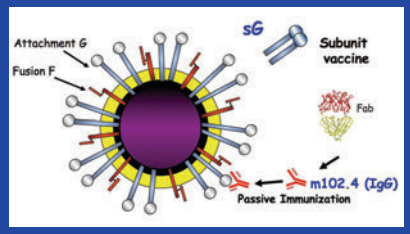
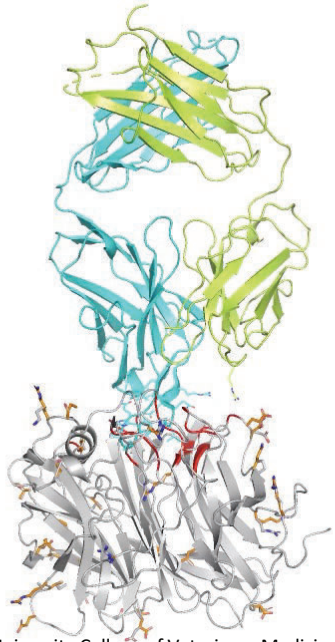


Comparison of the translated amino-acid sequence of this HeV-var and prototypic HeV RBP *in silico* revealed the ephrin-B2 entry receptor binding site and that of mAb m102.4 to be unchanged. Similarly, m102.4 neutralization was confirmed *in vitro* with HeV-var as for HeV. As such, it is expected that current PEP utilizing mAb m102.4 will remain effective against this HeV-var.





Dr (b) (6) Ohio State University College of Veterinary Medicine Department of Veterinary Biosciences, Columbus, Ohio, USA

Anticipated efficacy of sG vaccine for horses and post-exposure immunotherapy with m102.4 via *In silico* analysis of HeV variant receptor-binding protein structure based on established structure for HeV via x-ray crystallography.

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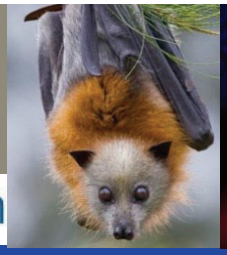

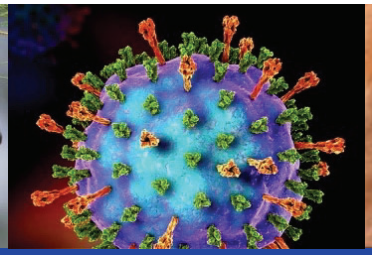

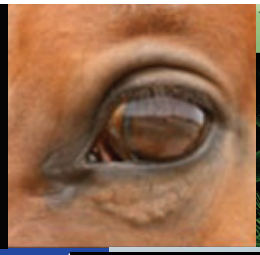

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Timely communication of equivalent pathogenicity and immunogenicity with updated qPCR testing capacity shared for routine priority disease investigation.

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EQUINE VETERINARIANS AUSTRALIA

Special Features

Hendra virus - Two Viruses, Same Fatal Disease

Edward J. Annand

University of Sydney | Sydney School of Veterinary Science |
Marie Bashir Institute for Infectious Diseases and Biosecurity, Sydney, NSW, AUS.
CSIRO | Health and Biosecurity, Black Mountain Laboratories, Canberra, ACT, AUS.
EquiEpiVet | Equine Veterinary and One Health Epidemiology, Airey's Inlet, Surf Coast, Vic., AUS.

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Title: **Novel Hendra virus variant detected by sentinel surveillance of Australian horses.**

Authors: Edward J. Annand[1#], Bethany A. Horsburgh[1], Kai Xu, Peter A. Reid, Ben Poole, Maximillian C. de Kantzow, Nicole Brown, Alison Tweedie, Michelle Michie, John D. Grewar, Anne E. Jackson, Nagendrakumar B. Singanallur, Karren M. Plain, Mary Tachedjian, Brenda van der Heide, David T. Williams, Cristy Secombe, Eric D. Laing, Spencer Sterling, Lianying Yan, Louise Jackson, Cheryl Jones, Raina K. Plowright, Alison J. Peel, Ibrahim Diallo, Andrew C. Breed, Christopher C. Broder, Philip N. Britton*, Navneet K. Dhand*, Ina Smith*, John-Sebastian Eden*

[1]**These authors contributed equally. *Shared senior authorship. [#]Corresponding author.**

Manuscript Number: EID-21-1245 Submitted Mon, May 31, 2021, *In Review*.

Article Summary Line: A novel variant of Hendra virus was discovered and isolated from an Australian horse with fatal disease.

Running Title: HeV variant associated with fatal disease in a horse

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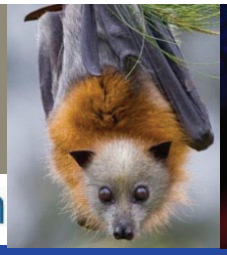
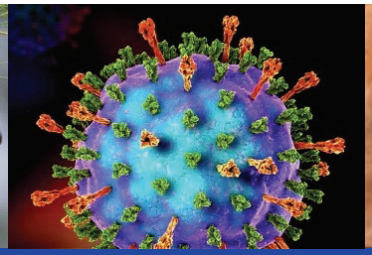
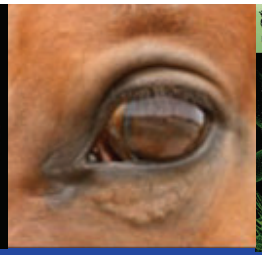

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Timely Prepublication Sharing of Duplex qPCR for Variant (HeVg2) and Original HeV Genotypes Results in Prospective Detection *(Southern-most HeV Spillover)*

Australian Government

The Hon David Littleproud MP
Minister for Agriculture and Northern Australia

(b) (6)

Joint media release: Researchers develop test for new Hendra variant

9 October 2021

Minister for Agriculture and Northern Australia, the Hon David Littleproud MP
Minister for Science and Technology, the Hon Melissa Price MP

- **New Hendra virus variant (HeV) confirmed in routine surveillance in New South Wales**
- **Researchers recently discovered this new Hendra virus variant in historical samples**
- **New test available nationally to identify Hendra virus variant**

NSW GOVERNMENT Department of Primary Industries

Hendra variant case confirmed near Newcastle

8 Oct 2021

A variant Hendra virus strain has been confirmed in a 7-year-old unvaccinated Clydesdale from West Wallsend, near Newcastle.

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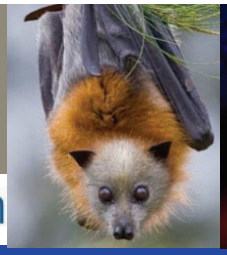
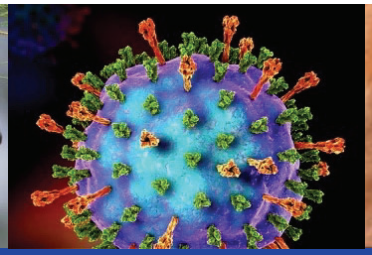
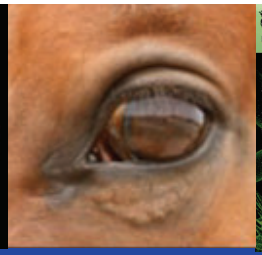

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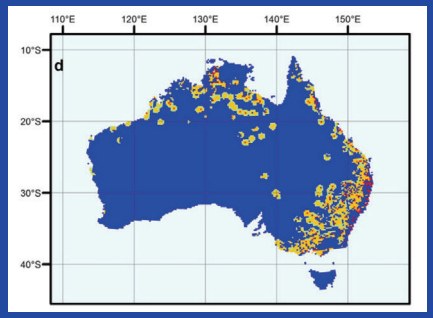
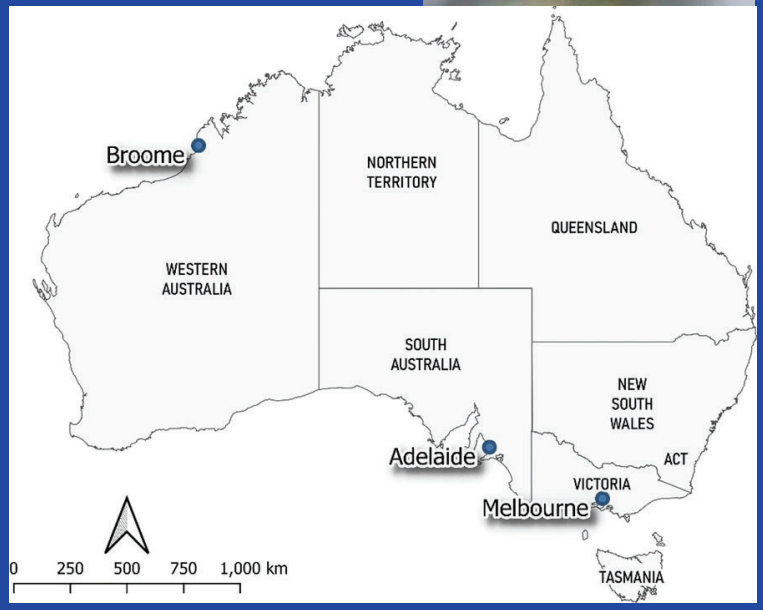
Numerous molecular detections of the same variant genotype of Hendra virus (HeVg2), in grey-headed flying foxes in South Australia and Victoria and a little red flying fox in Western Australia:

Wang, J., Anderson, D.E., Halpin, K. et al. A new Hendra virus genotype found in Australian flying foxes. Virol J 18, 197 (2021). https://doi.org/10.1186/s12985-021-01652-7.

The manuscript describes detections in bat samples (2013-2021) of the same consistent phenotypic second genotypic lineage detected retrospectively (2021) by this research to have caused fatal equine disease in QLD (2015).



East IJ, Wicks RM, Martin PAJ, Sergeant ESG, Randall LA, Garner MG. Use of a multi-criteria analysis framework to inform the design of risk based general surveillance systems for animal disease in Australia. Preventive Veterinary Medicine. 2013 Nov 1;112(3):230–47.



Map of Australia showing the locations of the HeV-g2 positive flying foxes collected between 2013 and 2021: one LRFF was from Broome, three GHFF were from Adelaide and 7 GHFF were from Melbourne.

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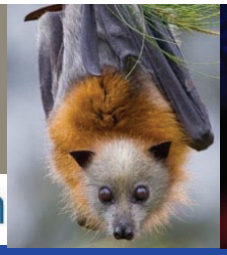
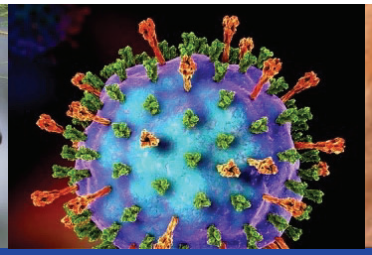
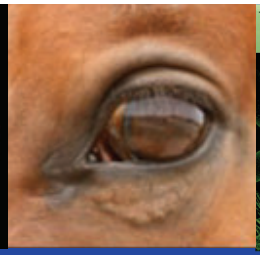

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Revised Geographic HeV Spillover Risk

Horses as Sentinels with BAT ONE HEALTH

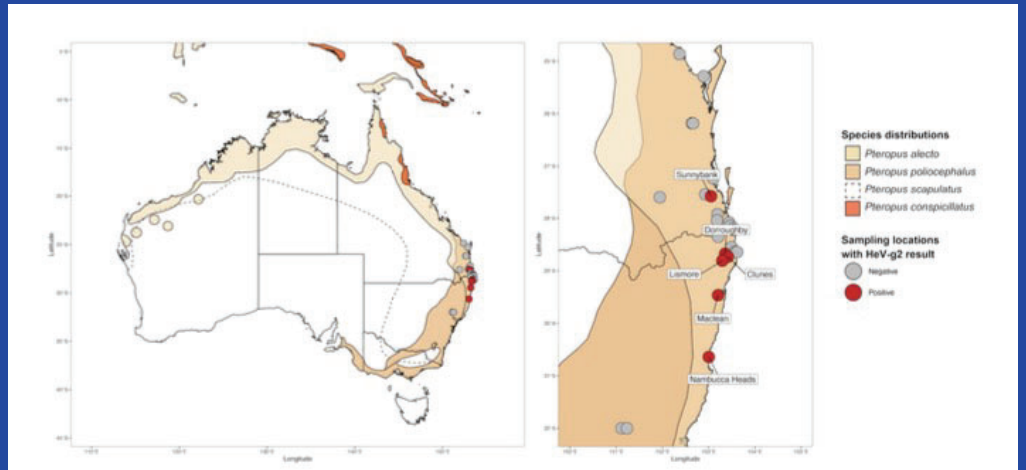

Figure 1: Map of Australia (left) and study area (right) showing the distribution of Australian flying foxes, the sampling locations and positive HeV-g2 detections.

A novel variant of Hendra virus circulates in black flying-foxes (*Pteropus alecto*) and grey-headed flying-foxes (*Pteropus poliocephalus*)

Alison J. Peel[1*], Claude Kwe Yinda[2*], [*], Edward J. Annand[3,4], Adrienne S. Dale[5], Peggy Eby[1,6], John-Sebastian Eden[7], Devin N. Jones[8], Maureen K. Kessler[9], Tamika J. Lunn[1], Tim Pearson[10], Jonathan E. Schulz[2], Ina L. Smith[11]Bat One Health[12], Vincent J. Munster[2#], Raina K. Plowright[8#]

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Manuscript Type:	Dispatch
Date Submitted by the Author:	16-Nov-2021

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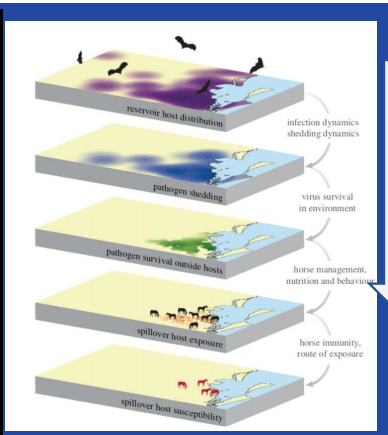

Plowright RK *et al.* 2015

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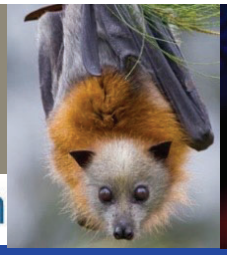
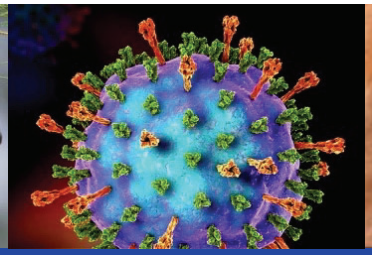
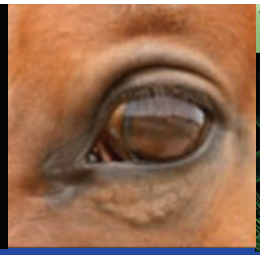

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HeV and ABLV are both severe zoonotic diseases that circulate amongst Australian bats and are lethal to both horses and humans.

While they are likely ancient viruses, our awareness of them is relatively recent and emerging.

(b) (6)

BVSc(Hons) MANZCVS (Equine Surgery) & (Epidemiology) CertAVP (Equine Stud Medicine) PgCertVPS MRCVS

Research Associate and PhD candidate | One Health Epidemiology and Virology
University of Sydney | Sydney School of Veterinary Science and Sydney Institute of Infectious Diseases, Sydney, NSW.
CSIRO | Health and Biosecurity, Black Mountain, ACT.
EquiEpiVet | Equine Veterinary and One Health Epidemiology, Aireys Inlet, Vic.

(b) (6)

Horses as Sentinels of Emerging Infectious Diseases Research
Co-leadership: Dr (b) (6)

Co-Hosted by: The University of Sydney (USYD) (Sydney School of Veterinary Science and Faculty of Medicine and Sydney Institute for Biosecurity and Infectious Diseases) and CSIRO (Health and Biosecurity Business unit). **Supported by:** Westmead Institute for Medical Research**:** Queensland Government Biosecurity Sciences Laboratory and Queensland Department of Agriculture and Fisheries**;** Australian Centre of Disease Preparedness Diagnostic Surveillance and Response Laboratory**;** University of Sydney, Sydney Medical School; EquiEpiVet; JData and the Broder lab - Uniformed Services, University of Health Sciences, USA.
Additional funding contribution from: Dalara Foundation, philanthropic donation - Horses and Human Health; Marie Bashir Institute of Biosecurity and Infectious Diseases - Internal USYD seed funding; USYD on behalf of Australian Government – Department of Education Skills and Employment - Research Training Program scholarship.
Co-investigators and contributors: Molecular and Bioinformatical co-investigators: (b) (6)
(b) (6)
Database, and data analysis contributors: Dr
(b) (6)
. Workshops and system processe (b) (6)
. Serology recombinant protein contributors: Prof. Christopher Broder, Dr Eric Laing, (b) (6)
Federal Government DAWE research partner: (b) (6) **; project manager:** (b) (6) and **research sponsor:** (b) (6) **. Queensland Government QDAF research partner:** (b) (6) **and project governance:** (b) (6)
Additional university research governance: (b) (6)
dditional Federal Government DAWE support/consulta (b) (6)
. Broader Scientific collab (b) (6)

From: (b) (6)
To: (b) (6) ; Broder, Christopher; (b) (6)
Cc: (b) (6)
Subject: Updating of One Health & Outbreak Surveillance Symposium 2021 lunchtime abstract presentation.
Date: Saturday, November 27, 2021 9:04:52 AM
Attachments: One-Health_Singapore_2021_(b) (6)_HeV-g2.pdf
image001.png

Hi (b) (6)

Updating here that I have had a late call up facilitated by (b) (6) (Cc'd - who is giving a keynote at the same event) to present the HeVg2 findings at:

One Health & Outbreak Surveillance Symposium 2021, Singapore (online) on Monday - https://blog.nus.edu.sg/ciderohs/ - in the lunchtime abstracts session.

One Health & Outbreak Surveillance Online Symposium

One Integrative Surveillance System for Pandemic Preparedness

29 November 2021 | Zoom (Online)

Organised by the Centre for Infectious Disease Epidemiology & Research,
Saw Swee Hock School of Public Health, National University of Singapore

The title of the abstract is: **Horses as Sentinels for Emerging Infectious Disease Research Discovery (2021) of a variant genotype of Hendra virus (HeVg2) affording prospective spillover detection (2021) and revision of geographical spillover risk.**

Sharing pdf of slides for your records.

I've had to send the slides in advance already but if anyone has any particular requests for edits let me know and I'll see if it can be accommodated. (b) (6) *– this is the same slide I used (while perhaps a little clearer) in the AVA VET Fest presentation on Thursday night which went very well by the way.*

Cheers

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From: (b) (6)
To: (b) (6) ; Broder, Christopher; (b) (6)

Subject: NIH Pandemic Preparedness discussions: draft slides
Date: Friday, November 5, 2021 10:54:22 AM
Attachments: Paramyxoviridae presentation.pptx

Dear All,

We promised to get you a draft of the slides for next week. (b) (6) and I are meeting this afternoon to finalize. We have been tag-teaming preparation, life and calendars.

Please don't worry about format just content.

ACTION

If anyone has useful slides to contribute to paramyxovirus background please can you send them to me – it would save us some time over the weekend

Please look at the summary slide we tried to capture what drover our recommendation, remembering not all columns are weighted equally. The colors are simply our best guess if anyone has strong opinions and how we have rated anything or if we have totally miscalled please let us know.

I have tried to really simplify the taxonomy … (b) (6) can you give this a good look at those slides.

If anyone finds it easier to chat I am around this afternoon from 14:00. Remember there's only 5 hours between EDT and CET at the minute.

Thanks so much in advance.

Kind regards

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From: (b) (6)
To: (b) (6) CHRISTOPHER BRODER
Cc: (b) (6)
Subject: RE: Project 1 RAViD version for editing
Date: Wednesday, November 3, 2021 3:16:36 PM
Attachments: Tulane_Res_strategy_Nipah-Final-for-revision .docx

---

Yes, thank you.

I changed it the text and fig 6

From: (b) (6)
Sent: Wednesday, November 3, 2021 2:06 PM
To: CHRISTOPHER BRODER (b) (6)
Cc: (b) (6)
Subject: Re: Project 1 RAViD version for editing

Please check the reference to the Aerobiology Delivery Core. I think that may be Core B and not Core A.

From: Broder, Christopher (b) (6)
Sent: Wednesday, November 3, 2021 1:56 PM
To: (b) (6)
Cc: (b) (6)
Subject: Re: Project 1 RAViD version for editing

if all OK with you ALL
then i am going to make the edits to
cut off 6 lines of text
and return to (b) (6)

On Wed, Nov 3, 2021 at 2:49 PM Broder, Christopher (b) (6) wrote:

> going over it now
>
> On Wed, Nov 3, 2021 at 2:40 PM (b) (6) wrote:
>
> > Please use this one for final review.

One more time…

From: (b) (6)
Sent: Wednesday, November 3, 2021 1:37 PM
To: (b) (6)
Cc: Broder, Christopher (b) (6)
Subject: RE: Project 1 RAViD version for editing

No we are cool
Thank you!

(b) (6)

From: (b) (6)
Sent: Wednesday, November 3, 2021 10:35 AM
To: (b) (6)
Cc: Broder, Christopher (b) (6)
Subject: Re: Project 1 RAViD version for editing

The current text looks great. Do we need to cut it further?

On Wed, Nov 3, 2021 at 9:41 AM (b) (6) wrote:

> Once (b) (6) has a new version, I can cut some in moa studies
>
> Sent from my iPhone
>
> > On Nov 3, 2021, at 8:24 AM, Broder, Christopher (b) (6) wrote:

Yes this is looking great guys.

We need to be orderly.
Perhaps (b) (6) shrink figures a bit and reduce font sizes in AIm 1 figures.
Figs in preliminary data could be a tad smaller. and the sG structure reduce a bit as well
Do anything else to cut down text. Make a final formatted version as soon as you can do it,

Then send to all of us. But in the meantime we can look at this version and mark your own changes in your own version
to get a head start, then move your edits into her version when we get it.

On Tue, Nov 2, 2021 at 11:57 PM (b) (6) wrote:

> Dear all,
>
> This is a well prepared document and I really like it. I did not try to cut it yet, but if we want to cut, we can shorten the MOA studies for small molecules. Maybe (b) (6) can shorten the MOA studies for nanobodies?
>
> Will work with you all to make a great project tomorrow.
>
> Best,
> (b) (6)
>
> [redacted]
>
> **From:** (b) (6)
> **Sent:** Tuesday, November 2, 2021 7:34 PM
> **To:** (b) (6) Broder, Christopher
> (b) (6)
> **Cc:** (b) (6)

(b) (6)

Subject: RE: Project 1 RAViD version for editing

Please use this one for editing.

We will format the tables and figures afterwards.

Thanks

From: (b) (6)
Sent: Tuesday, November 02, 2021 5:27 PM
To: (b) (6)
Cc: Broder, Christopher (b) (6)
Subject: Re: Project 1 RAViD

Hi (b) (6),

Pls send us the updated version with everything incorporated so we can edit. Thank you!

(b) (6)

Sent from my iPhone

> On Nov 2, 2021, at 4:41 PM, (b) (6) > wrote:
>
> I noticed that NiV-B was replaced with NiV_B in aim 3 (same way as "NiV Bangladesh (NiV_B)" in Doyle's publication).
> Should we change it everywhere in the text?
>
> ---
>
> **From:** (b) (6)
> **Sent:** Tuesday, November 02, 2021 4:03 PM
> **To:** (b) (6)
> **Cc:** Broder, Christopher (b) (6)

Subject: RE: Project 1 RAViD

Here is the intermediate version – for everyone to see what we have – where we keep everything as (b) (6) suggested.

my next task will be to replace the yellow section.

You can still use this version for any additional corrections and comments.

thanks

From: (b) (6)
Sent: Tuesday, November 02, 2021 3:44 PM
To: (b) (6)
Cc: (b) (6) Broder, Christopher (b) (6)
Subject: Re: Project 1 RAViD

(b) (6)

Do not worry about space now. We will do it once we have the entire doc.

(b) (6)

Sent from my iPhone

> On Nov 2, 2021, at 3:37 PM, (b) (6) wrote:
>
> I was thinking of having it near the description of the cryoEM work.
>
> As space is limited, I added headings to avoid adding more text. I can provide a caption but it will take space. Just let me know
>
> Sent from my iPhone
>
> > On Nov 2, 2021, at 3:58 PM, (b) (6) wrote:

I think once we have the text, we can decide how to cut it.
My understanding is (b) (6) will edit the first part of the strategy?
The project is looking better and better.

(b) (6)

[redacted]

From: (b) (6) [redacted]
Sent: Tuesday, November 2, 2021 11:54 AM
To: (b) (6) [redacted]
Broder, Christopher
(b) (6) [redacted]
Cc: norton peet
(b) (6) [redacted]
Subject: RE: Project 1 RAViD

Where do you want it to be? Pls indicate paragraph ID (ex. C1.2…..)
Do you have a fig legend or a short description to be incorporated in the text?

From: (b) (6) [redacted]
Sent: Tuesday, November 2, 2021 2:50 PM

To: (b) (6) Broder, Christopher
(b) (6)
Cc: Gaisina, Irina N
(b) (6)
Subject: RE: Project 1 RAViD

(b) (6),

I love it!
(b) (6), pls add it and will see how to shorten other parts. As (b) (6) said, Aim 1 is too long.

(b) (6)

From: (b) (6) >
Sent: Tuesday, November 2, 2021 11:29 AM
To: Broder, Christopher
(b) (6)
Cc: (b) (6)

Subject: Re: Project 1 RAViD

How about that?

<image001.jpg>

On Tue, Nov 2, 2021 at 2:53 PM Broder, Christopher (b) (6) > wrote:

> (b) (6)
> it would be awesome yes, but we have to cut text in AIM 1
> insert were you mention nAH1.3?
>
> On Tue, Nov 2, 2021 at 2:48 PM (b) (6) wrote:
>
> > Hi (b) (6),
> >
> > Is there room for me to include a figure about NiV G?
> >
> > (b) (6)
> >
> > On Tue, Nov 2, 2021 at 2:08 PM Broder, Christopher (b) (6) > wrote:
> >
> > > Hi all
> > >
> > > (b) (6),
> > >
> > > As I mentioned this AM, **please use this attached tracked version. There are many edits.** Incorporate (b) (6)'s into this one.
> > >
> > > We are down to details and every edit helps with space

and length

As soon as (b) (6) gets another version everyone should please at least check their sections. I have added the missing sections on nanobody development and sources to preliminary data and to Aim 2 (b) (6).

Then we need to remove all hanging sentences to tighten and save space

I am rewriting the background (Yellow highlighted here) as I said earlier. It is not the version from Sunday and has major errors.

General, Aim #1 needs to be cut down, either by detail or removing some figures, It is too long and out of balance with Aim 2 and 3 in scope.

Do you need refs for (b) (6) section Aim 3 ? I can do that also

Did all of you send your consortium letter of agreement to (b) (6) ?

(b) (6) is doing this lifting here now, if someone wants to make a list of bullets of the major AIMS and SubAIMS we can sent to (b) (6) and (b) (6) said

he make our missing Gantt chart for us
We need this or the attached ppt file list

[redacted]

--

(b) (6)
[redacted]

(b) (6)

--

(b) (6)

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(b) (6)

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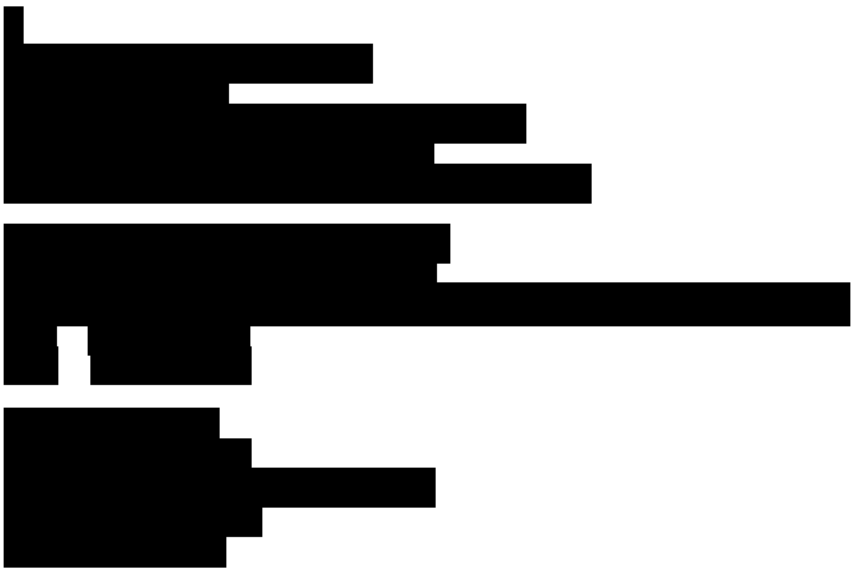
(b) (6)



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> intended recipient(s) and may contain confidential and privileged information. Any unauthorized use, disclosure or distribution is prohibited. If you are not the intended recipient, please contact the sender by replying to this e-mail and destroy all copies of the original message. (Uniformed Services University)

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[redacted]

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From: (b) (6)
To: (b) (6) ; Laing, Eric; Broder, Christopher; (b) (6)
Subject: comments on henipavirus RO1
Date: Wednesday, November 3, 2021 12:13:40 PM
Attachments: 1R01AI168287-01.pdf

Colleagues,

We've received scores and comments on our RO1. Score was [redacted] (b) (5) percentile. We need to be around (b) (5) percentile to be funded. So, not too bad for our first submission.

Two reviewers scored the proposal very high. Reviewer #2 is very cranky about biosafety issues. Hopefully, we can address these in the next submission. I'll meet with the Program Officer soon to see if she has any insights.

There are also some concerns about validating the serology tests, which was predictable. Perhaps we can think of ways to address that as well.

The plan is to resubmit at the next deadline in Feb.

Feeling hopeful.
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From: (b) (6)
To: Broder, Christopher; (b) (6)
Cc: (b) (6)
Subject: ResStrategy rCedV
Date: Monday, November 1, 2021 11:04:22 PM
Attachments: Tulane_Res_strategy_Nipah-11-1-revised.docx

---

please use this latest (for today) version for any corrections and additions. Thank you.
(b) (6)

From: Broder, Christopher (b) (6)
Sent: Monday, November 1, 2021 8:33 PM
To: (b) (6)
Cc: (b) (6)
Subject: rCedV

(b) (6)

try putting this rCedV tools into the preliminary data
it covers chimeras to replace the published rCedV-Luc description and existing figure 1 which we should remove. the chimera prelim data supports high throughput nanobody assessment, and synergy assay (which can be mentioned in Aim 1 drugs, and Aim 2 nanobodies.

also there is some text for resistance selection and genome sequencing for target identification if needed somewhere

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From: (b) (6)
To: Broder, Christopher; (b) (6)
Cc: (b) (6)
Subject: Re: rCedV
Date: Monday, November 1, 2021 9:56:06 PM
Attachments: Tulane_Res_strategy_Nipah-11-1-combined.docx

(b) (6), I'm still working on it, and this is my current version. I had to use an old one for (b) (6)'s edits.
Still need to fix refs in your "Background" portion to be able to put it in.
Although, it seems even longer than the one in the submitted Alabama grant.
Would it be possible to reduce it a little bit?

And I'll use the new figures that you just sent us.
Thanks.

From: Broder, Christopher (b) (6)
Sent: Monday, November 1, 2021 8:33 PM
To: (b) (6)
Cc: (b) (6)
Subject: rCedV

(b) (6)

try putting this rCedV tools into the preliminary data
it covers chimeras to replace the published rCedV-Luc description and existing
figure 1 which we should remove. the chimera prelim data supports high throughput
nanobody assessment, and synergy assay (which can be mentioned in Aim 1 drugs, and Aim 2
nanobodies.

also there is some text for resistance selection and genome sequencing for
target identification if needed somewhere

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From: Broder, Christopher (b) (6)
To: (b) (6)
Cc: (b) (6)
Subject: Re: Project 1 absrt
Date: Monday, November 1, 2021 9:36:40 PM
Attachments: SpecificAims-ResStrategy-HenipaRP1-RAViD-10312021.docx

---

i sent you all the Aims page on Sunday night--
it was page 1 of the document so that the references would be
a simple manner (attached here again)

i have not made any more changes.

On Mon, Nov 1, 2021 at 2:50 PM (b) (6) wrote:

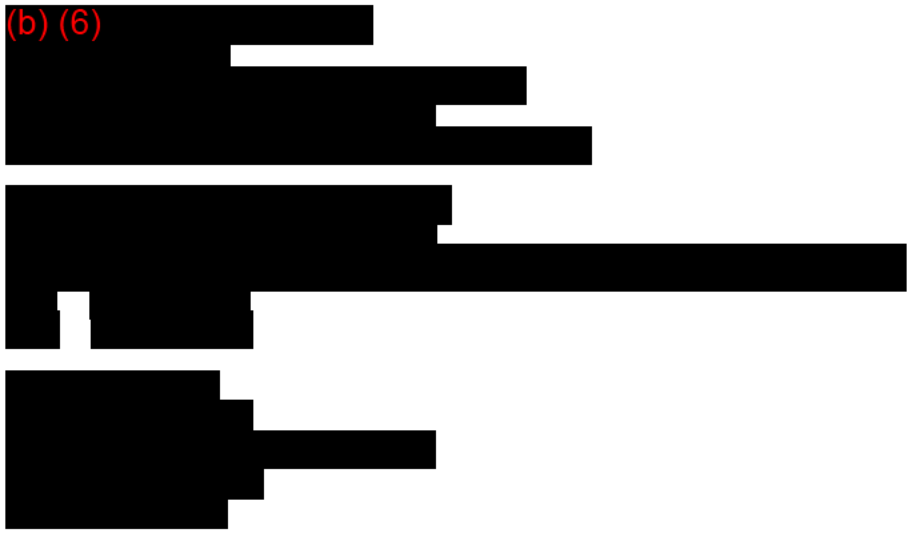
> Thank you (b) (6). Do you have an updated Aims page to share with us pls?
>
> (b) (6)
>
> [redacted]
>
> ---
>
> **From:** Broder, Christopher (b) (6)
> **Sent:** Monday, November 1, 2021 10:02 AM
> **To:** (b) (6) [redacted]
> **Subject:** Project 1 absrt

abstract draft attached

please edit and return and (b) (6) can upload

--

(b) (6)



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From: Broder, Christopher (b) (6)
To: (b) (6)
Cc: (b) (6)
Subject: RAViD
Date: Sunday, October 31, 2021 9:39:52 PM
Attachments: SpecificAims-ResStrategy-HenipaRP1-RAViD-10312021.docx

---

Dear (b) (6)
and All
I know you have been working on AIm 1 and (b) (6) on Aim 2

I said i was revising the Aims page, and working on the bkg and innovation.

Please integrate the attached, which is also a V3 revision to Aims page, into this Doc you just sent, before folks start editing.

Again. as we discussed. UIC will be the holder of the final format and reff generation. So the tedious bit here is the ref insertions into the BKG and Innovation.

I am not sure how all think to move forward from here, regards to editing and working on one document

I will work on Prelim data on nanos and SNT chimera assay tomorrow. with some figs to add
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From: (b) (6)
To: Broder, Christopher
Cc: (b) (6)
Subject: The Final DRAFT & everything else
Date: Thursday, March 7, 2019 10:13:27 AM
Attachments: Master List.Figures & Tables & PageCount.xlsx
CH14-HNV-Complete.190307.v9.0.docx
Figure Legends_Final.v5.Fields.docx
HNV_Fields Ref.enlp.zip
Henipavirus Tables 14.1-14.5-V10-190226.docx

HI Folks,

This is the final draft for now. I think it's still way too long by my counting, but at least everyone can see how everything hangs together.

The only edits and comments you see are new ones I added, mostly to (b) (6)s section, but I also took a couple sentences from (b) (6)s section to put into my pathogenesis part to avoid duplication. I clearly indicated where.

I removed two old figures and replace with two new ones.

Attached is the (1) Master Excel Spread sheet to keep everything straight, (2) the text manuscript file, (3) Figure Legend text file, (4) Master endnote file., (5) Final Table Text file

I will send you a Googlelink to the ppt file with all the figures. The tiff files are individually exported, can share them in a dropbox if you want.

Anyway, I'm happy to take out anything in my section - it's just too long, and I already cut out so much. Let me know what you think is important.

Thanks. ((b) (6) maybe you can tell (b) (6) we'll be uploading soon. I'm afraid to talk to him!) Gottta go and get some sleep now.

(b) (6)

> On Mar 6, 2019, at 11:00 PM, Broder, Christopher (b) (6) wrote:
>
> hey! Congrats (b) (6)! So happy for you!
>
> I just landed in NRT on way home to MD.
>
> HAHA I know what you mean about being afraid to get back online to email! and NO need to apologize! are you kidding. Life is way more important than this!
>
> I hope you have fun things planned the rest of the week and weekend! and not the damn chapter!

all very best wishes!
(b) (6)

On Wed, Mar 6, 2019 at 7:56 PM (b) (6) wrote:

> Hi Guys,
>
> The reason why I wanted things earlier on to put together is because . . . I just got (b) (6) . But my schedule is not your problem.
>
> I am also here at a GRC in Ventura (on Bioterrorism but speaking on CRISPR delivery, NOT NiV). Basically have been continuously working till 5 am or so every day since last week (except for flying here and on my (b) (6) day).
>
> This chapter is such a bear to put together. Reading the instructions, they really want the subsections constructed as in the outline, so the whole book has the same organization. I had re-do my original sections almost completely, and have to keep referring to both your sections (that's why I need your sections to fully complete mine) to make everything consistent and avoid duplication.
>
> I've added substantial new matter for (b) (6) 's into section on viral proteins properties, etc. To keep up with advances, I made an entirely original figure on B2/B3 expression across tissues using the original NGS data from the GTEx Portal. Then converting every figure into a high resolution tiff file for those that i don't have already is a nightmare.
>
> I'm going to send what I have even if the length is too long . . . to all of you by early am (Mar 7 EST). I may be upload it as well. We all have to revise the manuscript anyway, after review, and also after the substantial nomenclature changes are adopted later this month by ICTV.
>
> My husband is in L.A. all week and we are taking off for the weekend, so if I still have to work on this chapter this weekend, this will be shortest lived marriage. I can already tell the uploading process is a bear.
>
> So, I apologize. It's so much work than I thought! You see what I mean when i send everything. Thank you for your patience.
>
> Cheers,
>
> (b) (6)
>
> On Mar 6, 2019, at 1:42 AM, (b) (6) wrote:

A very nice updated figure!

(b) (6)
[redacted]

From: Christopher Broder (b) (6)
Date: Wednesday, 6 March 2019 at 5:35 PM
To: (b) (6)
Cc: (b) (6)
Subject: Re: GenBank AC#

this was replacement HeV vaccine map.

Must have been lost in the emails.

(b) (6)

On Sat, Mar 2, 2019 at 10:45 PM (b) (6) [redacted] wrote:

> Dear (b) (6)
>
> We have now got the GenBank AC# for the new NiV seuences:
>
> NiV/BD/Bat/2013/Sylhet-47(MK575070)
> NiV/BD/Bat/2013/Raypur-31(MK575060)
>
> I will let you to update in the figure.
>
> Thanks
>
> (b) (6)
>
> [redacted]

(b) (6)

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(b) (6)

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Christopher C. Broder, Ph.D.
(b) (6)

(b) (6) [redacted]

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From: (b) (6)
To: (b) (6) ; Broder, Christopher; (b) (6)
Cc: (b) (6)
Subject: RE: Proceedings from Nipah Virus International Conference
Date: Monday, March 30, 2020 12:30:25 AM
Attachments: Summary and Outline for mSphere_v1(b) (6).docx
image001.png
image002.png
image003.png

Hi there

I'm not sure if others have contributed to the *mSphere* article as requested by (b) (6) (email below), but please find my contribution.
Others are welcome to build on this, if they have time. It would be great to see a few more comments to round out the summary of this amazing (and somewhat prophetic!) conference.
I think (b) (6) wanted this by 30th March – so I'm cutting this a bit fine – sorry.

Thanks everyone
Kind regards
(b) (6)

From: (b) (6)
Sent: Thursday, 19 March 2020 9:57 PM
To: (b) (6) Broder, Christopher (b) (6)
Cc: (b) (6)
Subject: [WARNING : MESSAGE ENCRYPTED]Proceedings from Nipah Virus International Conference

Dear International Organizing Committee (IOC) members for the Nipah Virus International Conference,

Please find a ***draft*** (pdf) with the Proceedings from the Nipah Virus International Conference, which took place in Singapore last December. The password for this document is (b) (6). Please consider this document under 'embargo' until cleared by the the respective Communications departments from CEPI and its Conference organizing partners (WHO, NIAID, Duke-NUS).

We hope this email finds you well. We apologise for not sharing the Proceedings with you sooner, but we hope you will understand all of us have been swamped with the present COVID-19 situation. Although we do appreciate the Nipah Conference may be perceived as less of a global priority now

comparted to COVID-19, we believe the Proceedings contain relevant insights that should be shared with the global community in a pandemic situation.

Our plan is to upload a refined and cleared version of the Proceedings into a suitable online platform for distribution with our partners, ideally within 4 months of the Conference having taken place (i.e. before April 11th).

In addition, and thanks to (b) (6), the Editor-in-Chief for *mSphere* has also agreed to publish a *summary* version of this and we have started working on that. Please also find the *word* document with a *draft* summary of Conference. We would like to invite you to co-author this summary and edit/add comments to this **by Monday, March 30**. If you do not wish to co-author the summary, please let us know.

Again, we do recognise that this may be perceived as less of a priority now, but we hope the insights from the Conference can shed light in some of the scientific issues being discussed now. We would like to thank you again for your valuable contributions to this Conference, and we look forward to your participation in this process.

Best wishes,
(b) (6)

[redacted]

CEPI New vaccines for a safer world

[redacted]

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From: (b) (6)
To: (b) (6) Broder, Christopher
Cc: (b) (6)
Subject: FW: THELANCETID-D-19-01274
Date: Friday, October 18, 2019 6:19:56 PM
Attachments: THELANCETID-D-19-01274R1.pdf
Importance: High

Dear authors
We've had positive feedback from the Lancet senior editor on our resubmitted HeV manuscript that was uploaded on Wednesday of this week - - see comments below. The resubmission which includes reviewers comments and our responses, is attached for your records. It is now with the editor-in-chief who hopefully will render a final decision soon.
Will keep you posted.
Kind regards
(b) (6)

From: (b) (6)
Sent: Friday, 18 October 2019 12:12 PM
To: (b) (6)
Subject: FW: THELANCETID-D-19-01274
Importance: High

Hi (b) (6)

Good news thus far.

Please forward to the entire authorship group.

Regards

(b) (6)

From: (b) (6)
Sent: Friday, 18 October 2019 1:48 AM
To: (b) (6)
Subject: THELANCETID-D-19-01274
Importance: High

Dear Professor (b) (6),

I hope this email finds you well. I have received your revised version of the article THELANCETID-D-19-01274 "Safety, tolerability, pharmacokinetics, and immunogenicity of a human monoclonal antibody targeting the G glycoprotein of Henipaviruses in healthy adults: a randomised, first-in-human phase 1

study". I just wanted to let you know that I am happy with the revision and I have passed now the paper to our Editor-in-Chief who needs to make his check.
I had asked you about the delay between trial and submission because we always need to ask when we notice this (there have been instances when major issues were disclosed after probing authors for this issue): I had no doubt that there was a good reason in your case and I want to reassure that the explanation is fine and I am very happy that you sent the paper to us.
Kind regards,

(b) (6)

[redacted]

[redacted]

****

infected with a virus, other malicious computer programme or code that may occur as a consequence of receiving this email.

Unless stated otherwise, this email represents only the views of the sender and not the views of the Queensland Government.

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From: (b) (6)
To: (b) (6)
Cc: christopher.brode (b) (6)

Subject: RE: Revised Nipah R&D roadmap manuscript--please respond by June 25
Date: Friday, June 28, 2019 7:30:41 AM
Attachments: Nipah Roadmap manuscript June 2019V3 (b) (6) .docx
image001.png

Dear (b) (6),

Thank you for sharing the revised draft.

I have attached some minor comments for your consideration.

I concur with the content of the roadmap, reviewed the draft and agree to be listed as a co-author on the manuscript.

Regards,
(b) (6)

icddr,b

From: (b) (6)
Sent: Monday, June 24, 2019 1:11 AM
To: christopher.brode (b) (6)

Cc: (b) (6)
Subject: FW: Revised Nipah R&D roadmap manuscript--please respond by June 25
Importance: High

Dear all. Please read the email below. This is just a reminder to send me any comments on the June 2019 version of the Nipah roadmap paper and to confirm that you are willing to be a co-author on the manuscript as outlined below.

Thank you so much for your time!

Warm regards,

(b) (6)

From: (b) (6)
Sent: Saturday, June 15, 2019 1:26 PM
To: christopher.broder(b)(6) (b) (6)

Cc: (b) (6)
Subject: Revised Nipah R&D roadmap manuscript--please respond by June 25
Importance: High

Dear Colleagues: I hope this message finds each of you well. Per the email that you received in May, I have provided an updated version of the Nipah R&D roadmap manuscript. I have also provided the full roadmap—just in case you want to refer back to that.

Our current plan is to submit this to the NEJM as a Sounding Board article per advice from (b) (6). They will also provide a Supplemental Appendix with the full roadmap. WHO will then publish the roadmap once it has been published in NEJM.

Sounding Board articles have a 2,000 word limit and are structured as commentaries rather than as reviews, so the article has been substantially shortened—please keep that in mind as you go through it.

The process for submission is as follows:

1. Please review this version of the manuscript and **send any comments or edits to me by June 25** (through replying to this email). Please send a response even if you have no comments.
2. In your email response, also please state that you are willing to be a co-author on the manuscript and that you agree you fulfill the criteria for authorship, which for NEJM are:
    - Substantial contributions to conception and design; or acquisition, analysis, or interpretation of data AND
    - Drafting of the article or critical revision for important intellectual content AND
    - Final approval of the version to be published AND
    - Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the article are appropriately investigated and resolved

3. If NEJM decides to request a revised version and move to the next step of accepting the manuscript, there will be three forms each co-author will need to complete—one on conflict of interest, one on copyright transfer, and one stating that you fulfill the authorship requirements. Those will be sent to you at a later time.
4. You will also need to sign off on the final version of the manuscript.

Again, thank you for your time in this important process.

Warm regards,

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From: (b) (6)
To: (b) (6)
Cc: christopher.brode (b) (6)

Subject: Re: Revised Nipah R&D roadmap manuscript--please respond by June 25
Date: Tuesday, June 25, 2019 12:39:48 AM
Attachments: Nipah Roadmap manuscript.June 2019,V3 comments (b) (6) .docx

---

Dear (b) (6),

Greetings from Bangladesh !!!

Please find attached herewith the manuscript with my comments for your consideration. However, I concur with the content agree to be a co-author.

Best regards,

(b) (6)

On Sun, Jun 16, 2019 at 1:26 AM (b) (6) > wrote:

> Dear Colleagues: I hope this message finds each of you well. Per the email that you received in May, I have provided an updated version of the Nipah R&D roadmap manuscript. I have also provided the full roadmap—just in case you want to refer back to that.
>
> Our current plan is to submit this to the NEJM as a Sounding Board article per advice from (b) (6) . They will also provide a Supplemental Appendix with the full roadmap. WHO will then publish the roadmap once it has been published in NEJM.
>
> Sounding Board articles have a 2,000 word limit and are structured as commentaries rather than as reviews, so the article has been substantially shortened—please keep that in mind as you go through it.
>
> The process for submission is as follows:
>
> 1. Please review this version of the manuscript and **send any comments or edits to me by**

June 25 (through replying to this email). Please send a response even if you have no comments.

2. In your email response, also please state that you are willing to be a co-author on the manuscript and that you agree you fulfill the criteria for authorship, which for NEJM are:

- Substantial contributions to conception and design; or acquisition, analysis, or interpretation of data AND
- Drafting of the article or critical revision for important intellectual content AND
- Final approval of the version to be published AND
- Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the article are appropriately investigated and resolved

3. If NEJM decides to request a revised version and move to the next step of accepting the manuscript, there will be three forms each co-author will need to complete—one on conflict of interest, one on copyright transfer, and one stating that you fulfill the authorship requirements. Those will be sent to you at a later time.

4. You will also need to sign off on the final version of the manuscript.

Again, thank you for your time in this important process.

Warm regards,

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From: (b) (6)
To: (b) (6)
Cc: Broder, Christopher
Subject: Your Sections
Date: Tuesday, February 19, 2019 8:18:31 AM
Attachments: CH14-Henipavirus-LW190215 v(b) (6).docx
HNV_Fields Ref.enlp.zip

Dear (b) (6)

I started reading through your sections and adding/editing what I could but ran into some problems and inconsistencies with our agreed upon outline, I had to stop. I simply can't do everything myself - I also have meetings to chair and conferences to go to.

For now, I'm sending back to you what I have done and ask for your help in giving me back a document that I can deal with more easily. I almost finished everything up to my own section on Pathogenesis _ I can't add my own section yet because of the **referencing conflicts** - in fact I hardly have had time for my own section.

(1) I have added all the new references you requested and then some more (these are are highlighted in GREEN in the main text). This was easy using my endnote file.

- The most important thing is that all the old references in big brackets/parentheses (ref)(ref)(ref)(ref) are not linked to the master endnote file I sent out. So, when I configure my bibliography, I can only see the new refs I added in, which is about 16 for now.
- I simply do **not** have the time or energy to go through 168 (ref)(ref)(ref)(ref) by myself and individually replace them with linked citations from my endnote file (I'm sure you weren't expecting me to).
- When (b) (6) gave me his section, I could easily format and configure the bibliography, which will help when I put everything together. That was the plan.
- PLEASE replace all the (ref) (ref) (ref) (ref) in the main text with the refs from my Endnote file. Or have someone do it for you. You and (b) (6) will see what I mean when you open the document and see my comments/notes.

(2) To make things easier to see, I have accepted all your comments (on the side column) but left your original changes (it appears orange/peach colored in my word document. My changes/edits are dark red/maroon. It'll be obvious. I added the most text about the 3'IUTR and on the RBP section. Then, I ran out of steam. It's extremely distracting and hard not to be able to format all those (ref) (ref) (ref) (ref) !

(3) I made some suggestions about what Tables to put online, etc.

(4) Finally, parts of what you wrote does not conform to the section outline you sent us, and which I painstakingly clarified and confirmed multiple times by sending out clearly color coded outline and Figure/Table numbering. For example, I thought the genetic diversity section was suppose to be in Section D5 (Epidemiology), so your **Fig. 14.4 in the text** is actually **Fig. 14.11** in my master powerpoint. I thought we al agreed to the figure and Table numbering and order.

Basically, I was a bit frustrated and confused to continue. I felt like all my efforts in

organizing the figures and sticking to your outline have been wasted. I am more than happy to edit and compile, but we must all agree to the same rules and do our parts.

I am sending back **(A)** your doc which I edited so far, and **(B)** the latest master endnote file, which you should use to replace all (ref) (ref) (ref) (ref) in your document. (b) (6), I hope you understand what I mean when you open the document.

There is no way I can compile the final documents unless everyone uses the same endnote file. I had no problems with compilingeveryone's Tables, so I was surprised at receiving your doc where all the (ref) (ref) (ref) (ref) have not been replaced.

(b) (6) **(One word doc and one Endnote file attached).**

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From: (b) (6)
To: (b) (6)
Cc: christopher.brode (b) (6)
Subject: Re: Revised Nipah R&D roadmap manuscript--please respond by June 25
Date: Monday, June 24, 2019 2:48:44 PM
Attachments: Nipah Draft Roadmap.revised for taskforce review.April 2018 (b) (6) docx

---

Hi (b) (6)

This looks great. I attach a couple minor suggestions.

I am willing to be listed as a co-author on the manuscript. I have fulfilled the criteria for authorship.

(b) (6)

wrote on 6/15/2019 12:25 PM:

> Dear Colleagues: I hope this message finds each of you well. Per the email that you received in May, I have provided an updated version of the Nipah R&D roadmap manuscript. I have also provided the full roadmapï¿½just in case you want to refer back to that.
> ï¿½
> Our current plan is to submit this to the NEJM as a Sounding Board article per advice from (b) (6). They will also provide a Supplemental Appendix with the full roadmap. WHO will then publish the roadmap once it has been published in NEJM.
> ï¿½
> Sounding Board articles have a 2,000 word limit and are structured as commentaries rather than as reviews, so the article has been substantially shortenedï¿½please keep that in mind as you go through it. ï¿½
> ï¿½
> The process for submission is as follows:

<!--[if !supportLists]-->1.ï¿½ï¿½ï¿½ï¿½ï¿½ï¿½ <!--[endif]-->Please review this version of the manuscript and **send any comments or edits to me by June 25** (through replying to this email). Please send a response even if you have no comments.

<!--[if !supportLists]-->2.ï¿½ï¿½ï¿½ï¿½ï¿½ï¿½ <!--[endif]-->In your email response, also please state that you are willing to be a co-author on the manuscript and that you agree you fulfill the criteria for authorship, which for NEJM are:

<!--[if !supportLists]-->ï¿½ï¿½ï¿½ï¿½ï¿½ï¿½ï¿½ï¿½ï¿½ <!--[endif]-->Substantial contributions to conception and design; or acquisition, analysis, or interpretation of data AND

<!--[if !supportLists]-->ï¿½ï¿½ï¿½ï¿½ï¿½ï¿½ï¿½ï¿½ï¿½ <!--[endif]-->Drafting of the article or critical revision for important intellectual content AND

<!--[if !supportLists]-->ï¿½ï¿½ï¿½ï¿½ï¿½ï¿½ï¿½ï¿½ï¿½ <!--[endif]-->Final approval of the version to be published AND

<!--[if !supportLists]-->ï¿½ï¿½ï¿½ï¿½ï¿½ï¿½ï¿½ï¿½ï¿½ <!--[endif]-->Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the article are appropriately investigated and resolved

<!--[if !supportLists]-->3.ï¿½ï¿½ï¿½ï¿½ï¿½ï¿½ <!--[endif]-->If NEJM decides to request a revised version and move to the next step of accepting the manuscript, there will be three forms each co-author will need to completeï¿½one on conflict of interest, one on copyright transfer, and one stating that you fulfill the authorship requirements. Those will be sent to you at a later time.

<!--[if !supportLists]-->4.ï¿½ï¿½ï¿½ï¿½ï¿½ï¿½ <!--[endif]-->You will also need to sign off on the final version of the manuscript.

ï¿½

Again, thank you for your time in this important process.

ï¿½

Warm regards,

ï¿½

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From: (b) (6)
To: (b) (6) christopher.broder(b)(6) (b) (6)

Subject: Re: Revised Nipah R&D roadmap manuscript--please respond by June 25
Date: Monday, June 24, 2019 1:19:03 PM
Attachments: Nipah Roadmap manuscript.June 2019,V3 (b)(6).docx

---

(b) (6)

Thanks for the update and very happy to see this work move ahead in a manuscript forma.

I've attached a couple of minor comments, in case they are useful.

I agree to coauthor this work and attest that I have made significant contributions to the content of the roadmap, its interpretations, and have contributed to and reviewed this draft.

Best,
(b) (6)

From: (b) (6)
Sent: Sunday, June 23, 2019 3:10:33 PM
To: christopher.broder(b)(6) (b) (6)
Cc: (b) (6)
Subject: FW: Revised Nipah R&D roadmap manuscript--please respond by June 25

Dear all. Please read the email below. This is just a reminder to send me any comments on the June 2019 version of the Nipah roadmap paper and to confirm that you are willing to be a co-author on the manuscript as outlined below.

Thank you so much for your time!

Warm regards,

(b) (6)

From: (b) (6)
Sent: Saturday, June 15, 2019 1:26 PM
To: christopher.broder(b)(6) (b) (6)
Cc: (b) (6)
Subject: Revised Nipah R&D roadmap manuscript--please respond by June 25
Importance: High

Dear Colleagues: I hope this message finds each of you well. Per the email that you received in May, I have provided an updated version of the Nipah R&D roadmap manuscript. I have also provided the full roadmap—just in case you want to refer back to that.

Our current plan is to submit this to the NEJM as a Sounding Board article per advice from (b) (6). They will also provide a Supplemental Appendix with the full roadmap. WHO will then publish the roadmap once it has been published in NEJM.

Sounding Board articles have a 2,000 word limit and are structured as commentaries rather than as reviews, so the article has been substantially shortened—please keep that in mind as you go through it.

The process for submission is as follows:

1. Please review this version of the manuscript and **send any comments or edits to me by June 25** (through replying to this email). Please send a response even if you have no comments.
2. In your email response, also please state that you are willing to be a co-author on the manuscript and that you agree you fulfill the criteria for authorship, which for NEJM are:
    - Substantial contributions to conception and design; or acquisition, analysis, or interpretation of data AND
    - Drafting of the article or critical revision for important intellectual content AND
    - Final approval of the version to be published AND
    - Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the article are appropriately investigated and resolved
3. If NEJM decides to request a revised version and move to the next step of accepting the manuscript, there will be three forms each co-author will need to complete—one on conflict of interest, one on copyright transfer, and one stating that you fulfill the authorship requirements. Those will be sent to you at a later time.
4. You will also need to sign off on the final version of the manuscript.

Again, thank you for your time in this important process.

Warm regards,

(b) (6)
[redacted]

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From: (b) (6)
To: christopher.brode (b) (6)
Cc: (b) (6)
Subject: FW: Revised Nipah R&D roadmap manuscript--please respond by June 25
Date: Sunday, June 23, 2019 3:11:07 PM
Attachments: Nipah Roadmap manuscript.June 2019,V3.docx
Nipah RD Roadmap.January2019.docx
Importance: High

Dear all. Please read the email below. This is just a reminder to send me any comments on the June 2019 version of the Nipah roadmap paper and to confirm that you are willing to be a co-author on the manuscript as outlined below.

Thank you so much for your time!

Warm regards,

(b) (6)

From: (b) (6)
Sent: Saturday, June 15, 2019 1:26 PM
To: christopher.brode (b) (6)
Cc: (b) (6)
Subject: Revised Nipah R&D roadmap manuscript--please respond by June 25
Importance: High

Dear Colleagues: I hope this message finds each of you well. Per the email that you received in May, I have provided an updated version of the Nipah R&D roadmap manuscript. I have also provided the full roadmap—just in case you want to refer back to that.

Our current plan is to submit this to the NEJM as a Sounding Board article per advice from (b) (6). They will also provide a Supplemental Appendix with the full roadmap. WHO will then publish the roadmap once it has been published in NEJM.

Sounding Board articles have a 2,000 word limit and are structured as commentaries rather than as reviews, so the article has been substantially shortened—please keep that in mind as you go through it.

The process for submission is as follows:

1. Please review this version of the manuscript and **send any comments or edits to me by June 25** (through replying to this email). Please send a response even if you have no comments.

2. In your email response, also please state that you are willing to be a co-author on the manuscript and that you agree you fulfill the criteria for authorship, which for NEJM are:

    - Substantial contributions to conception and design; or acquisition, analysis, or interpretation of data AND
    - Drafting of the article or critical revision for important intellectual content AND
    - Final approval of the version to be published AND
    - Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the article are appropriately investigated and resolved

3. If NEJM decides to request a revised version and move to the next step of accepting the manuscript, there will be three forms each co-author will need to complete—one on conflict of interest, one on copyright transfer, and one stating that you fulfill the authorship requirements. Those will be sent to you at a later time.
4. You will also need to sign off on the final version of the manuscript.

Again, thank you for your time in this important process.

Warm regards,

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From: (b) (5)
To: (b) (5) ; Broder, Christopher
Subject: Fwd: Thank you for your submission to Wolters Kluwer Health: Medical Practice
Date: Saturday, March 16, 2019 2:04:05 AM
Attachments: Fields-Chapter 14.WKMEDPRAC-S-19-00583.pdf

It's done! Here's the entire pdf file.

I'm worried that only one of our tiff figures appear to pass their initial QC, but we'll wait till we hear back from them since we haven't even seen the professional rendering that is required for the 4 figures we requested help on.

Phew.

(b) (5)

Begin forwarded message:

From: (b) (5)
Subject: Thank you for your submission to Wolters Kluwer Health: Medical Practice
Date: March 16, 2019 at 1:56:48 AM EDT
To: (b) (5) >
Reply-To: (b) (5)

16 Mar 2019

RE: Chapter 14 by Professor Benhur Lee for Field's Virology: Emerging Viruses

Dear (b) (5)

Your submission entitled "Chapter 14" has been received by the editorial office.

You may check on the progress of your submission at any time by logging in to Editorial Manager as an author.

https://www.editorialmanager.com/wkmedprac/

Your username is: (b) (5)
(b) (5)

You will be notified of the reference number assigned to your submission in due course. Please note that we have the following information on file for you and any coauthors on file. Please verify if this information is correct. If this is not correct, please respond and provide the correct information.

1. Professor (b) (5), MD
Secondary Name:

E-mail Address: (b) (5)
Position: Ward-Coleman Chair in Microbiology
Institution: Icahn School of Medicine at Mount Sinai
Department: Microbiology
Street Address: (b) (5)
City: New York
State: NY
Zip or Postal Code: 10001
Country or Region: UNITED STATES

2. Professor (b) (5) PhD
Secondary Name:
E-mail Address: (b) (5)
Position: Professor and Chair
Institution: Uniformed Services University of the Health Sciences
Department: Microbiology and Immunology
Street Address: 4301 Jones Bridge Rd
City: Bethesda
State: MD
Zip or Postal Code: 20814-4799
Country or Region: UNITED STATES

3. Professor (b) (5), PhD
Secondary Name:
E-mail Address: (b) (5)
Position: Professor and Director
Institution: Duke-NUS Medical School
Department: Programme in Emerging Infectious Diseases
Street Address: 8 College Road
City: Singapore
State: Singapore
Zip or Postal Code: 169857
Country or Region: SINGAPORE

Thank you for submitting your work to Wolters Kluwer Health: Medical Practice.

Kind Regards,

Wolters Kluwer Health: Medical Practice Editorial Office

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From: (b) (6)
To: (b) (6)
CHRISTOPHER BRODER; (b) (6)
Cc: (b) (6)
Subject: Re: Project Order, PDP, and Project drafts
Date: Friday, October 8, 2021 3:40:30 PM
Attachments: AD3C.2 Project_1_10.8.21_Aims_Plan_Biblio.docx
SA and Research Strategy- Henipavirus-UAB-7October21.docx
Admin Core SA Research Strategy Oct 7 Final.docx
P21.0410 Screening Core (Specific Aims-Research Strategy-References) FinalV2.docx

---

Hello all,

I'm sending drafts for Project 1: coronavirus and Project 2: Henipavirus. As well as the screening core (SC) and admin core for your input. I will send drafts from Overall and Med Chem Core, once they can incorporate the remaining two project. If you can get comments back by Tuesday, Oct. 12 at the latest, that would be helpful.

Also, just to make sure we are consistent with our core titles:
Administrative Core (Admin Core)
Screening Core (SC)
Medicinal Chemistry Core (MCC)
Structural Biology Core (SBC)

Thank you,

(b) (6)

From: (b) (6)
Date: Tuesday, October 5, 2021 at 2:55 PM
To: '(b) (6)
CHRISTOPHER BRODER (b) (6)

(b) (6)

Cc: "(b) (6)

Subject: Re: Project Order, PDP, and Project drafts

Hello all,

Please find the current draft of the Structural Biology Core from Dr. (b) (6) and team. The file is quite large - please find the file here.

Please let me know if you have questions or need help sending edits back to him.

Thank you,

(b) (6)

From: (b) (6)

Sent: Monday, October 4, 2021 3:57:21 PM

To: (b) (6)
; CHRISTOPHER BRODER; (b) (6)

Cc: (b) (6)

Subject: Project Order, PDP, and Project drafts

Hello all project and core leaders,

Thank you for your continued progress. We have a few immediate items to bring to your attention.

While working on the overall specific aims, it became necessary to reorder the projects. Therefore, here is the new project order:

1. Coronavirus
2. Henipavirus
3. Flavivirus
4. Bunyavirus
5. Alphavirus

(b) (6) can help address the change of numbers on the documents and will change the component order in ASSIST to reflect the new project order.

Another point we want to address is that we would like to use a product development profile (PDP) within the "overall" section to apply to all projects. **We would like the attached paragraph that references the PDP to be added to all projects and for you to include a timeline for your project please refer to Bunya write up for an example timeline**.

Finally, please see the attached current versions of Alpha and Bunya projects. We would appreciate any comments or edits by **Thursday, Oct. 7** that we can share with them. The other project drafts will be sent as they are received.

Thank you,

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From: (b) (6)
To: (b) (6) Broder, Christopher; (b) (6)
Cc: (b) (6)
Subject: Nipah Task Force (confirmed)
Attachments: CEPI Nipah Task Force_September 2021_final.pdf

Dear Members of CEPI's Task Force on Nipah Standards, Assays & Animal Models,

Please find the attached pre-reads ahead of our call next week. We look forward to our discussions.

Best wishes,
(b) (6)

Topic Presenter Allocated time Objective
Introduction & welcome (b) (6), CEPI 5 min
Updates from henipavirus-affected countries (b) (6), Australia
(b) (6), Bangladesh
(b) (6), India
(b) (6), Malaysia
10 min each
+ 5 min Q&A To gain visibility on recent henipavirus work and on how COVID-19 has affected R&D in country
NiV antibody standard development I (b) (6), University of Malaya 10 min To share information on objectives, including NiV assays in the pipeline
NiV antibody standard development II (b) (6), NIBSC 10 min To gain visibility on the plans and timelines for the feasibility study leading to the development of an international NiV antibody standard
10 min Q&A on antibody standard development
Natural history studies (b) (6), CEPI 10 min To share information/updates on objectives, status and expected timelines.

Updates from CREID network (b) (6), NIAID 10 min
Updates from CEPI Epi RfP (b) (6), CEPI 10 min
5 min Q&A on CEPI & NIAID initiatives
Action items & closing (b) (6), CEPI 5 min

Microsoft Teams meeting
Join on your computer or mobile app
Meeting options

Sensitivity: CEPI Internal

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From: (b) (6)
To: (b) (6)
Cc: (b) (6) Broder, Christopher; Eric Laing; (b) (6)
Subject: Re: Updated draft of Nipah R01 proposal
Date: Sunday, May 16, 2021 3:41:51 PM
Attachments: Nipah R01 pitch 2021 05 16 sl.docx

Hi (b) (6)

It is exciting to see this develop further. I downloaded the Google doc, so that I had the most recent version, but then made my comments in the attached MS Word document.

(b) (6)

(b) (6) wrote on 5/1/2021 10:49 PM:

> Dear colleagues,
>
> Attached is a new version of the science proposal. Your comments on the last draft were great and I've made the suggested changes or written a response. I have proposed sample sizes using prior data and assumptions to do power calculations, plus a little bit of brainstorming about logistical effort. Please make or suggest changes as you see fit, especially when it comes to staying within our budget.
>
> I still have some edits to do on the inferential models section of the Aim 3 strategy, but that isn't vital to budgeting, so I will get to it tomorrow.
>
> The main document is on Google Docs in case folks would prefer to do their comments there as I continue to make changes.
>
> (b) (5)
>
> All the best,
> (b) (6)

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From: (b) (6)
To: (b) (6) Eric Laing; (b) (6)
Cc: Broder, Chris (b) (6)
Subject: Statement of work for Munster Lab; ethics approval
Date: Tuesday, November 3, 2020 1:17:12 PM
Attachments: IDCRP-085_CORE_AM14_Approval_20201016.pdf
IDCRP-085_CORE_Protocol_v11_EIRB v1.15_20200914.pdf
IDCRP-085_CORE_Adult_ICD_HIPAA_MTF Inpatient_v2.0_20200715_STAMPED.pdf
IDCRP-085_CORE_Adult_ICD_HIPAA_MTF Outpatient_v2.0_20200715_STAMPED.pdf
IDCRP-085_CORE_Adult_ICD_HIPAA_Online_v2.0_15JUL2020_STAMPED.pdf
IDCRP-085_CORE_Assent_v4.0_15JUL2020_STAMPED.pdf
IDCRP-085_CORE_Parental ICD_v4.0_20200715_STAMPED.pdf

Dr (b) (6) and all,

We are enthused to start this collaboration on the EPICC project. See below for the next two steps:

1. Below is a description of the proposed scope of this work so we can get the agreements in place to send specimens and data to the (b) (6) Lab. Is the below accurate?
    a. IDCRP/USU sends up to 500 uL of sera from 50 subjects and associated metadata from the EPICC study.
    b. (b) (6) Lab perform wild type MERS, SARS-CoV-1 and SARS-COV2 neutralization on up to 50 SARS-CoV-2 positive sera with and without cross binding to MERS and SARS-1 (as determined by the (b) (6) lab) with an objective to examine how cross-binding correlates with wildtype cross-neutralization.
2. For your NIH NHSR determination, please find the protocol, CV, and ICF attached.

Thanks, let me know if you have any questions

(b) (6)

[redacted]

From: (b) (6)
Sent: Wednesday, October 21, 2020 11:54 AM
To: (b) (6) Eric Laing
(b) (6)

Cc: 'Broder, Chris (b) (6)

Subject: RE: nhps and CoV-2 question

Hi all

Following up on the below:

1/ See current protocol and IRB approval to inform NIAID NHSR determination

2/ CCing in (b) (6) for agreements

3/ (b) (6) : to confirm, is the proposed project synopsis essentially wt VNT of n = ?25 SARS-CoV-2 positive sera with and without cross binding to MERS and SARS-1 with an aim to examine how cross-binding correlates with wildtype cross-neutralization?

Thanks

(b) (6)

From: (b) (6)
Sent: Monday, October 5, 2020 7:59 AM
To: (b) (6) Eric Laing (b) (6)
Cc: Broder, Chris (b) (6)
Subject: RE: nhps and CoV-2 question

Dr (b) (6) ,

I am one of the AIs on the EPICC study and the director of the COVID19 research area at IDCRP. Pleased to meet you and looking forward to this EPICC subproject. Dr (b) (6) is the EPICC PI and deputy science director of IDCRP and (b) (6) the CRM of EPICC. (b) (6) is our IDCRP Agreements Officer (among other roles). Below are the relatively simple next logistic steps required to execute this really interesting serology subproject.

1. Ethics review (NIAID NHSR determination) - Attached is the EPICC protocol v 9 for your preliminary review. I will send ICF and protocol for version 10. (b) (6) are you able to send the relevant DoD determination?

2. I discussed with (b) (6) and this should just require a SLA between USU and NIAID. We can initiate this on our side.

3. Study synopsis – for the purposes of #2, we'd need a brief synopsis. (b) (6) , this sounds like wt VNT of n = ?25 SARS-CoV-2 positive sera with and without cross binding to MERS and

SARS-1 with an aim to examine how cross-binding correlates with wildtype cross-neutralization?

Happy to assist on all the above as needed

(b) (6)

[redacted]

From: (b) (6) [redacted]
Sent: Tuesday, September 29, 2020 3:09 PM
To: Eric Laing (b) (6) [redacted]
Cc: Broder, Chris (b) (6) [redacted]
Subject: [EXTERNAL] RE: nhps and CoV-2 question

Thanks (b) (6),

Sounds good, would be easy to determine any neutralizing cross-reactivity with MERS, SARS-2/2

Cheers,

(b) (6) [redacted]

From: Laing, Eric (b) (6) [redacted]
Sent: Tuesday, September 29, 2020 12:42 PM
To: (b) (6) [redacted]

(b) (6)
Cc: Broder, Chris (b) (6)
Subject: Re: nhps and CoV-2 question

Hi (b) (6),

Looping back to this topic regarding MERS/SARS-CoV VNs/PRNTs and bringing in the IDCRP leadership that can begin an approval process for non-human research determination.

We have a handful of SARS-CoV-2 IgG positive serum samples (~15 - 20) with fairly high cross-reactivity to SARS-CoV-1 and MERS-CoV. Some of these serum samples are longitudinal collections from the same subject; over time, the cross-reactivity decreases as the IgG response matures and becomes monotypic for SARS-CoV-2. It would be interesting to write a short letter about cross-reactivity and cross-neutralization between SARS-CoV-2 IgG antibodies and high priority zoonotic bCoVs.

(b) (6)

On Thu, Sep 10, 2020 at 9:47 AM (b) (6) wrote:

> Hi (b) (6)
>
> Would be easy to do some MERS and SARS-CoV-2 VNs or prnts.
>
> Cheers,
>
> (b) (6)

From: Laing, Eric (b) (6)
Sent: Wednesday, September 9, 2020 8:34 PM
To: (b) (6)
Cc: Broder, Chris (b) (6)
Subject: Re: nhps and CoV-2 question

Hi (b) (6),

Any interest or bandwidth for testing SARS-CoV-2 patient serum samples that are highly cross-reactive with MERS-CoV and SARS-CoV-1 spike proteins to assess cross-neutralization potential? We have a handful of SARS-CoV-2 seroconverts that have a strong polyclonal response to SARS-CoV-1 and MERS-CoV, could be an interesting short translational letter if the antisera retains neutralizing antibodies.

(b) (6)

On Fri, Sep 4, 2020 at 9:42 AM (b) (6) wrote:

> Hi (b) (6) no plans for that,
>
> Housing the animals for 6 months after challenge into high containment would make it unfeasible. There are reports of back challenge after a month or so, but I think we should start getting data from humans soon,
>
> Cheers,
>
> (b) (6)

(b) (6)

From: "Broder, Christopher"(b) (6)
Date: Thursday, September 3, 2020 at 10:01 AM
To: (b) (6)
Cc: Eric Laing (b) (6)
Subject: nhps and CoV-2 question

hi (b) (6) .

hope all is continuing to go so great.

are you all going to try an NHP SARS-2 back challenge exp after
waiting 6 or more months after primary infection and recovery? so expensive i know,
might provide very informative data on the ab response / longevity / anamnestic response ect..

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From: (b) (6)
To: Broder, Christopher
Cc: (b) (6)
Subject: Master Endnote file
Date: Friday, February 15, 2019 4:17:30 PM
Attachments: HNV_Fields Ref.enlp.zip

O.K, I hope this is what you are asking for.

This is the Endnote "Package" file (i.e. it is self-contained **".enlp"** that does not require accompanying "Endnote Library.Data Folders", rdg, tdb, etc.). It is NOT the default ",enl" endnote file which Endnote generates.

Feel free to search directly from this **.enlp** file to add anything that is not here. Right now, it has 1,193 ref with no duplications and contains PubMed Refs with Nipah OR Hendra in any field.

Thanks!

(b) (6)

[redacted]

[redacted]

[redacted]

On Feb 15, 2019, at 10:21 AM, Broder, Christopher (b) (6) wrote:

Do we need to add edits to the WORD file they sent us? As you ([redacted]) have done?

I was under the impression that we need to make a new WORD file of the Text body, there
will be so many edits and sections and reff changes.

My sections are considerably re-written. As soon as I get a master Endnote file from you
I will start putting in all the correct reffs to the sections i am writing now/.

On Thu, Feb 14, 2019 at 9:18 PM (b) (6)

wrote:

> Dear (b) (6),
>
> See attached with all my edits and changes of the text.
>
> I will look into the Endnot files later …. Has to go to meetings now.
>
> Thanks
>
> (b) (6)
>
> [redacted]

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From: (b) (6)
To: christopher.brode (b) (6)
Cc: (b) (6)
Subject: Nipah virus R&D roadmap and manuscript as submitted to WHO for final clearance
Date: Friday, January 11, 2019 8:05:42 PM
Attachments: Nipah RD Roadmap.January2019.docx
Nipah Roadmap manuscript.20190111.docx

Dear Nipah virus R&D taskforce members:

First, thanks to all of you who reviewed the manuscript and roadmap and provided input. We very much appreciate your thoughtful comments and time.
We have incorporated your feedback and have sent the documents to WHO for their final review. I have attached them here as an FYI. The roadmap contains track changes so our WHO colleagues can see the edits and make final decisions regarding the content.

We hope that that the roadmap will be published in the next few weeks and that we can submit the manuscript for publication soon. We're currently planning to submit to Lancet ID, but the final decision and next steps rest with WHO. We will likely need you to sign off on the manuscript once it is accepted for publication.

We'll keep you posted as this effort progresses.

Again, thank you for your time and support.

Kind regards,

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From: (b) (6)
To: christopher.brode (b) (6)b) (6)
Cc: (b) (6)
Subject: Next version of the Nipah virus R&D roadmap and manuscript--please review changes by January 7
Date: Wednesday, December 19, 2018 12:57:13 PM
Attachments: Nipah Roadmap manuscript.V2.docx
Nipah RD Roadmap.December2018.docx
Importance: High

Dear Nipah virus R&D taskforce members: Thank you to those who submitted comments on the versions that were sent earlier. I have attached a revised manuscript and a slightly revised roadmap; both incorporate feedback that we received.

Please take another look, if you have time. IF WE DON'T HEAR FROM YOU BY JANUARY 7, WE WILL ASSUME THAT THESE VERSIONS ARE ACCEPTABLE TO YOU.

After that date, I will forward the manuscript and roadmap to WHO for their final review and approval process. Any changes to the roadmap will be at the discretion of WHO. They will also be moving the manuscripts forward. We'll keep you posted on that process. I assume that, at some point, we'll need to obtain an authorship form from you, but that can wait for now.

Thank you again for your time and effort regarding this important work!

Happy holidays to all of you!

Warm regards,

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From: (b) (6)
To: Broder, Christopher; (b) (6)
Subject: RE: CEPI and CETR U19
Date: Tuesday, December 18, 2018 4:26:25 PM
Attachments: CEPI Nipah Full Proposal FINAL.pdf

Hi (b) (6),

Please find attached the Final Nipah vaccine Technical Proposal submitted by Profectus to CEPI. At the request of our attorney I have deleted any reference to the cost of the program and have marked the document Profectus confidential. As you correctly point out, the Specific Aims of the CEPI and NIAID contracts are completely different, with the exception of developing your Cedar virus-based assay. Let me know if this is adequate to address all overlap concerns.

We will get you BioSketches with updated other support by COB tomorrow.

Best,

(b) (6)

From: Broder, Christopher (b) (6)
Sent: Thursday, December 13, 2018 1:02 PM
To: (b) (6)
Subject: CEPI and CETR U19

hi (b) (6) (b) (6) cc'd)

NIAID was satisfied with my revised SAC for the CETR U19. We are uploading today. I will send another msg to all partners today.

(b) (6) **(here) will be reaching out to our CETR partners (PIs and their POCs) for JIT other support docs of all**
key personnel today/tomorrow.

(b) (6) sent msg below this AM. This specifically relates to only me (USU), Profectus and UTMB - as we are all parties to the CEPI-funded Nipah vaccine
so you both as key personnel need to put together your other support pages. and address any overlap more specifically.

Scientifically. as far as the Profecus CETR project (RP1) goes (and UTMB/GNL) (Core C) there is no scientific overlap with CEPI project (the goals and proposed studies are totally different.
CEPI project is just gearing up HeV-sG production as GMP material and alum vaccine formulation and assay development, and clinical trials.

There is only slight overlap with USU CETR project (RP3) regards to making just two Cedar chimeric viruses which i will address.

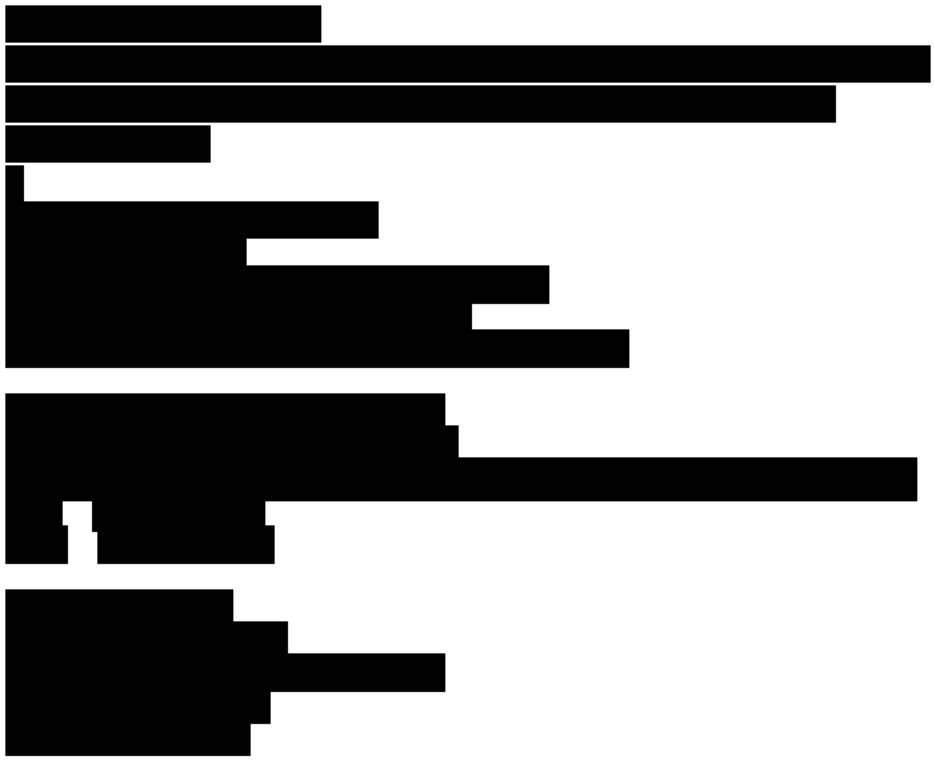
(b) (6), as you can see from the msg from NIAID, I need the final funded CEPI application for them., Is this possible? ASAP.

I will be email (b) (6) back shortly and give him a heads up.

v/r
(b) (6)

Hi (b) (6),

We are aware that you and additional key personnel of your proposed CETR are engaged in a recently funded CEPI project to develop a Nipah virus vaccine. At this stage of CETR payplan processing, we need to assess potential scientific and budgetary overlap between the CEPI and CETR projects. Accordingly, please provide a copy of the funded CEPI application (uploaded as a JIT document vie eCommons) as soon as possible. Feel free to contact me if you have any questions.
Regards,
(b) (6)



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From: (b) (6)
To: (b) (6)
Cc: christopher.brode (b) (6)

Subject: Re: Nipah R&D roadmap and draft manuscript
Date: Monday, December 10, 2018 8:34:32 AM
Attachments: Nipah Roadmap manuscript.V1.2(b) (6).docx
ATT00002.bin
Nipah TF members.degrees and affiliations (b) (6) .docx
ATT00004.bin
Importance: High

(b) (6), MD, MPH

Medical Director

Dear Prof. (b) (6):

Sorry for responding late. I had been traveling therefore could not send my comments.

I have gone through the manuscript on the roadmap for R & D on Nipah. It is well-written and provides a comprehensive coverage of the topic. I have a few suggestions, indicated in-line in the attached manuscript.

With best regards,

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From: (b) (6)
To: (b) (6)
Cc: christopher.brode (b) (6)

Subject: RE: Nipah R&D roadmap and draft manuscript
Date: Tuesday, December 4, 2018 2:24:41 PM
Attachments: Nipah Roadmap manuscript.V1.2 (b) (6).docx
Nipah TF members.degrees and affiliations (b) (6).docx

Dear (b) (6),

I agree that the manuscript reads well. I've attached a couple of minor comments and edits and my affiliation information.

I also agree with (b) (6)'s suggestion about how to address the limitations for development of therapeutics.

All the best,
(b) (6)

From: (b) (6)
Sent: Friday, November 30, 2018 10:08 PM
To: (b) (6)
Cc: christopher.broder (b) (6)
Subject: Re: Nipah R&D roadmap and draft manuscript

(b) (6),

The manuscript reads well. Thanks for all of your effort in pulling these ideas together and communicating this so clearly.

I agree with the Wellcome reviewer that there is some risk of favoring one therapeutic by including a specific milestone to advance it.

If the R&D roadmap is successful in in catalyzing the development of multiple therapeutic agents a limiting constraint to assessing their effectiveness will be the number of patients with Nipah who can be enrolled. A broader discussion on how best to allocate such a scarce resource seems more appropriate than assuming that we should focus all resources on the first available agent.

Personally, I would favor an R&D roadmap for therapeutics that noted this issue, and proposed an approach to engage in a broad open conversation on how we allocate multiple scarce resources, donor interest, donor funding, and patients identified early enough to be candidates for therapeutic

trials.

I have attached my identifying information.

(b) (6)

(b) (6) wrote on 11/20/2018 2:07 AM:

Dear Nipah R&D Roadmap Taskforce members:

On behalf of CIDRAP, Wellcome, and WHO, attached please find the final version of the Nipah R&D Roadmap, a draft manuscript for publication, and a table for you to complete with personal information.

- The roadmap is undergoing final review and clearance at WHO.
- The draft manuscript is also being reviewed by WHO; their edits are not yet reflected in this version, so it may change. Before submission, once we have incorporated all comments, we will send a final version to you again for your approval and sign off.

Here are some high-level thoughts regarding the manuscript:

- We thought it was essential to provide some context for where things stand with the current status of diagnostics, therapeutics, and vaccines, so we added a few short paragraphs in order to provide this perspective. This was done for the MERS-CoV roadmap and we all agreed that this was the right approach.
- We also decided to highlight some of the key challenges and barriers, as we did in each of the roadmaps—so we used our best judgement to focus on what we thought were the most critical issues. We also did quite a bit of reworking to put the material into a more readable format.
- With regard to the roadmap itself, we decided to include tables of the goals and milestones, since it wasn't possible to cover everything and these seem to be the most critical. We should be able to provide a link to the full roadmap once it's posted, assuming each is posted before publication.
- We decided that the manuscripts should be no more than about 3,500 words, so we aimed for that length.

We would very much appreciate your help by doing the following:

1. Review the manuscript and submit your comments via track changes on the Word document.
2. Complete the attached table, so you are cited correctly in the manuscript.
3. If, as you review the manuscript, you find anything glaring that needs to

change in the roadmap, please let us know. We still have time to make minor changes to the roadmap. Again, you can use track changes on the Word document of the roadmap.

Please also address the following question from Wellcome regarding the roadmap: For Nipah therapeutics we mention “By 2020 generate a reliable source of m102.4 to be used in clinical trials. We have not mentioned a specific vaccine or therapeutic for Lassa—is there a risk of promoting one candidate over others? This may be a redundant question since the Nipah field is far less advanced than other diseases.”

WE WOULD VERY MUCH APPRECIATE YOUR COMMENTS AND FEEDBACK BY MONDAY, DECEMBER 3.

Thank you again for your support of this important effort, and thank you in advance for reviewing the manuscript. Please send any comments to me at (b) (6) .

Warm regards,

(b) (6)

notified that you have received this communication in error and that any review, disclosure, dissemination, distribution or copying of it or its contents is prohibited. If you have received this communication in error, please notify me immediately by replying to this message and deleting it from your computer. Thank You.

B O H R N

The Bat / One Health Research Network

Overview

There is a long tradition of international cooperation in scientific research. Scientific networks can be instrumental to bridge cultural boundaries and build trust, addressing the global threat of emerging infectious diseases. Current trends in scientific research funding, specifically competition for ever-decreasing research budgets, necessitate international collaborations focused around specific and prioritized research questions.

Scientists posit that the Ebola outbreak of 2014 began with a Guinean toddler playing in a bat roost amongst fruit bats that had migrated 2,500 miles from Central Africa. Understanding bat migration patterns, the effect of humans on those patterns, and the challenges of conducting disease surveillance in free-range bat populations, will enable relevant policy makers to better identify, plan, and prepare for the next pandemic. Additionally, research coordinated networks have the ability to significantly impact threat reduction by identifying and prioritizing coordinated approaches to close these and other pressing knowledge gaps.

The Defense Threat Reduction Agency (DTRA) Biological Threat Reduction Program (BTRP) is sponsoring a multi-regional disease surveillance research coordinated network to mitigate the threat of bat-associated pathogens of security concern. This threat reduction network will identify and connect interdisciplinary expertise, convening an agile group to adapt to a wide spectrum of arising challenges and threats. The Bat / One Health Research Network (BOHRN) will enable shared learning and research opportunities, establish new research projects, and facilitate joint applications for funding; thus increasing the opportunity for peer review, especially if a cross-regional and multi-disciplinary team of authors are involved

The BOHRN kick-off meeting coincided with the 2nd International Symposium on Infectious Diseases of Bats in Fort Collins, CO on 29 June 2017. During this meeting, the group began preliminary actions to build a self-sustainable disease surveillance network and identified initial network objectives needed to develop a comprehensive research strategy to address bat-associated disease threats and mitigation solutions.

Network Objectives

- Facilitate interdisciplinary collaboration to identify research goals and needs for bat-borne disease research and broader threat reduction
- Create a common action plan that yields collaborative and sustainable projects which: (1) better inform policy makers; (2) better inform scientific community regarding funding targets and gaps in areas of research and development; (3) better define threat to global health security from bat-associated pathogens; and (4) improve national, regional, and global capacity to detect and respond to pathogens of security concern
- Enable better communication, coordination, and outreach at the research and conservation interface

Approach and Impact

BOHRN has four thematic focus areas, which were characterized and developed into research Working Groups at the kick-off meeting. These Working Groups (described below) will operationalize the network objectives by serving as subdivisions to the overall network to foster multi-national and multidisciplinary participation and mentorship. Each member of the BOHRN will identify with at least one Working Group based on field of research/practice. Working Group members will identify and prioritize research gaps and needs, and research project ideas will be solicited from BOHRN membership to address the identified gaps and needs.

Working Group 1: Host / pathogen biology interactions; specifically: (1) Bat physiology and immunology; (2) Bat pathogen community biology (e.g., co-infections and co-morbidities); and (3) Distribution of pathogens among species

Working Group 2: Pathogen surveillance, diagnostic capacity, and epidemiology; specifically: (1) Molecular epidemiology; (2) Distribution of pathogens geographically and phylogenetically; and (3) Detection, diagnosis, and reporting of bat-associated pathogens

Working Group 3: Ecology (bat, domesticated animal, and wildlife interface); specifically: (1) Bat behavior; (2) Domesticated animal and wildlife behavior, distribution, and movement impact; and (3) The effect of anthropogenic disturbance and modification on pathogen dynamics and spillover risks

Working Group 4: Human-bat interactions; specifically: (1) Hunting and commodity chain (e.g., bushmeat, guano, and pet trade); (2) Ecotourism; and (3) Interactions in human dwellings

Why Bats?

Bats act as natural reservoirs for over 60 pathogens, including some of the world's most deadly viruses, such as Nipah, Hendra, Marburg, and SARS viruses. Understanding the role of bats as a reservoir and the risk of pathogen transmission from bats to humans and other animals could be a key to discovering novel pathogens, mitigating the impact of emerging and re-emerging pathogens, and preventing future pandemics.

There are a number of factors which make bats unique disease reservoirs, including their social behavior, distinct physiology and metabolism, ability to travel long distances, nocturnal activity, species diversity, and long life span (10-20 years). These specific bat characteristics, coupled with the impact of human-mediated interactions and environmental changes, create research challenges to understanding the role of bats in global zoonotic disease ecology. BOHRN will create opportunities for policy makers, scientists, conservationists, funders, and students to identify community challenges, develop priority research lists and implement associated action plans that target needs and gaps. The opportunities created will work at all levels to build awareness of bat-associated disease burden and transmission risks and improve the prevention, detection, diagnosis, and reporting of pathogens of security concern.

U.S. DoD and Health Security

BOHRN outcomes will also support the Global Health Security Agenda (GHSA) Zoonotic Disease Action Package, which has a five-year target for countries to adopt measured behaviors, policies, and/or practices that minimize the spillover of zoonotic diseases from lower animals into human populations.

Although not directly involved in implementation, DTRA BTRP supports the GHSA goals and milestones, and synchronizes with GHSA country projects through the DoD GHSA Coordination Cell. DTRA BTRP is the DoD' premier biological nonproliferation division protecting the United States and its allies from especially dangerous pathogens by collaborating with partner countries and the international community to minimize the threat of deliberate, accidental, and natural infectious disease outbreaks through enhanced biosafety, security, and surveillance measures. DTRA BTRP investments build capacity to detect, diagnose, and report disease events and help reduce the magnitude and response costs of biological incidents.

Additionally, DTRA BTRP promotes scientific and technical collaborations among partner nations and the international community in the disciplines of biological safety, security, and surveillance to build constructive and sustainable international partnerships that address threats posed to health security. These science diplomacy-based activities engage scientists in peaceful application of biotechnology; building partner country disease surveillance capabilities; promoting adherence to international codes of conduct, security, and safety; and enhancing transparency and confidence building.

Although DTRA BTRP is committed to supporting BOHRN, there is no guarantee or obligation for DTRA BTRP to fund projects resulting from the network or its members.

Point of Contact

Dr. (b) (6), DTRA BTRP
Pacific Region Science Lead
(b) (6)

BHORN Website

https://www.bohrn.net

Fact Sheet References

Hayman, D. 2016. *As the Bat Flies*. Science 02 Dec 2016: Vol. 354, Issue 6316, pp. 1099-1100`
DOI: 10.1126/science.aaj1818

GHSA. 2017. *Global Health Security Agenda: Zoonotic Disease Action Package (GHSA Action Package Prevent 2)*. https://www.ghsagenda.org/packages/p2-zoonotic-disease

Kingston T., et. al. 2016. *Networking networks for global bat conservation. Bats in the Anthropocene: Conservation of Bats in a Changing World*. Springer Open.

Saez AM, et.al. 2014. *Investigating the zoonotic origin of the West African Ebola epidemic*. EMBO Mol Med. Vol 7, Iss 1:17-23. DOI: 10.15252/emmm.201404792

Quad Chart Instructions: Please fill out all four portions of the quad chart. The chart is read in a clockwise direction starting with the technical description. This activity is intended to provide a big picture overview and not an in-depth report of your research. Please limit the text to provide only the most important aspects of each quad. You will be given 5 minutes to present the information within this chart. Refer to the questions in each box for more guided assistance.

TECHNICAL DESCRIPTION AND OBJECTIVES

Briefly describe the research you are currently conducting and why. What questions are you trying to answer and what is the importance of this research to your field and the region? What are the specific aims of your research? What is currently known about your research? Consider the following:

1. **Objectives of research**
2. **Current state of understanding**
3. **Location of research (city/country and or coordinates)**

KEY PARTNERS AND REGIONS OF STUDY

Briefly describe the key partners and funders in your research. List the region(s) your research is being conducted in and any networks you are working with formally or informally. Consider the following:

1. **Who are the key partners involved in your research?**
2. **Who are the key funders involved in your research?**
3. **What region(s) is your research being conducted in?**
4. **Are you working with any networks (formally or informally)?**

MILESTONES, STATUS, AND CHALLENGES

Briefly explain the timeline of your research. When do you anticipate your research to be completed? Are there deliverables or steps along the way that will show substantial progress? Identify the challenges you will face. Consider the following:

1. **Provide timeline for delivery**
2. **Overview on project status**
3. **List challenges or needs in your research**

REGIONAL IMPACT

Describe the potential regional impact of your research. What will the impact be for the regional area? Will this lead to the need for future studies? Consider the following:

1. **Define the quantitative impact of project.**
2. **Define the regional impact.**

TECHNICAL DESCRIPTION AND OBJECTIVES

KEY PARTNERS AND REGIONS OF STUDY

MILESTONES, STATUS, AND CHALLENGES

REGIONAL IMPACT

From: (b) (6)
To: christopher.brode (b) (6)
Cc: (b) (6)
Subject: Invitation to the Bat One Health Research Network Workshop in Vienna
Date: Monday, October 29, 2018 11:57:55 AM
Attachments: PAOBOHRNFactSheet copy.pdf
BOHRN_QUADCHART[2].docx
image001.png

Dear Dr. (b) (6),

I wanted to follow up on your earlier discussion with Dr. (b) (6), the Southeast Asia Science Manager for the Defense Threat Reduction Agency, Biological Threat Reduction Program (formerly Cooperative Biological Engagement Program or CBEP), regarding the Bat One Health Research (BOHRN) Workshop in Vienna.

On behalf of Dr. (b) (6) we would like to extend an invitation for you to the BOHRN 1st Annual Workshop in Vienna, Austria on 8-9 November 2018. The two day meeting will be held at the Imlauer Wien hotel.

Established 2016, BOHRN convenes multi-disciplinary and One Health-focused scientists, policy makers, research scientists, and medical/veterinary practitioners with interests in bat-related research involving pathogens of security concern. The network builds on community standards and best practices for research. BOHRN identifies and shares information on research funding opportunities offered by multiple institutions. Most importantly, this network fosters international relationships among collaborators, agencies, and organizations, which can produce long-term, sustainable partnerships that withstand changes in government and organization budgets, priorities, postures, and policies. Please see the attached fact sheet for more information on BOHRN.

DTRA BTRP sponsors BOHRN a global networks that aims to characterize global threats of bat-borne pathogens and formalize community standards and conservation- conscious practices for One Health disease research. We hope to achieve the following objectives during the 1st Annual BOHRN Workshop:

(1) Facilitate a multi-disciplinary forum for discussion on research methods and practices to characterize bat-borne pathogen spillover threats

(2) Engage available funding program representatives with interests in conservation-conscious bat-borne pathogen research

(3) Characterize global research interests and priorities, and align them with network research focus areas and available funding programs

(4) Discuss upcoming planning opportunities

Attached you will find the network fact sheet; you can also visit www.bohrn.net for more information.

We have secured a hotel room for you the nights of 7-8 November at the Imlauer hotel in Vienna prior to the IMED meeting. In addition, **please find a quad chart attached, we ask that you please fill in the attached quad chart and return as soon as possible.**

Each participant will be asked to present the attached quad chart during the workshop day 1 event (about five minutes per chart). The information presented will be used to aid breakout group and large group discussions. Along with the quad chart, we are requesting that you send a picture of yourself. The quad charts and pictures will be printed to display around the room during the two day workshop.

Please let us know if you have any questions regarding this meeting or the quad chart.

Kind Regards,

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[redacted]

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From: (b) (6)
To: Broder, Christopher; (b) (6)
Subject: Table 14.3 Henipavirus Cell Tropism and Receptor Expression
Date: Sunday, February 17, 2019 12:07:46 AM
Attachments: Table 14.3.Henipavirus Cell Tropism.docx
TABLE 14-5-Formatted-(b) (6).docx
HNV_Fields Ref.enlp.zip

Hi Folks,

I forgot to send you my completed **Table 14.3** on HNV Cell tropism and Receptor Expression.

Please check the Table if you have time. Refs have been formatted. I will remove the name/date when we are ready to put everything together (since only Ref numbers are allowed in the Tables).

- Please see the **footnotes (a, b, c, d)** to the Table for an explanation of how to interpret the various items in the Table.
- It's obviously not comprehensive. I had to make judgement calls about what to put in and what to leave out. My criteria is for the table to be useful, so there is a combination of positive and negative data.
- It's divided into human and non-human cell lines/primary cell types, and then listed alphabetically.
- Let me know if I missed anything major.

(b) (6), can you put the reference number in (or ask your asst to do it ;-) for your tables. It'll take me too much time to replace all the (name/date) references that you have in your Tables by myself.

(b) (6), I was so pleased that I could format your Table 14.5 with no problems using the Master endnote file (**HNV_Fields Ref.enlp**). So, the system works! I have attached your formatted Table 14.5. Hopefully everything looks correct.

Finally, I have added the refs you all have asked me to. The Master endnote file I'm attaching to this email has **1,208 references**.

Thanks!

(b) (6)

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From: (b) (6)
To: (b) (6) ; Eric Laing; (b) (6)
Cc: Broder, Chris (b) (6)
Subject: RE: nhps and CoV-2 question
Date: Wednesday, October 21, 2020 11:53:54 AM
Attachments: IDCRP-085_CORE_AM14_Approval_20201016.pdf
IDCRP-085_CORE_Protocol_v11_EIRB v1.15_20200914.pdf

Hi all

Following up on the below:

1/ See current protocol and IRB approval to inform NIAID NHSR determination

2/ CCing in (b) (6) for agreements

3/ (b) (6): to confirm, is the proposed project synopsis essentially wt VNT of n = ?25 SARS-CoV-2 positive sera with and without cross binding to MERS and SARS-1 with an aim to examine how cross-binding correlates with wildtype cross-neutralization?

Thanks

(b) (6)

From: (b) (6)
Sent: Monday, October 5, 2020 7:59 AM
To: (b) (6) Eric Laing
(b) (6)
Cc: Broder, Chris (b) (6)
Subject: RE: nhps and CoV-2 question

Dr (b) (6),

I am one of the AIs on the EPICC study and the director of the COVID19 research area at IDCRP. Pleased to meet you and looking forward to this EPICC subproject. Dr (b) (6) is the EPICC PI and deputy science director of IDCRP and (b) (6) the CRM of EPICC. (b) (6) is our IDCRP Agreements Officer (among other roles). Below are the relatively simple next logistic steps required to execute this really interesting serology subproject.

1. Ethics review (NIAID NHSR determination) - Attached is the EPICC protocol v 9 for your preliminary review. I will send ICF and protocol for version 10. (b) (6) are you able to send the relevant DoD determination?

2. I discussed with (b) (6) and this should just require a SLA between USU and NIAID. We can initiate this on our side.

3. Study synopsis – for the purposes of #2, we'd need a brief synopsis (b) (6), this sounds like wt VNT of n = ?25 SARS-CoV-2 positive sera with and without cross binding to MERS and SARS-1 with an aim to examine how cross-binding correlates with wildtype cross-neutralization?

Happy to assist on all the above as needed

(b) (6)

[redacted]

From: (b) (6) [redacted]
Sent: Tuesday, September 29, 2020 3:09 PM
To: Eric Laing (b) (6) [redacted]
Cc: Broder, Chris (b) (6) [redacted]
Subject: [EXTERNAL] RE: nhps and CoV-2 question

Thanks (b) (6),

Sounds good, would be easy to determine any neutralizing cross-reactivity with MERS, SARS-2/2

Cheers,

(b) (6) [redacted]

From: Laing, Eric (b) (6) [redacted]
Sent: Tuesday, September 29, 2020 12:42 PM

To: (b) (6)

Cc: Broder, Chris (b) (6)

Subject: Re: nhps and CoV-2 question

Hi (b) (6),

Looping back to this topic regarding MERS/SARS-CoV VNs/PRNTs and bringing in the IDCRP leadership that can begin an approval process for non-human research determination.

We have a handful of SARS-CoV-2 IgG positive serum samples (~15 - 20) with fairly high cross-reactivity to SARS-CoV-1 and MERS-CoV. Some of these serum samples are longitudinal collections from the same subject; over time, the cross-reactivity decreases as the IgG response matures and becomes monotypic for SARS-CoV-2. It would be interesting to write a short letter about cross-reactivity and cross-neutralization between SARS-CoV-2 IgG antibodies and high priority zoonotic bCoVs.

(b) (6)

On Thu, Sep 10, 2020 at 9:47 AM (b) (6) > wrote:

> Hi (b) (6),
>
> Would be easy to do some MERS and SARS-CoV-2 VNs or prnts.
>
> Cheers,
>
> (b) (6)

(b) (6)

From: Laing, Eric (b) (6)
Sent: Wednesday, September 9, 2020 8:34 PM
To: (b) (6)
Cc: Broder, Chris (b) (6)
Subject: Re: nhps and CoV-2 question

Hi (b) (6),

Any interest or bandwidth for testing SARS-CoV-2 patient serum samples that are highly cross-reactive with MERS-CoV and SARS-CoV-1 spike proteins to assess cross-neutralization potential? We have a handful of SARS-CoV-2 seroconverts that have a strong polyclonal response to SARS-CoV-1 and MERS-CoV, could be an interesting short translational letter if the antisera retains neutralizing antibodies.

(b) (6)

On Fri, Sep 4, 2020 at 9:42 AM (b) (6) wrote:

> Hi (b) (6) no plans for that,
>
> Housing the animals for 6 months after challenge into high containment would make it unfeasible. There are reports of back challenge after a month or so, but I think we should start getting data from humans soon,
>
> Cheers,
>
> (b) (6)

(b) (6)

From: "Broder, Christopher"(b) (6)
Date: Thursday, September 3, 2020 at 10:01 AM
To: (b) (6)
Cc: Eric Laing(b) (6)
Subject: nhps and CoV-2 question

hi(b) (6) .

hope all is continuing to go so great.

are you all going to try an NHP SARS-2 back challenge exp after
waiting 6 or more months after primary infection and recovery? so expensive i know,
might provide very informative data on the ab response / longevity / anamnestic response ect..

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From: (b) (6)
To: (b) (6) Broder, Christopher; (b) (6)
Subject: proofs for mSphere paper
Date: Thursday, June 25, 2020 6:57:46 PM
Attachments: mSphere00602-20 (2) proof (b)(6).pdf
image007.png
image008.png
image009.png

Dear All,

Please find the proof for our mSphere paper, which will be published in the July/August issue of the journal. Kindly have a quick look for any last minute *editing* changes. The Editor is asking for a 48 hour turnaround; so I will send your compiled changes (if any) on Saturday 5pm, CET(Oslo). In addition:

1. (b) (6), please see the question on funding sources for cross-referencing and kindly advise.
2. (b) (6), please advise if anything else should be included in the Acknowledgements section.

Also, please note that the Editor will include an accompanying editorial piece. He is currently writing this.

Many thanks to all for the final push in this.

Best wishes,

(b) (6)

[redacted]

CEPI New vaccines for a safer world

[redacted]

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From: (b) (6)
To: (b) (6)
Cc: (b) (6) Broder, Christopher; (b) (6)
Subject: final version for mSphere
Date: Wednesday, June 10, 2020 9:00:10 AM
Attachments: Summary for mSphere_v9_FINAL_withproceedingslink.docx
image007.png
image008.png
image009.png

Dear (b) (6), Dear All,
Thank you all for your final comments.
All co-authors have now agreed to submit the attached version to *mSphere*. (b) (6) has kindly agreed to submit this and will let us know if anything else is needed.
Thanks again to all for your continued patience and contributions in this effort.
Best wishes,
(b) (6)

[redacted]

[redacted]

CEPI New vaccines for a safer world

[redacted]

[redacted]

[redacted]

From: (b) (6) [redacted]

Sent: onsdag 3. juni 2020 08:28
To: (b) (6)
Cc: (b) (6)
Broder, Christopher (b) (6)
Subject: Re: final version for mSphere
Importance: High

Thanks, (b) (6). I'm acknowledging receipt and will standby.

(b) (6)

On Jun 2, 2020, at 9:00 AM, (b) (6) wrote:

USE CAUTION: External Message.

Dear All,
I hope this email finds you well. Here is the latest version of our summary to *mSphere*. It has now incorporated ***all*** your comments. It has also undergone significant internal review at CEPI and should be considered the near final version. Could you please:

1. Review and indicate any ***major*** changes that absolutely need to be made prior to submission
2. Approve the authorship order and this near final version for submission

(b) (6) and I will follow up with *mSphere*. Apologies this has taken so long (nearly 6 months after the Nipah Conference!), but we have all been swamped with COVID-19 activities – and so have been most Nipah researchers, I understand…Kindly send your reply ***by this Friday, June 5.***
Best wishes,
(b) (6)

<image002.png>

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<Summary for mSphere_v8.docx>

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From: (b) (6)
To: (b) (6)
Cc: (b) (6) Broder, Christopher; (b) (6)
Subject: Re: final version for mSphere
Date: Wednesday, June 3, 2020 7:11:12 AM
Attachments: Summary for mSphere_v8 comments (b) (6) .docx
image002.png
image004.png
image006.png

Dear (b) (6),

Greetings from Bangladesh !!!

Thanks for sharing the final version. I have one issue to raise, please check the year of Nipah outbreak in Malaysia. To my knowledge is was in 1998.

I concur with the content and agree to be a co-author and is also in agreement with the authorship order.

Best regards,

(b) (6)

[redacted]

On Tue, Jun 2, 2020 at 7:00 PM Raul (b) (6) wrote:

> Dear All,
>
> I hope this email finds you well. Here is the latest version of our summary to *mSphere*. It has now incorporated ***all*** your comments. It has also undergone significant internal review at CEPI and should be considered the near final version. Could you please:
>
> 1. Review and indicate any ***major*** changes that absolutely need to be made prior to submission
> 2. Approve the authorship order and this near final version for submission
>
> (b) (6) and I will follow up with *mSphere*. Apologies this has taken so long (nearly 6 months after the Nipah Conference!), but we have all been swamped with COVID-19 activities – and so have been most Nipah researchers, I understand…Kindly send your reply ***by this Friday, June 5.***
>
> Best wishes,
>
> (b) (6)

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[redacted]

CEPI New vaccines for a safer world

[redacted]

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From: (b) (6)
To: (b) (6) Broder, Christopher;
(b) (6)
Cc: (b) (6)
Subject: RE: final version for mSphere
Date: Tuesday, June 2, 2020 7:50:21 PM
Attachments: Summary for mSphere_v8(b) (6).docx
image001.png
image002.png
image003.png

Hi (b) (6)

Thanks for sending through the final version. This article is looking great. I just found one grammatical error:
Page 2 – Lesson 3: it should be the "midst" of an epidemic (not mist). I have also captured this in the attached document for tracking changes.

I am happy with the authorship order.

Kind regards
(b) (6)

From: (b) (6)
Sent: Tuesday, 2 June 2020 11:00 PM
To: (b) (6)
; Broder, Christopher (b) (6)
Cc: (b) (6)
Subject: final version for mSphere
Importance: High

Dear All,
I hope this email finds you well. Here is the latest version of our summary to *mSphere*. It has now incorporated ***all*** your comments. It has also undergone significant internal review at CEPI and should be considered the near final version. Could you please:

1. Review and indicate any ***major*** changes that absolutely need to be made prior to submission
2. Approve the authorship order and this near final version for submission

(b) (6) and I will follow up with *mSphere*. Apologies this has taken so long (nearly 6 months after the Nipah Conference!), but we have all been swamped with COVID-19 activities – and so have been most Nipah researchers, I understand…Kindly send your reply ***by this Friday, June 5.***
Best wishes,
(b) (6)

CEPI New vaccines for a safer world

(b) (6)
[redacted]

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From: (b) (6)
To: (b) (6)
Cc: (b) (6) ; christopher.brode(b)(6)

Subject: RE: Revised Nipah R&D Roadmap and CPA table for taskforce member review
Date: Saturday, August 18, 2018 9:59:59 AM
Attachments: Nipah Roadmap August 2018 with track changes For Review(b) (6).doc
Nipah revised CPA table August 2018 for Review(b) (6).docx

Dear(b) (6)
Please see the comments in the attached file. My comments in Nipah roadmap document are combined with(b) (6)s one. For Nipah CPA table I included my comment with(b) (6) one.

Regards,
(b) (6)

From: (b) (6)
Sent: Friday, August 17, 2018 6:18 PM
To: (b) (6)
Cc: christopher.broder(b)(6)b) (6)
Subject: Re: Revised Nipah R&D Roadmap and CPA table for taskforce member review

Dear(b) (6):

Attached are my comments. I apologize that these are not combined with(b) (6)'s. I started working on this a couple of days ago, but only completed it after(b) (6)'s comments.

(b) (6)

(b) (6)

Wednesday, August 08, 2018 5:54 AM

Dear Nipah WHO R&D roadmap taskforce members:

Attached please find the following:

- A revised version of the Nipah WHO R&D roadmap, with track changes so you can see the edits that were made on the documents in response to discussions that took place at the July meeting in London. We DID NOT use track changes on the strategic goals and milestones, since these were substantially changed; the goals and milestones are highlighted in gray and we would like you to pay particular attention to those.
- A revised Critical Path Analysis (CPA) table that incorporates the new goals and

> milestones and also looks at critical dependencies among milestones, rather than looking at possible mitigation steps. Please be aware that this version is still in draft and the final version may not use this format.

To those of you who were able to attend the meeting in person, we're very grateful for you time and input—the meeting was extremely valuable. To those of you who could not be there, we're still very interested in your edits and written comments on this next version.

We would very much appreciate your review of the roadmap and the current version of the CPA table. Please send us any edits or comments that you have by **AUGUST 17, 2018.** If we don't hear from you by then, we'll assume that the current drafts are acceptable to you.

Once again, thank you so much for your ongoing involvement and participation in this important process. It's a pleasure to work with all of you.

Warm regards,

(b) (6)

communication in error, please notify me immediately by replying to this message and deleting it from your computer. Thank You.

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From: (b) (6)
To: (b) (6) ; christopher.broder(b) (6)
Cc: (b) (6)
Subject: RE: Revised Nipah R&D Roadmap and CPA table for taskforce member review
Date: Thursday, August 16, 2018 5:18:19 PM
Attachments: Nipah Roadmap.August 2018.with track changes.For Review_eg.docx

(b) (6),

Please find my comments on this draft attached.

Best,
(b) (6)

From: (b) (6)
Sent: Tuesday, August 7, 2018 8:24 PM
To: christopher.broder(b) (6)
Cc: (b) (6)
Subject: Revised Nipah R&D Roadmap and CPA table for taskforce member review

Dear Nipah WHO R&D roadmap taskforce members:

Attached please find the following:

- A revised version of the Nipah WHO R&D roadmap, with track changes so you can see the edits that were made on the documents in response to discussions that took place at the July meeting in London. We DID NOT use track changes on the strategic goals and milestones, since these were substantially changed; the goals and milestones are highlighted in gray and we would like you to pay particular attention to those.
- A revised Critical Path Analysis (CPA) table that incorporates the new goals and milestones and also looks at critical dependencies among milestones, rather than looking at possible mitigation steps. Please be aware that this version is still in draft and the final version may not use this format.

To those of you who were able to attend the meeting in person, we're very grateful for you time and input—the meeting was extremely valuable. To those of you who could not be there, we're still very interested in your edits and written comments on this next version.

We would very much appreciate your review of the roadmap and the current version of the

CPA table. Please send us any edits or comments that you have by **AUGUST 17, 2018.** If we don't hear from you by then, we'll assume that the current drafts are acceptable to you.

Once again, thank you so much for your ongoing involvement and participation in this important process. It's a pleasure to work with all of you.

Warm regards,

(b) (6)
[redacted]

Nipah WHO R&D Roadmap Taskforce Meeting

July 9 and 10, 2018

DAY 1	
8:00 am	**REGISTRATION**
	Session 1: Welcome, Introductions, and Overview
8:30 am	Welcome ([redacted] *[WHO] and* [redacted] *[Wellcome])*
8:35 am	Introductions ([redacted] *[CIDRAP])*
8:40 am	Overview and meeting objectives ([redacted] *[CIDRAP])*
	Session 2: Nipah Cross-Cutting Issues
8:50 am	Comments on current roadmap draft: cross-cutting issues
9:05 am	Milestones for cross-cutting issues
9:35 am	Critical path analysis for cross-cutting issues
10:15 am	**BREAK**
10:30 am	Critical path analysis for cross-cutting issues (cont.)
	Session 3: Nipah Diagnostics
11:20 am	Comments on current roadmap draft: diagnostics
11:30 am	**LUNCH**
12:15 pm	Milestones for diagnostics
12:45 pm	Critical path analysis for diagnostics
2:15 pm	**BREAK**
	Session 3: Nipah Therapeutics
2:30 pm	Comments on current roadmap draft: therapeutics
2:45 pm	Milestones for therapeutics
3:15 pm	Critical path analysis for therapeutics
	Session 4: Nipah Vaccines
4:45 pm	Comments on current roadmap draft: vaccines
5:00 pm	Milestones for vaccines
5:30 pm	**ADJOURN DAY 1**
6:00 pm	**DINNER**
DAY 2	
8:00 am	Welcome: Day 2 ([redacted] *[CIDRAP])*
8:05 am	Critical path analysis for vaccines
9:35 am	**BREAK**
	Session 5: Roadmap Implementation and Next Steps
9:50 am	Discussion: Roadmap implementation ([redacted] *[Wellcome]*)
11:50 am	Discussion: Publication process ([redacted] *[WHO])*
12:20 pm	Wrap up and next steps ([redacted] *[CIDRAP])*
12:30 pm	**ADJOURN DAY 2**
12:30 pm	**LUNCH**

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From: (b) (6)
To: christopher.brode (b) (6)
Cc: (b) (6)
Subject: Revised Nipah R&D Roadmap and Critical Path Analysis table for your review
Date: Wednesday, June 27, 2018 2:08:33 PM
Attachments: Agenda.Nipah R&D TF Meeting.final.062618.docx
Nipah Roadmap.with edits from public comment.062618.docx
Nipah.CPA table.for taskforce review. June 27.docx
Importance: High

Dear Nipah taskforce members,

We have revised the Nipah R&D draft roadmap based on comments received during the public comment period; I have attached it here for your review. The new edits are in track changes, so you can see them easily. You will also note that we have included a set of milestones for implementation under each main topic area (these are not in track changes). The milestones don't yet have dates attached to them—we need your input for that.

I have also attached a draft of the Critical Path Analysis (CPA) table that builds on the milestones.

We have structured the upcoming taskforce meeting to be relatively informal. Our primary goal is to walk through the roadmap and the CPA table in detail over the course of our time together. We very much need your input on these documents before we can move them forward.

These materials will form the basis of all of our discussions during the meeting, so please review them carefully before you come to London. I have attached a draft agenda as well, so you can see how the time will flow.

If you can't attend the meeting, we would still very much appreciate your review and hope that you can find time to send us some written comments.

Again, thank you so much for your time on this important project and I look forward to seeing you again in London!

Warm regards,

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From: (b) (6)
To: (b) (6) ; christopher.broder@(b) (6) [redacted]
Subject: 2nd TC with Experts to discuss therapeutic protocols for the current Nipah situation; 14:00 Geneva Time, May 25
Attachments: Nipah_Draft_Roadmap_publiccomment_MAY2018.pdf
WHO_NIPAH_baseline_situation_analysis_27Jan2018.pdf

Dear All,

Thank you very much for your time on the call today. A special thanks to our chair, Dr (b) (6), and to our country colleagues for their valuable updates.

As agreed, we are inviting you all for a second teleconference tomorrow, Friday 25 May 2018, at the same time (14:00-15:00 CET).
The call will be 60 min long and will mainly focus on the development of a PEP protocol. Dial in details below:

Please join my meeting from your computer, tablet or smartphone.
website).

Many thanks and looking forward to our discussion tomorrow,
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From: (b) (6)
To: (b) (6) ; christopher.broder(b)(6) (b) (6)
Cc: (b) (6)
Subject: RE: Revised Nipah virus R&D roadmap for taskforce review and comment--please send written comments by April 25
Date: Wednesday, April 25, 2018 4:30:47 PM
Attachments: Nipah Draft Roadmap.revised for taskforce review.April 2018(b) (6) edits.docx

Hello (b) (6),
I found the document very inclusive of the ideas discussed at the meeting. I don't have any substantive changes to suggest for the majority of the document but had a few suggestions for the vaccine section. The document is very nicely done.
(b) (6)

From: (b) (6)
Sent: Friday, April 13, 2018 5:19 PM
To: christopher.broder(b)(6) (b) (6)
Cc: (b) (6)
Subject: Revised Nipah virus R&D roadmap for taskforce review and comment--please send written comments by April 25
Importance: High

Dear Nipah Virus R&D Roadmap Taskforce members:

Attached please find a revised version of the Nipah virus R&D roadmap for your review. This version incorporates comments made during the in-person consultation in early March, comments we received after the consultation, and recent literature. We also consolidated all of the cross-cutting issues near the beginning of the roadmap.

We would very much appreciate your written feedback/comments on this version **no later than Wednesday April 25.**

We're sending this to you as a Word doc to make editing and commenting easier. We suggest that you cc the whole group when you provide edits/comments—to ensure that you all see each other's perspectives.

Once we incorporate your additional feedback, the next step will be to post the document for public comment. We also plan to begin working on a set of measurable and achievable milestones and will start considering ways to approach the critical path analysis.

Again, we very much appreciate your ongoing support and involvement with this important project.

Warm regards,

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From: (b) (6)
To: (b) (6) ; christopher.broder(b)(6)(b) (6)
Cc: (b) (6)
Subject: RE: Revised Nipah virus R&D roadmap for taskforce review and comment--please send written comments by April 25
Date: Wednesday, April 25, 2018 3:49:09 PM
Attachments: Nipah Draft Roadmap.revised for taskforce review.April 2018_(b)(6).docx

(b) (6),

Many thanks for your efforts to put this draft together. I think the draft does a great job of summarizing our conversations in London. I've included some comments and suggestions, attached.

All the best,
(b) (6)

From: (b) (6)
Sent: Friday, April 13, 2018 6:19 PM
To: christopher.broder(b)(6)(b) (6)
Cc: (b) (6)
Subject: Revised Nipah virus R&D roadmap for taskforce review and comment--please send written comments by April 25
Importance: High

Dear Nipah Virus R&D Roadmap Taskforce members:

Attached please find a revised version of the Nipah virus R&D roadmap for your review. This version incorporates comments made during the in-person consultation in early March, comments we received after the consultation, and recent literature. We also consolidated all of the cross-cutting issues near the beginning of the roadmap.

We would very much appreciate your written feedback/comments on this version **no later than Wednesday April 25.**

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Once we incorporate your additional feedback, the next step will be to post the document for public comment. We also plan to begin working on a set of measurable and achievable

milestones and will start considering ways to approach the critical path analysis.

Again, we very much appreciate your ongoing support and involvement with this important project.

Warm regards,

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From: (b) (6)
To: (b) (6)
Cc: christopher.brode (b) (6)
Subject: Re: Revised Nipah virus R&D roadmap for taskforce review and comment--please send written comments by April 25
Date: Wednesday, April 25, 2018 1:48:58 PM
Attachments: Nipah Draft Roadmap.revised for taskforce review.April 2018 (b) (6) .docx

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Hi (b) (6),

Attached are a few additional comments.

(b) (6)

(b) (6) Wednesday, April 25, 2018 8:17 AM

Dear (b) (6),

Greetings from Bangladesh !!!

Hope you all are doing fine.

Please find attached herewith the "Nipah Research and Development (R&D) Roadmap" with my comments in track change mode.

Best regards,

(b) (6)

(b) (6) Friday, April 13, 2018 3:19 PM

Dear Nipah Virus R&D Roadmap Taskforce members:

Attached please find a revised version of the Nipah virus R&D roadmap for your review. This version incorporates comments made during the in-person consultation in early March, comments we received after the consultation, and recent literature. We also consolidated all of the cross-cutting issues near the beginning of the roadmap.

We would very much appreciate your written feedback/comments on this version **no later than Wednesday April 25.**

We're sending this to you as a Word doc to make editing and commenting easier. We suggest that you cc the whole group when you provide edits/comments—to ensure that you all see each other's perspectives.

Once we incorporate your additional feedback, the next step will be to post the document for public comment. We also plan to begin working on a set of measurable and achievable milestones and will start considering ways to approach the critical path analysis.

Again, we very much appreciate your ongoing support and involvement with this important project.

Warm regards,

(b) (6)
[redacted]

or its contents is prohibited. If you have received this communication in error, please notify me immediately by replying to this message and deleting it from your computer. Thank You.

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From: (b) (6)
To: (b) (6)
Cc: christopher.brode (b) (6)
Subject: Re: Revised Nipah virus R&D roadmap for taskforce review and comment--please send written comments by April 25
Date: Wednesday, April 25, 2018 11:17:33 AM
Attachments: Nipah Draft Roadmap.revised for taskforce review.April 2018 Comments from Prof. (b) (6) .docx

---

Dear (b) (6),

Greetings from Bangladesh !!!

Hope you all are doing fine.

Please find attached herewith the "Nipah Research and Development (R&D) Roadmap" with my comments in track change mode.

Best regards,

(b) (6)

On Sat, Apr 14, 2018 at 4:19 AM, (b) (6) wrote:

> Dear Nipah Virus R&D Roadmap Taskforce members:
>
> Attached please find a revised version of the Nipah virus R&D roadmap for your review. This version incorporates comments made during the in-person consultation in early March, comments we received after the consultation, and recent literature. We also consolidated all of the cross-cutting issues near the beginning of the roadmap.
>
> We would very much appreciate your written feedback/comments on this version **no later than Wednesday April 25.**
>
> We're sending this to you as a Word doc to make editing and commenting easier. We suggest that you cc the whole group when you provide edits/comments—to ensure that you all see each other's perspectives.
>
> Once we incorporate your additional feedback, the next step will be to post the document for

public comment. We also plan to begin working on a set of measurable and achievable milestones and will start considering ways to approach the critical path analysis.

Again, we very much appreciate your ongoing support and involvement with this important project.

Warm regards,

(b) (6)

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this communication in error and that any review, disclosure, dissemination, distribution or copying of it or its contents is prohibited. If you have received this communication in error, please notify me immediately by replying to this message and deleting it from your computer. Thank You.

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From: (b) (6)
To: christopher.brode (b) (6)
Cc: (b) (6)
Subject: Call notes from the Nipah Roadmap Taskforce Conference Call which was held on Tuesday, January 9
Date: Thursday, January 18, 2018 8:48:13 PM
Attachments: Nipah Taskforce Conference Call Summary 20180109.final.doc
Nipah Draft Roadmap.V5.docx
Importance: High

Dear Nipah roadmap taskforce members:

First, I want to thank all of you who were able to participate in our conference call on Tuesday January 9. The discussion was very helpful and we very much appreciate you input.

Second, please send us any written edits or comments on the draft document as soon as possible, if you have a moment. I have attached the draft again here. We plan to revise the draft and send it on to WHO for review soon, in order to meet our current timelines. Any additional written comments you may have would be most appreciated.

Finally, I have attached meeting notes from the conference call for your records.

Again, thank you for all of your support in this important effort. We will be in contact again soon regarding next steps in preparing for the in-person March meeting in London.

Warm regards,

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From: (b) (6)
To: (b) (6) "christopher.broder(b) (6)
Cc: (b) (6)
Subject: RE: Save the Date - January 9 and 10 (depending on time zone)- WHO R&D roadmap taskforce conference call for Nipah virus
Date: Monday, January 15, 2018 5:32:45 PM
Attachments: Nipah Draft Roadmap.V5(b) (6) .docx

My comments on the document. See you tomorrow.

(b) (6)

From: (b) (6)
Sent: Friday, December 8, 2017 11:47 AM
To: christopher.broder(b) (6)
Cc: (b) (6)
Subject: Save the Date - January 9 and 10 (depending on time zone)- WHO R&D roadmap taskforce conference call for Nipah virus
Importance: High

Dear Colleagues:

Thank you to those who provided your availability for a Nipah WHO Roadmaps taskforce conference call.

Based on majority availability, the taskforce call will be held on **Tuesday, January 9 at 7 pm CST.** The date and time for this call in the different time zones is as follows:

- PST (US): 5:00 pm (Tuesday January 9)
- MST (US): 6:00 pm (Tuesday January 9)
- CST (US): 7:00 pm (Tuesday January 9)
- EST (US): 8:00 pm (Tuesday January 9)
- GMT (London): 1:00 am (Wednesday January 10)
- CET (Geneva): 2:00 am (Wednesday January 10)
- BST (Bangladesh): 7:00 am (Wednesday January 10)
- SGT (Singapore): 9:00 am (Wednesday January 10)

Call-in information:

- Conference Code for all numbers listed below: 998-3378#
  - USA Toll Free: 866-767-9978
  - UK Toll Free: 0808 1017 535

    - Switzerland Toll Free: 0800 5537 12
    - Singapore Toll Free: 800 101 2395
- For Bangladesh, we do not have a toll free line; we will contact you separately to make logistical arrangements.
- As a BACKUP: International callers may use the following conference call information: Conference Line 205-254-8650 Conference Code: 998-3378#. However, please note that this is NOT a toll-free number.

An agenda and other meeting materials will be shared closer to the conference call date. A meeting summary will be developed and shared with the group after the call. As we anticipated, we were unable to accommodate everyone's schedules, so we apologize if you are not able to participate. If you are unable to participate, we still very much want your input, and we will look at either obtaining input via email or possibly through an individual call, if time allows.

We deeply appreciate your ongoing support and value your expertise and involvement. Please do not hesitate to contact me with any questions.

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From: (b) (6)
To: (b) (6) ; Broder, Christopher; (b) (6)
Subject: Re: Version 5 henipavirus partnership grant
Date: Thursday, January 11, 2018 9:15:47 PM
Attachments: henipavirus science 5_LZ_pictures deleted.docx

(b) (6)

Looks like its coming together…some edits/comments for your consideration.

Good luck
(b) (6)

From: (b) (6)
Date: Thursday, January 11, 2018 at 4:33 PM
To: (b) (6) Christopher Broder
(b) (6)
Subject: Version 5 henipavirus partnership grant

This is a little cleaned up compared the version i sent earlier.
If you haven't started, use this to edit
Its getting big so i zipped it
It you need to , you can just send edited pieces back, or share a dropbox or something

---

From: (b) (6)
Date: Thursday, January 11, 2018 at 2:39 PM
To: (b) (6) Chris Broder
(b) (6)
Subject: Re: nipah

(b) (6)
What is the drop dead deadline to get edits back to you?
Best
(b) (6)

From: (b) (6)
Date: Thursday, January 11, 2018 at 12:26 PM
To: Christopher Broder (b) (6)
Subject: Re: nipah

Here is what I have. It's a little bit of a shambles, but it has most of the pieces and parts.
Each of you could make your parts much better i am sure
Also, i haven't really integrated it top to bottom to make it smooth, and formatting is bad and inconsistent

Sorry!!!! Embarrassed

From: Chris Broder (b) (6)
Date: Thursday, January 11, 2018 at 1:44 PM
To: (b) (6)
Cc: (b) (6)
Subject: Re: nipah

yup, on board as well

On Thu, Jan 11, 2018 at 2:18 PM, (b) (6) wrote:

> Me too
>
> ---
>
> **From:** (b) (6)
> **Sent:** Thursday, January 11, 2018 1:02 PM
> **To:** (b) (6)
> **Cc:** Broder, Christopher
> **Subject:** Re: nipah
>
> I'll be available this afternoon and in the morning for any edits you need from me.
>
> Safe travels
> (b) (6)
>
> ---
>
> **From:** (b) (6)
> **Date:** Thursday, January 11, 2018 at 10:57 AM
> **To:** (b) (6) >
> **Cc:** Christopher Broder (b) (6)
> **Subject:** Re: nipah
>
> Not really. I'm struggling. I am boarding a plane for 4 hours so hope to make some progress if my battery lasts
>
> If I can get WiFi on plane I will send you all he current draft
>
> The places that each of you would be responsible for need help. But hey are not long and you'll be able to fix them quickly
>
> Sent from my iPhone
>
> On Jan 11, 2018, at 12:12 PM, (b) (6) wrote:

(b) (6)

Are you OK on everything?

(b) (6)

From: (b) (6)
Sent: Tuesday, January 09, 2018 11:55 AM
To: Broder, Christopher; (b) (6)
Cc: (b) (6)
Subject: Re: nipah

Thanks (b) (6)

From: Chris Broder (b) (6)
Date: Tuesday, January 9, 2018 at 10:56 AM
To: (b) (6)
Cc: (b) (6)
>
Subject: Re: nipah

(b) (6)

for pt #4 to (b) (6), here is an additional section of grant text. Also., you can capture the issue of new SNT idea from the document i sent before xmas.
I highlighted in blue. and added some red text.

(b) (6)

On Tue, Jan 9, 2018 at 10:02 AM, (b) (6) wrote:

> (b) (6),
>
> See below. (b) (6) and I are providing the requested info. Do you have any time to help (b) (6) with this? On number 4 to me below I sent him a few of our pubs. Not sure if you have anything from grants that could be co-opted here.
>
> Many thanks!
>
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>
> **From:** (b) (6)
> **Sent:** Monday, January 08, 2018 9:42 PM
> **To:** (b) (6)

Subject: Re: nipah

I'm kinda hurting bad on this. I made the mistake/choice/blessing of taking ~ 8 days off at the end of the year, and have been completely underwater ever since. I still want to submit this, but it's going to be a scramble. A lot (but not all) of the admin stuff is done. But the science is still in shambles. I need to put it together over the next day.

What I need, or could benefit from, from you guys:
(b) (6)

1. Select agent section, since there is Hendra and Nipah
2. Biosafety section
3. Vertebrate Animals section

4. Any basic (even extended) text or old grant sections on "HeV and NiV are xxx viruses", "bad for you", and "neut test is done by x, with these strains: yy", "animal models are . . . "

(b) (6)

1. Anything you would do for characterizing manufacturability or development of the lead mabs, for the science section
2. A "product development plan section" on human mAbs for these viruses. Kinda boilerplate, which i am guessing you have already

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From: (b) (6)
Date: Sunday, January 7, 2018 at 3:57 PM
To: "(b) (6)
Subject: RE: nipah

(b) (6),

Likewise. Please let me know if you need anything on the proposal.

Best,

(b) (6)

From: (b) (6)

Sent: Thursday, January 04, 2018 1:40 PM
To: (b) (6)
Cc: (b) (6)
Subject: nipah

Hi (b) (6),

Just checking on what you'll need from me for the proposal.

Best wishes for 2018

(b) (6)

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A roadmap for MERS-CoV research and product development: report from a World Health Organization consultation

Kayvon Modjarrad, Vasee S Moorthy, Peter Ben Embarek, Maria Van Kerkhove, Jerome Kim & Marie-Paule Kieny

As part of the World Health Organization (WHO) R&D Blueprint initiative, leading stakeholders on Middle East respiratory syndrome coronavirus (MERS-CoV) convened to agree on strategic public-health goals and global priority research activities that are needed to combat MERS-CoV.

The emergence and persistence of MERS-CoV as a cause of severe respiratory disease 10 years after the outbreak of severe acute respiratory syndrome coronavirus (SARS-CoV) highlights the need for the rapid development of effective interventions against highly pathogenic human coronaviruses. As MERS-CoV grows in global importance—causing disease and death in more than 1,700 and 600 people, respectively, across 27 countries[1]—research and development (R&D) efforts to design diagnostic, prophylactic and therapeutic products are gaining momentum. In the aftermath of the 2014–16 Ebola epidemic in West Africa and the current Zika virus outbreak, it has become clear that more strategic investments are needed in the early development of diagnostics, therapeutics and preventives against pathogens of pandemic potential[2]. The ultimate goal is to reduce delays between the identification of a public-health emergency and the deployment of effective medical interventions that will save lives and minimize socioeconomic disruption. Toward this objective, the WHO is developing a blueprint for emergency R&D to prevent, or at least mitigate, the impact of infectious-disease outbreaks. MERS-CoV is one of eight pathogens prioritized in the WHO blueprint (http://www.who.int/medicines/ebola-treatment/WHO-list-of-top-emerging-diseases/en/), and it was selected as a case study to demonstrate how accelerated basic and applied research, as well as product development, could be better supported and coordinated. The WHO therefore convened a consultation of leading experts (**Supplementary Note 1**) on 10–11 December 2015 to develop a roadmap for MERS-CoV activities as part of the blueprint agenda.

Kayvon Modjarrad is in the US Military HIV Research Program, Walter Reed Army Institute of Research, Silver Spring, Maryland, USA. Vasee S. Moorthy, Peter Ben Embarek and Marie-Paule Kieny are in the World Health Organization, Geneva, Switzerland. Maria Van Kerkhove is at the Institut Pasteur, Center for Global Health, Paris, France. Jerome Kim is at the International Vaccine Institute, Seoul, Republic of Korea.
e-mail: moorthyv@who.int

Baseline assessment and epidemiology

The WHO's assistant director-general for health systems and innovation, Marie-Paule Kieny, opened the consultation by framing the meeting in the context of the broader blueprint for R&D preparedness and emergency response for known priority pathogens. The first author of this report then presented a landscape analysis of MERS-CoV diagnostics, preventives and therapeutics, highlighting the major gaps and advances in ongoing research and product-development activities. This baseline analysis summarized a report that was written for the consultation and that can be accessed on the WHO website (http://www.who.int/csr/research-and-development/mers-landscape.pdf?ua=1). After these overviews, a series of presentations described current knowledge about MERS-CoV epidemiology.

There is already broad consensus within the scientific community that dromedary camels are the main animal reservoir and source of zoonotic transmission to human populations[3]. However, the dynamics of transmission events from dromedaries to humans and between humans are poorly understood. Although studies have demonstrated that individuals with close and frequent contact (i.e., occupationally exposed) with dromedaries are at much higher risk for MERS-CoV infection than the general population[4], it remains unclear what routes of exposure mediate viral transmission most efficiently. Since the recognized introduction and entrenchment of MERS-CoV in human populations, the majority of MERS-CoV outbreaks have occurred in the nosocomial setting[5]. Although surveillance programs, observational studies and enhanced infection-control systems are now being prioritized and implemented throughout the Middle East, there is still wide variability across the region in the reach of surveillance efforts, the depth of case investigations and adherence to infection-control protocols. Improvements in these efforts will require political will, coordination across multiple sectors within countries at highest risk for future outbreaks, more streamlined communication, engagement with affected communities and cross-validation of diagnostics already in use and in development.

Diagnostic-assay development and standardization

During the early stages of infection, MERS-CoV cannot be clinically distinguished from other types of respiratory infections. Thus,

Table 1 MERS-CoV monoclonal antibodies in development. Several groups have identified monoclonal antibodies that have at least shown potent neutralization against MERS-CoV, and in some cases, that have protected transgenic mice and NHPs from MERS-CoV disease after viral challenge.

Institution	Name	Source	Target	R&D
Chinese Academy of Sciences, China	4C2, 2E6	RBD-immunized mouse	RBD	Mouse efficacy
Dana-Farber Cancer Institute, USA; Abviro, USA	3B11 (AV-3)	Human-antibody library	RBD	Mouse and NHP efficacy
HUMABS Biomed, Switzerland	LCA60	Human survivor	RBD	Mouse and NHP efficacy
New York Blood Center, USA; Fudan University, China	Mersmab1	S1 immunized mouse	RBD	*In vitro*
Organic Vaccines, USA	m336, m337, m338	Human-antibody library	RBD	Mouse, rabbit, and NHP efficacy
National Institutes of Health, USA	D12, F11, G2, G4	S/S1 immunized mouse	RBD, S1, S2	NHP efficacy
Regeneron, USA	REGN3048/REGN3051	Humanized mouse	RBD	Mouse and NHP efficacy
Tsinghua University, China	MERS-4, MERS-27	Human-antibody library	RBD	*In vitro*

S1, spike-domain-containing RBD; S2, spike-domain-containing fusion machinery.

as the current case definition of MERS-CoV infection is based on laboratory confirmation[6], the development and harmonization of sensitive, specific and easily administered diagnostic assays are crucial to the success of surveillance systems, epidemiologic studies and efficacy assessments in clinical trials. One of the principal challenges of developing useful diagnostic assays is that they are dependent on high levels of virus replication and thus cannot detect infection until several days after viral exposure. During this time, the individual is infectious and poses great risk to others. Speaking to these issues, several participants surveyed the diagnostics in use and commented on their current utility and future viability for both clinical and research purposes.

Nucleic acid–amplification tests (NAATs) are currently the gold standard of MERS-CoV diagnostic platforms[7]. Although these tests have become substantially easier to implement, their performance is still dependent on specimen quality and technician training, because environmental contamination can easily confound accurate interpretation of results. Serologic assays—such as those based on immunofluorescence, immunochromatography, enzyme-linked immunosorbence and live-virus or pseudovirus neutralization—vary in their performance characteristics, but provide benefits over NAATs in the form of easier implementation and more functionally relevant readouts[8]. The performance of any assay, particularly with respect to MERS-CoV, depends on when it is administered during the natural history of disease. A more detailed understanding of the key features of the clinical course of MERS-CoV infection is, therefore, needed to inform the optimization of existing assays and the development of next-generation diagnostics. It was generally agreed that, even in the absence of these data, there is an urgent need for rapid, point-of-care diagnostics for both human and animal populations. The suggestion was made to develop commercially available—or at least adequately validated—simple, dipstick immunochromatographic assays that are suitable for use on livestock and humans (before confirmatory testing of positive results in humans). It was also recommended that more-advanced diagnostics, such as real-time reverse-transcriptase polymerase chain reaction (rRT–PCR) assays, be developed as part of a multivalent respiratory panel. There is also a need for more coordinated efforts to sequence circulating viruses and to correlate those data with phenotypic outcomes, such as viral fitness, virulence and structure–function relationships of the surface Spike (S) and other MERS-CoV proteins. There was a general call for the development of commercial tests and quality assurance of existing assays, although some of this work has already been started[7]. The validation of diagnostic tests will be essential for the execution and interpretation of epidemiologic studies that can better define viral reservoirs, transmission dynamics and correlates of protection.

Therapeutics

There are currently no licensed treatments for MERS-CoV. The discovery of antivirals for MERS-CoV has been limited to the repurposing of compounds already licensed or in development for other diseases. Some of the experimental treatments used sporadically during this outbreak are the same as those used for SARS-CoV. As in the SARS-CoV epidemic, however, the use of treatments such as ribavirin, interferons and corticosteroids have yielded little to no clinical benefit, despite showing efficacy in nonhuman primates (NHPs)[9]. High-throughput screens of large libraries have uncovered pharmaceutical agents across several classes that inhibit MERS-CoV replication *in vitro* and a few that improve survival in marmoset models[10]. Investigational agents are also being repurposed from other infectious diseases, such as Ebola virus disease, for potential use against MERS-CoV. Data were presented on the antiviral furthest along in development: GS-5734, an adenine analog that incorporates into viral RNA to disrupt replication. It has been shown to protect NHPs from Ebola virus disease and is now advancing through a phase 1 dose-escalation trial[11]. So far, its activity against MERS-CoV has been tested only in cell lines.

Antibodies, both monoclonal and polyclonal, have eclipsed antivirals as the focus of MERS-CoV therapeutic R&D. Initially, convalescent plasma administration, which had been used in other emerging infection outbreaks, was seen as a potentially expeditious and effective means of post-exposure prophylaxis in the setting of cluster outbreaks[12]. A regional protocol was developed, but ultimately could not be implemented, owing to a lack of sufficient convalescent donors[13]. Meanwhile, a different formulation of polyclonal antibodies, derived from a transchromosomal humanized bovine model, has been moving forward in development. Both prophylactic and therapeutic use of this polyclonal preparation significantly reduces viral lung titers in mice that were intranasally transduced with adenoviral vectors that expressed the human MERS-CoV cognate protein receptor, DPP4 (ref. 14). However, it was noted by several experts that therapeutic studies conducted thus far in animals may not be relevant to human outbreaks, given that products are administered only hours after challenge, probably before symptoms in humans would appear.

Among products being researched for pre- or post-exposure prophylaxis, monoclonal antibodies (mAbs) targeting the receptor-binding domain (RBD) of S are furthest

Table 2 MERS-CoV vaccine candidates in development. There are five general vaccine platforms in development for MERS-CoV. At the time of this report, all candidates are still in different preclinical stages of development.

Vaccine platform	Institution	Product	Stage of preclinical development			Stage of clinical development		
			In vitro	Immunogenicity	Efficacy	Phase 1	Phase 2	Phase 3
Live attenuated	Universidad Autonoma de Madrid, Spain	Recombinant MERS-CoV						
Subunit	Novavax, USA	Full-length S trimers						
	Central South University, China	RBD fused with human Fc						
	New York Blood Center, USA Fudan University, China	RBD fused with human Fc						
	Chinese CDC, China	Truncated RBD						
DNA	GeneOne Life Sciences, South Korea	Full-length S						
Prime-boost	National Institutes of Health, USA	Full length S DNA prime, S1 subunit protein boost						
Recombinant vector	Greffex, USA	Ad5 S						
	Chinese CDC, China	Ad5 S or S1						
	University of Pittsburgh, USA Erasmus Medical Center, the Netherlands	Ad5 or Ad41						
	University of Oxford, UK	ChAd5 S						
	Paul Ehrlich Institute, Germany	Measles S						
	Ludwig Maximilian University of Munich, Germany	MVA S						

Fc, crystallizeable fraction of a human antibody; RBD, receptor-binding domain of the spike glycoprotein; Ad, adenovirus; Ad41, adenovirus serotype 41; MVA, modified vaccinia Ankara virus.

along in the product-development pipeline[15] (**Table 1**), some of which were presented at the meeting. Although representatives from each of the groups developing MERS-CoV mAbs presented data on the origin, potency, breadth and animal efficacy of their respective mAbs, some common themes emerged from the session as a whole and from the discussion that followed. Because most of the antibodies that have been developed target the RBD, there is a potential for viral escape from any one mAb. Thus, there should be greater efforts to (i) monitor circulating strains to assess viral evolution; (ii) define and measure phenotypic correlates of viral sequences; (iii) investigate the use of combination mAbs or polyclonal sera to overcome the potential emergence of therapeutic resistance; and (iv) study the serum of human survivors to better understand the response to natural infection and to develop reference reagents.

Vaccines

The global will to develop a coronavirus vaccine faded in the aftermath of the SARS-CoV pandemic, but has since gained renewed momentum in the face of the current MERS-CoV outbreak. This session started with a broad overview of vaccine-development pipelines and stressed that most vaccines fail to advance beyond phase 1 testing because of a lack of interest from funders and limited industrial support. In the realm of emerging infectious diseases with suspected or known pandemic potential, governmental agencies and nongovernmental organizations might have a key role in the development of interventions against diseases that do not provide a strong incentive for private-sector investments, but that are still relevant to public health and global security.

There are currently a dozen vaccine candidates in preclinical development (**Table 2**). Seven of the groups presented their products at the meeting. All developers are basing their immunogen designs on the S surface glycoprotein, the primary target for neutralizing antibodies during natural MERS-CoV infection[16]. Multiple platforms can be used to produce S, including but not limited to those presented at the meeting, such as live-attenuated viruses, DNA vectors, protein subunits and viral vectors (i.e., adenovirus, modified vaccinia virus Ankara and measles virus)[17]. Several of the products presented have demonstrated protection in at least one animal model. Although most of the vaccine candidates in the pipeline are being developed for human use, two of the vaccines have been tested in camels, which, if effective, would interrupt transmission of the virus to humans. A successful example of this "OneHealth" strategy—in which human, animal and environmental concerns are all considered—was described for the vaccine against Hendra virus in horses[18]. One of the lessons learned from the Hendra experience is that preclinical development and animal-model testing in relevant smaller animal models should be extensive before efficacy trials are commenced in larger-animal target populations, such as horses, or in the case of MERS-CoV, dromedary camels. Downselection of vaccine candidates in smaller animals increases the likelihood of success in large animals, which is crucial because the costs are much higher and logistical challenges much greater in the latter models. An additional lesson to take from previous experiences with animal vaccines for human health is to engage affected populations and educate them on the potential benefits and risks of a vaccine for their animals (i.e., camels) and communities.

One of the difficulties facing the development of effective vaccines for MERS-CoV is the absence of an animal model that recapitulates the pathogenesis and natural history of severe human disease. Two presentations and

a robust discussion centered on this limitation in the field. Several mouse models that are transgenic for the human DPP4 protein receptor have now been developed[19]. Despite their manifestation of clinical disease, data from these transgenic mice might need to be supplemented with that of other, larger animal models for clinical advancement and ultimate licensing. Semi-permissive NHPs have been used as an animal surrogate in vaccine-efficacy testing thus far[20], but it is not clear whether either the rhesus or marmoset NHP models will serve as an accurate proxy for human disease, given that knowledge of the human pathology of MERS-CoV infection is limited to a single autopsy[21]. The development of more relevant animal models requires parallel investigation and elucidation of the virus's pathogenesis in humans. Additionally, there is some concern that a vaccine developed against a new coronavirus may induce antibody-dependent enhancement of infectivity and eosinophilic pulmonary infiltrates, as was observed among mice vaccinated with a virus-like particle (VLP) or killed–inactivated SARS-CoV vaccine[22]. However, none of the vaccines currently being developed for MERS-CoV includes the VLP or killed-inactivated platforms.

The roadmap for MERS-CoV R&D (**Supplementary Note 2**) will focus on vaccines that are indicated for populations and purposes of priority to the WHO perspective. Three broad indications for vaccination were discussed. The first two indications are for human use. They include a single-dose vaccine to be deployed for individuals at acute risk during outbreaks and a two-dose vaccine to induce durable protection for those at continual risk, such as camel handlers and health-care workers. The third vaccine indication proposed is for dromedaries, particularly juvenile camels, which pose a greater risk than older camels of transmitting virus to humans. The endpoint for a veterinary vaccine is likely to be the reduction or prevention of viral shedding, rather than sterilizing immunity. Despite the great potential for a camel vaccine to interrupt the epidemic, there has been a gap in funding for the development of a camel vaccine from conventional sources that support veterinary vaccines. One reason may be that a MERS-CoV vaccine for camels would be used differently than conventional veterinary vaccines. Usually, animals are vaccinated to prevent illness and death within veterinary populations. In the case of a MERS-CoV camel vaccine campaign, however, the primary intent is to prevent infection and disease in human populations. It will be important to shore up funds for a camel vaccination option, as this may be the fastest developmental and regulatory route toward licensing a product that can prevent human MERS-CoV infections and deaths.

Drafting a research and product development roadmap for MERS-CoV

R&D roadmaps have been used successfully in many sectors in which large-scale, collaborative efforts are required to deliver outcomes related to the innovation and development of new products. In all such processes, it is essential to start by clearly articulating and understanding the goals and markets for these products. High-level priority areas are first identified, after which an agreement on specific activities is decided. It is also crucial to first map out the baseline knowledge gaps and then to develop a strategic plan to address those deficiencies. This also requires an assessment of capacity needs that can support these activities. Project management and implementation structures are subsequently established to pursue agreed-upon activities to reach these goals. An example of this process can be found in the WHO Malaria Vaccine Technology Roadmap, which has culminated in a first-generation malaria vaccine and catalyzed the development of second-generation products (http://www.who.int/immunization/topics/malaria/vaccine_roadmap/en/).

At the meeting, four strategic goals were agreed upon in principle. The first is to establish a surveillance network of coronavirus laboratories as an early warning system to identify circulating species and strains in animal populations, new outbreaks in human populations and emerging strains in all populations. The second is to acquire a better understanding of MERS-CoV pathogenesis, natural history and veterinary and human epidemiology. The third is to develop, manufacture, test, license and use improved diagnostics, preventives and therapeutics that enable the interruption of transmission between humans and from dromedary camels to humans. The fourth, and perhaps most important, is for the global donor community to establish a mechanism that provides a line-of-sight for manufacturers from preclinical proof-of-concept studies to post-licensing procurement of MERS-CoV products, by initiating a public-health financial model for emerging pathogens prioritized by the WHO blueprint process.

Priority activities to be pursued through the MERS-CoV roadmap

A series of activities was prioritized and divided into the following categories: (i) basic, translational, epidemiological and social research; (ii) cross-cutting product development that includes refining more relevant animal models, developing reference reagents and designing tools and methods for rational prioritization between products; (iii) improved diagnostics; (iv) therapeutics, mAbs and polyclonal-antibody preparations; (v) vaccines for humans and camels; (vi) capacity development; and (vii) policy and commercialization. These will be further developed in consultation with potential funding stakeholders such as the Biomedical Advanced Research and Development Authority (BARDA), the US National Institutes of Health (NIH), the Wellcome Trust, the European Commission and the International Vaccine Initiative, among others, and with the broader MERS-CoV research community, to include public-health officials, manufacturers, regulators and product-development partnerships. The draft roadmap was posted for public consultation on WHO's website throughout the month of February 2016 and was finalized in May 2016 (http://www.who.int/csr/research-and-development/roadmap-consultation/en/).

Summary

The 2014 Ebola epidemic in West Africa revealed both great potential and pernicious deficiencies within existing mechanisms for rapid medical-product development and deployment. In the aftermath of the epidemic, global health communities coalesced around the realization that a multifaceted plan was required to respond quickly and efficiently to the next outbreak. The WHO is currently developing an R&D blueprint by which such preparation and response can follow, highlighting MERS-CoV as a case study. Although global coordination has resulted in the maturation of the preclinical pipeline for novel interventions for MERS-CoV, products will have to be developed along faster than normal timelines, with greater investments by multiple agencies for development, manufacturing and preclinical and clinical testing, as well as preparations for timely efficacy testing in affected populations if the incidence of disease rises sharply. As the global community takes lessons from the recent Ebola crisis, applies them to the current Zika virus outbreak and prepares for the potential of another regional epidemic or broader pandemic, stakeholders in research and product development on emerging pathogens must set out a sound strategy now for where to best target their investments in anticipation of future outbreaks. The current consultation is a first step toward that end,

having opened a forum for global dialogue between public-health agencies, scientists, product developers and funders to engage in joint planning of MERS-CoV R&D activities.

Note: Supplementary Information and Source Data files are available in the online version of the paper.

ACKNOWLEDGMENTS
We gratefully acknowledge input to the roadmap-development process from all those who attended the WHO consultation in December 2015. A supplementary file lists all those who attended. The views, findings and conclusions within are those of the authors and should not be construed to represent the positions of the US Department of Defense or the World Health Organization.

COMPETING FINANCIAL INTERESTS
The authors declare competing financial interests: details are available in the online version of the paper.

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From: (b) (6)
To: (b) (6) ; christopher.broder(b) (6)
Cc: (b) (6)
Subject: RE: Agenda and materials for the Nipah WHO R&D Roadmap Taskforce conference call to be held on Tuesday, January 9 or Wednesday January 10, depending on time zone
Date: Wednesday, January 10, 2018 4:39:58 AM
Attachments: Nipah Draft Roadmap.V5(b) (6).docm

Dear Dr(b) (6),

Please find the attached file having some of my suggestions.

Thanks.

(b) (6)

From: (b) (6)
Sent: Thursday, 4 January, 2018 9:51 AM
To: christopher.broder(b) (6)
Cc: (b) (6)
Subject: Agenda and materials for the Nipah WHO R&D Roadmap Taskforce conference call to be held on Tuesday, January 9 or Wednesday January 10, depending on time zone
Importance: High

Dear Colleagues:

I am writing to remind you of our Nipah Roadmap Taskforce conference call **on Tuesday, January 9 at 5:00 pm PST, 7:00 pm CST, 8:00 pm EST, and Wednesday, January 10 at 1:00 am GMT, 2:00 am CET, 7:00 am BST, and 9:00 am SGT.**

Each of you should have received a calendar invite with the call-in information and I have repeated that information below:

- Conference code: 998-3378#
  - USA Toll Free: 866-767-9978
  - UK Toll Free: 0808 1017 535
  - Switzerland Toll Free: 0800 5537 12
  - Singapore Toll Free: 800 101 2395
- For Bangladesh, we do not have a toll free line; we will contact you separately to make logistical arrangements.
- As a BACKUP: International callers may use the following conference call information: Conference Line 205-254-8650 Conference Code: 998-3378#. However, please note that this is NOT a toll-free number.

This email includes the following attachments:

- The Nipah draft roadmap outline. **Please review this document before the call** because most of our call will be devoted to discussing the document. Also, we would very much appreciate any written edits or comments that you have on the document, particularly if you are not able to participate in the call. In order to keep to our development timeline, we need to receive any written comments from you no later than Friday, **January 19.** Please send comments directly to (b) (6)
- A short slide deck for our call on Tuesday.
- Supplemental materials on the MERS-CoV Roadmap as examples, including a Nature Medicine article on that Roadmap and an outline of the roadmap, similar to the outline we have developed for the Nipah Roadmap.

The agenda for the call is as follows (most of the time will be devoted to discussing the draft Nipah Roadmap outline):

1. Welcome and roll call
2. R&D roadmap architecture
3. Role of roadmap taskforce members
4. Roadmap development and timeline
5. Discussion of draft R&D roadmap for Nipah
6. Next steps

Thank you again for agreeing to be part of this important work. We look forward to speaking with you next week and to receiving your comments on the draft Roadmap outline by January 19.

Warm regards,

(b) (6)

[redacted]

whom it is addressed. If you are not the intended recipient, please notify the sender, and please delete the message and any other record of it from your system immediately.

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From: (b) (6)
To: (b) (6)
Cc: christopher.brode (b) (6)

Subject: RE: Agenda and materials for the Nipah WHO R&D Roadmap Taskforce conference call to be held on Tuesday, January 9 or Wednesday January 10, depending on time zone
Date: Monday, January 8, 2018 8:40:10 PM
Attachments: Nipah Draft Roadmap.V5 (b) (6) .docx

(b) (6) and colleagues,

I very much look forward to working with you all on this endeavor.

I will be on a flight to Dhaka during the call, so unable to participate. I'll look forward to hearing the updates.

I've attached a few comments to the draft that (b) (6) sent.

All the best,
(b) (6)

From: (b) (6)
Sent: Saturday, January 6, 2018 1:38 AM
To: (b) (6)
Cc: christopher.broder (b) (6)

Subject: Re: Agenda and materials for the Nipah WHO R&D Roadmap Taskforce conference call to be held on Tuesday, January 9 or Wednesday January 10, depending on time zone

(b) (6),

Thanks so much for moving this forward. I am looking forward to the call.

I will be in Bangladesh, but I should be able to connect.

Attached are some comments on the roadmap outline.

(b) (6)

Thursday, January 04, 2018 8:50 AM

Dear Colleagues:

I am writing to remind you of our Nipah Roadmap Taskforce conference call **on Tuesday, January 9 at 5:00 pm PST, 7:00 pm CST, 8:00 pm EST, and Wednesday, January 10 at 1:00 am GMT, 2:00 am CET, 7:00 am BST, and 9:00 am SGT.**

Each of you should have received a calendar invite with the call-in information and I have repeated that information below:

- Conference code: 998-3378#
  - USA Toll Free: 866-767-9978
  - UK Toll Free: 0808 1017 535
  - Switzerland Toll Free: 0800 5537 12
  - Singapore Toll Free: 800 101 2395
- For Bangladesh, we do not have a toll free line; we will contact you separately to make logistical arrangements.
- As a BACKUP: International callers may use the following conference call information: Conference Line 205-254-8650 Conference Code: 998-3378#. However, please note that this is NOT a toll-free number.

This email includes the following attachments:

- The Nipah draft roadmap outline. **Please review this document before the call** because most of our call will be devoted to discussing the document. Also, we would very much appreciate any written edits or comments that you have on the document, particularly if you are not able to participate in the call. In order to keep to our development timeline, we need to receive any written comments from you no later than Friday, **January 19.** Please send comments directly to (b) (6) .
- A short slide deck for our call on Tuesday.
- Supplemental materials on the MERS-CoV Roadmap as examples, including a Nature Medicine article on that Roadmap and an outline of the roadmap, similar to the outline we have developed for the Nipah Roadmap.

The agenda for the call is as follows (most of the time will be devoted to discussing the draft Nipah Roadmap outline):

1. Welcome and roll call
2. R&D roadmap architecture

3. Role of roadmap taskforce members
4. Roadmap development and timeline
5. Discussion of draft R&D roadmap for Nipah
6. Next steps

Thank you again for agreeing to be part of this important work. We look forward to speaking with you next week and to receiving your comments on the draft Roadmap outline by January 19.

Warm regards,

(b) (6)

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From: (b) (6)
To: (b) (6)
Cc: christopher.brode (b) (6)

Subject: Re: Agenda and materials for the Nipah WHO R&D Roadmap Taskforce conference call to be held on Tuesday, January 9 or Wednesday January 10, depending on time zone
Date: Saturday, January 6, 2018 4:49:10 AM
Attachments: Nipah Draft Roadmap.V5 .docx

(b) (6),

Thanks so much for moving this forward. I am looking forward to the call.

I will be in Bangladesh, but I should be able to connect.

Attached are some comments on the roadmap outline.

(b) (6)

Thursday, January 04, 2018 8:50 AM

Dear Colleagues:
ï¿½
I am writing to remind you of our Nipah Roadmap Taskforce conference call **on Tuesday, January 9 at 5:00 pm PST, 7:00 pm CST, 8:00 pm EST, and Wednesday, January 10 at 1:00 am GMT, 2:00 am CET, 7:00 am BST, and 9:00 am SGT.**
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<!--[if !supportLists]-->ï¿½ï¿½ï¿½ï¿½ï¿½ï¿½ï¿½ï¿½ï¿½ï¿½ <!--[endif]-->Conference code: 998-3378#

<!--[if !supportLists]-->oï¿½ï¿½ <!--[endif]-->USA Toll Free: 866-767-9978

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<!--[if !supportLists]-->oï¿½ï¿½ <!--[endif]-->Switzerland Toll Free: 0800 5537 12

<!--[if !supportLists]-->oï¿½ï¿½ <!--[endif]-->Singapore Toll Free: 800 101 2395

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<!--[if !supportLists]-->ï¿½ï¿½ï¿½ï¿½ï¿½ï¿½ï¿½ï¿½ï¿½ï¿½ <!--[endif]-->As a BACKUP: International callers may use the following conference call information: Conference Line 205-254-8650 Conference Code: 998-3378#. However, please note that this is NOT a toll-free number.

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This email includes the following attachments:

<!--[if !supportLists]-->ï¿½ï¿½ï¿½ï¿½ï¿½ï¿½ï¿½ï¿½ï¿½ï¿½ <!--[endif]-->The Nipah draft roadmap outline. **Please review this document before the call** because most of our call will be devoted to discussing the document. Also, we would very much appreciate any written edits or comments that you have on the document, particularly if you are not able to participate in the call. In order to keep to our development timeline, we need to receive any written comments from you no later than Friday, **January 19.** Please send comments directly to (b) (6) .

<!--[if !supportLists]-->ï¿½ï¿½ï¿½ï¿½ï¿½ï¿½ï¿½ï¿½ï¿½ï¿½ <!--[endif]-->A short slide deck for our call on Tuesday.

<!--[if !supportLists]-->ï¿½ï¿½ï¿½ï¿½ï¿½ï¿½ï¿½ï¿½ï¿½ï¿½ <!--[endif]-->Supplemental materials on the MERS-CoV Roadmap as examples, including a Nature Medicine article on that Roadmap and an outline of the roadmap, similar to the outline we have developed for the Nipah Roadmap.

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The agenda for the call is as follows (most of the time will be devoted to discussing the draft Nipah Roadmap outline):

1. Welcome and roll call
2. R&D roadmap architecture
3. Role of roadmap taskforce members
4. Roadmap development and timeline
5. Discussion of draft R&D roadmap for Nipah
6. Next steps

ï¿½

Thank you again for agreeing to be part of this important work. We look forward to speaking with you next week and to receiving your comments on the draft Roadmap outline by January 19.

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Warm regards,

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(b) (6)

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(b) (6)

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Science Program Review
February 08 - 10, 2017

Department of Defense
Cooperative Threat Reduction Program

DEFENSE THREAT REDUCTION AGENCY
8725 JOHN J. KINGMAN ROAD, STOP 6201
FORT BELVOIR, VA 22060-6201

Dear Colleagues,

Welcome to the 9th Science Program Review of the U.S. Defense Threat Reduction Agency's Cooperative Biological Engagement Program (DTRA CBEP). The CBEP carries out its mission by collaborating with partner countries and the international community to minimize the threat of deliberate, accidental and natural infectious disease outbreaks through enhanced biosecurity, biosafety, and biosurveillance measures. Cooperative Biological Research (CBR) is an integral element of the threat reduction mission by supporting and informing operational biosurveillance, enhancing global health security, and establishing and fostering sustainable research partnerships. CBEP's achievements rely on successful collaboration with partner-country scientists, and we thank you for your dedication and cooperation in our shared efforts to better understand the biological threats that face our world today.

We have in attendance nearly 120 researchers from over 30 countries representing 70 CBR projects, and this three-day event will feature briefings by CBEP partner-country scientists and collaborators conducting CBEP-funded research. We look forward to engaging with researchers from around the globe and evaluating the spectrum of CBEP research activities, in order to strengthen the quality of projects supported by the program and ensure that current efforts meet research priorities and objectives.

A significant benefit of the CBEP Science Review is networking amongst fellow scientists. During the next few days, you will have the opportunity to meet with scientific and program colleagues from CBEP partner nations, the U.S. Department of Defense, other U.S. Government stakeholders, and international and U.S. program partners. With such a broad range of scientific expertise present, we hope that this environment will create new networks among subject matter experts and facilitate new partnerships within the scientific community.

Again, I want to thank you for your participation in this 2017 CBEP Science Program Review. I hope that you will enjoy your experience and find opportunities to expand your collaborative research network.

Sincerely,

(b) (6)

Division Chief
Cooperative Biological Engagement
Program, J3CTB

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CBEP Mission

The Defense Threat Reduction Agency's Cooperative Biological Engagement Program is the Department of Defense's premier biological nonproliferation division protecting the United States and its allies from especially dangerous pathogens by collaborating with partner countries and the international community to minimize the threat of deliberate, accidental, and natural infectious disease outbreaks through enhanced biosafety, security, and surveillance measures.

The Defense Threat Reduction Agency's Cooperative Biological Engagement Program utilizes Science Diplomacy to promote scientific and technical collaborations among partner nations and the international community in [the disciplines of] biological safety, security and surveillance to build constructive and sustainable international partnerships that address threats posed to health security from deliberate, accidental, and natural infectious disease outbreaks.

General Information

- Posters may be displayed on the available poster boards in the Foyer area. An informal networking and poster session will be held following the "Doing Science in Difficult Places" session.
    - Posters displayed in the Foyer area must be removed by 1:00 PM on Friday, February 10, 2017. Posters that are not removed by this time will not be kept by DTRA.
- A designated Prayer Room is available next to the Registration area in the Dogwood Room. Please see attendants at the Registration desk for additional information.
- If temporary luggage storage is required, please see the hotel Front Desk.
- Meeting space is available during the event for collaborative discussions. Please see the Registration desk for more information.
- Please see the Registration Desk for any questions or concerns during the event.

Acknowledgments

The following individuals were instrumental in the development and implementation of this event.

CBEP Science Program Review Steering Committee

Dr. (b) (6)
Chief Scientist, Cooperative Biological Engagement Program (CBEP)

Dr. (b) (6)
Southeast Asia Science Manager, CBEP

Dr. (b) (6)
Africa Science Manager, CBEP

Contractor Support Steering Committee Members:

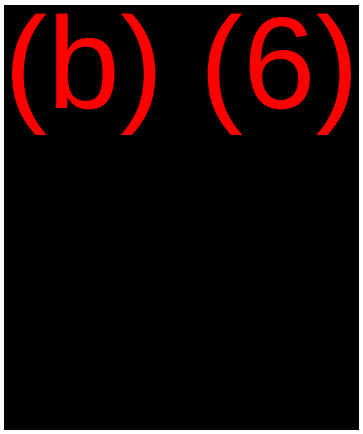
- Dr. (b) (6)
- Ms
- Dr.
- Dr.
- Dr.
- Dr.

Special Thank You to those who helped organize this event:

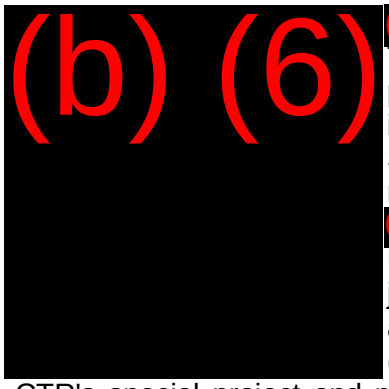
- Dr. (b) (6)
- Dr.
- Mr.
- Dr.
- Ms
- Mr.
- Mr
- Ms
- Ms

Science Program Review Panel

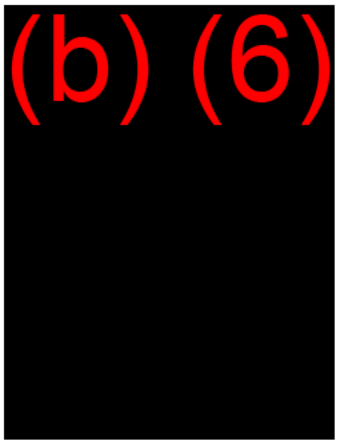
CBEP would like to acknowledge the following individuals for serving on the Review Panel for the 2017 CBEP Science Program Review. The Review Panel is comprised of experts with varied backgrounds in a broad range of scientific disciplines and expertise in public health security and program implementation. The Review Panel will provide CBEP with an external technical and programmatic evaluation of the CBEP-sponsored research portfolios to identify strengths and weaknesses in CBEP research engagements, along with recommendations for improving how the portfolios are designed and implemented.



(b) (6) **, PhD**, is the Associate Director, Portfolio Development for the Biomedical Advanced Research and Development Authority (BARDA) Modeling and Visualization Hub within BARDA's Division of Analytic Decision Support at the U.S. Department of Health and Human Services (HHS). She oversees the development of a portfolio focused on assessing the public health and medical consequences for chemical, biological, radiological, and nuclear (CBRN) incidents, emerging infectious diseases, and pandemic influenza events. Prior to joining HHS, she was at the Deputy Undersecretary of the Army for Test and Evaluation CBRN Defense Division, where her portfolio included oversight of CBRN test and evaluation programs, as well as the testing and standards development for the Transport Isolation Systems during the 2014-2015 West African Ebola crisis. Previously, she was a member of the Chemical and Biological Defense Division of Department of Homeland Security's Science and Technology Directorate, where she provided subject matter expertise and strategic vision for existing and planned biosurveillance programs. She was part of the interagency team that identified critical needs and gaps across the government in biological detection and diagnostic technology for the National Biosurveillance Science & Technology Roadmap. Dr. (b) (6) earned her Ph.D. in Neurobiology from Harvard University, and her B.S. in biochemistry from the University of Southern California.

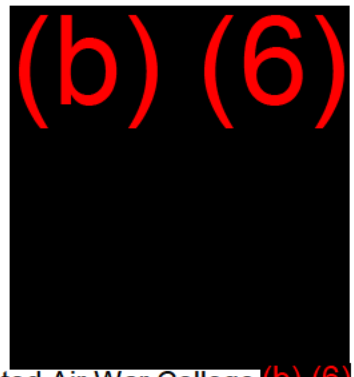


(b) (6) **, PhD**, is the Director for Countering Biological Threats on the National Security Council (NSC) staff. Dr. (b) (6)'s portfolio includes the Global Health Security Agenda, domestic and international biosecurity issues, implementation of the President's Strategy for Countering Biological Threats, biological threat reduction, and policies related to Dual Use Research of Concern. Dr. (b) (6) is also an adjunct Professor at the George Washington University where she lectures on Global Health Diplomacy. Prior to joining NSC staff, Dr. (b) (6) was the Senior Advisor for Biosecurity and a Team Chief in the Office of Cooperative Threat Reduction (CTR) at the U.S. Department of State. In this role, Dr. (b) (6) led CTR's special project and management teams to develop policy and implement programs to combat global biological, chemical, and nuclear threats. Prior to this role, Dr. (b) (6) served as the Acting Deputy Team Chief for the Biosecurity Engagement Program where she oversaw health security programs to reduce global biological risks. Dr (b) (6) received a Ph.D. in Cell and Developmental Biology from Vanderbilt University, where she studied intracellular transport during early development. Dr. (b) (6) holds a B.S. in Neuroscience with a minor in Spanish from Vanderbilt University.



(b) (6), **DVM**, has 15 years working experience in the field of animal health and epidemiology in both developed and less developed countries. He received a Degree in Veterinary Medicine (DVM) from Bologna University (IT) in 2002 and a MSc degree in Veterinary Epidemiology from the Royal Veterinary College and the London School of Hygiene and Tropical Medicine, UK in 2008. He is currently enrolled in the residency training (Population Medicine) of the European College of Veterinary Public Health. Following his DVM he practiced as a veterinary clinician in dairy farms in Italy. He then worked as a veterinary consultant and project coordinator for international organizations (non-governmental organizations and the Food and Agriculture Organization (FAO)) in East Africa from 2004 to 2007. In 2009-2013, he worked on research and capacity building programs at the "World Organization for Animal Health (OIE) and FAO Reference Laboratory for Avian Influenza" and the "FAO Reference Centre for Rabies" at Istituto Zooprofilattico Sperimentale delle Venezie (IZSVe) in Italy. In 2011, he coordinated the European Food Safety Authority (EFSA)-funded project "Flurisk," assessing the pandemic risk posed by animal influenza viruses.

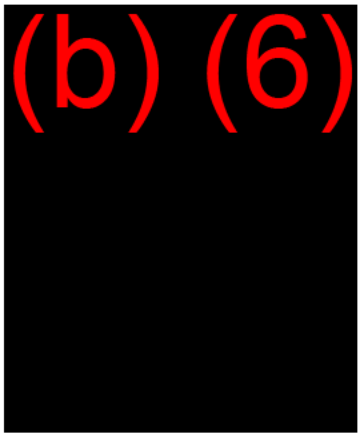
In 2013 he joined the private veterinary consultancy company SAFOSO (www.safoso.com), where he is responsible for the development and implementation of consultancy, research projects and capacity building programs in the area of risk assessment, surveillance, veterinary epidemiology, animal health and food safety. As a SAFOSO consultant he worked in different projects in Europe, U.S., Ukraine, Armenia, Georgia, Kazakhstan, Mongolia and Vietnam.



(b) (6), **PhD**, is as a Senior Executive at the National Science Foundation. Hearing the call to service in 2001, he escaped the bounds of a traditional scientific career by seeking out organizations undergoing change. He oversaw the development of new drugs, negotiated new international agreements, and provided entrepreneurs training on transforming their ideas into business ventures.

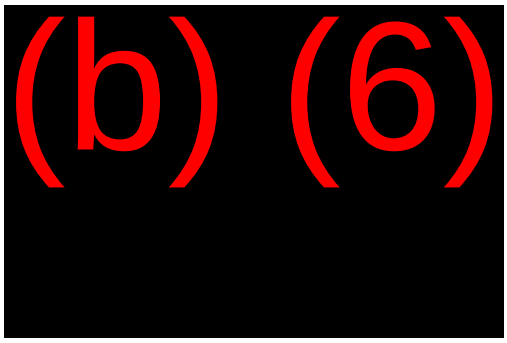
With a healthy appetite for risk, (b) (6) is known for his 'first unto the breach' leadership and willingness to try most any food.

Possessed of a strong imagination and an inquisitive mind, he earned a Ph.D. in Chemistry-Environmental Toxicology and completed Air War College. (b) (6) most important job is raising his sons to be good Southern gentlemen.



(b) (6), **PhD**, is the executive director of The Pathogen and Microbiome Institute, which uses genomic tools for understanding infectious diseases and the microbiome. This is a joint institute between TGen and Northern Arizona University, where Dr (b) (6) holds the Cowden Endowed Chair in Microbiology. His work has employed genetic and genomic analyses for understanding bacterial pathogen population structure and evolution for more than 30 years. Linking populations to their ecology has also been a critical part of his program. His laboratory served as the evidence repository and genetic analysis lab for the Federal Bureau of Investigation during the 2001 anthrax-letter investigation. He has been a leader in the field of microbial forensics which uses evolutionary analysis to understand close relationships among pathogen isolates. This work was foundational for his pursuit in public health investigations and the development of novel clinical diagnostic tests. He is an elected

fellow of both the American Association for the Advancement of Science and the American Academy for Microbiology. The National Institutes of Health appointed him to the National Science Advisory Board for Biosecurity in 2004 and served as its chairman for two years. He has published over 400 scientific research articles that have been cited over 20,000 times.



(b) (6), **DrPh, SM**, is a Senior Associate at the Johns Hopkins Center for Health Security (formerly the University of Pittsburgh Medical Center for Health Security), Visiting Faculty in the Department of Environmental Health and Engineering and Associate in the Department of Epidemiology at the Johns Hopkins Bloomberg School of Public Health. An epidemiologist by training, her work focuses on international and domestic biosurveillance, infectious disease diagnostics, and disease mitigation strategies. She also has worked on issues related to the Affordable Care Act, tuberculosis control, foodborne outbreaks, and water security. Dr. (b) (6) is an Associate Editor of the peer-reviewed journal *Health Security* (formerly *Biosecurity and Bioterrorism*).

In addition to her work at the Center, Dr. (b) (6) has advised national governments and nonprofit organizations. She has served as a consultant to the National Biosurveillance Advisory Subcommittee, as a member of the US Environmental Protection Agency's National Drinking Water Advisory Council (NDWAC), and as a member of the NDWAC's Water Security Working Group. She has also served as a project advisor for the American Water Works Association Research Foundation (now called the Water Research Foundation), a primary funding organization for drinking water research in the United States. She has also been consulted on pandemic planning efforts in the Republic of Indonesia and Taiwan.

Dr. (b) (6) received a Doctor of Public Health degree (DrPH) in epidemiology from the Johns Hopkins Bloomberg School of Public Health, an SM in environmental health from Harvard University, and a BS in environmental sciences from Rutgers University.

(b) (6), **PhD**, a native of the Czech Republic, obtained a Bachelor of Arts (BA) in Biological Science from Goucher College and a Doctorate of Philosophy in Molecular Microbiology and Immunology from the Johns Hopkins Bloomberg School of Public Health. She has extensive research experience in HIV vaccine research and human immunity and infectious diseases, especially viral pathogens. Dr. (b) (6) has worked in support of the Department of Defense since 2010. She started at the Joint Program Executive Office (JPEO) as the Technical Director for the Critical Reagents Program (CRP) and as a subject matter expert in infectious diseases and especially dangerous pathogens. Dr. (b) (6) eventually transitioned to serving as the Liaison Officer coordinating various inter- and intra-agency efforts and organizations, including the Armed Forces Health Surveillance Branch (AFHSB), the Centers for Disease Control and Prevention (CDC), the Defense Threat Reduction Agency (DTRA), and many others. She assumed her role as the Chief Scientist for the Global Emerging Infections Surveillance (GEIS) Section at the AFHSB in 2014. There she provides scientific guidance and oversight for emerging infectious disease surveillance projects conducted by or in conjunction with US military research laboratories in continental United States (CONUS) as well as outside of the continental United States (OCONUS) settings.

Program Agenda

Wednesday, February 8, 2017		
Time	**Session**	**Notes**
1:30 PM - 2:00 PM	**CBEP EUCOM Overview**	Overview Briefs by DTRA/CBEP for Review Panel (Note: **Closed Session** for US Government Personnel and Review Panelists only)
2:00 PM - 2:30 PM	**CBEP AFRICOM Overview**	
2:30 PM - 3:00 PM	**CBEP CENTCOM Overview**	
3:00 PM - 3:30 PM	**CBEP PACOM Overview**	
3:30 PM - 4:00 PM	**Coffee Break**	Open to all Attendees
4:00 PM - 5:00 PM	**Session 1: Special Presentation** **"Doing Science in Difficult Places"**	Open to all Attendees
5:00 PM - 6:30 PM	**Poster Session & Networking Event**	Open to all Attendees

Thursday, February 9, 2017			
Time	**Speaker**	**Country**	**Project**
8:30 AM - 8:50 AM	**Opening Remarks**		
8:50 AM - 10:10 AM	**Session 2: Chiroptera (Bats) - Important Reservoirs Hosts of Emerging Viruses**		
	(b) (6)	Georgia & Jordan	(BAA) Understanding the Risk of Bat-Borne Zoonotic Disease Emergence in Western Asia
		Cambodia	(BAA) Investigating the Risk of Human Disease from Parasites of Small Mammals and Bats
		Uganda	(Call) Arthropod-borne Viruses Associated with the Chiroptera of Uganda: Isolation and Characterization
		India	(BAA) Bat Harvesting in India: Detection, Characterization and Mitigation of Emerging Infectious Disease Risk
		Tanzania	(BAA) Evaluating Zoonotic Viral Sharing Among Bats, Primates and People in High Risk Transmission Interface in Southern Tanzania.
10:10 AM- 10:30 AM	**Coffee Break**		
10:30 AM- 11:40 AM	**Session 3: Epidemiology & Biosurveillance - Group 1**		
	(b) (6)	Georgia	GG-19: Epidemiology and Ecology of Human Tularemia in Georgia
		Azerbaijan	TAP-10: Ecological and Epidemiological Study of *Yersinia pestis* and *Francisella tularensis* in the Northern Part of Azerbaijan Regions of Gusar and Khachmaz
		Tanzania	(BAA) One Health Approach to Brucellosis and Rift Valley Fever Surveillance in Tanzania
		Thailand	(Call) Acute Febrile Illness Study Among Patients in Nakhon Phanom and Tak Province, Thailand
		Kazakhstan	TAP-8: Especially Dangerous Pathogen Differential/Rule-Out Elimination Assays & Modeling (EDP-DREAM) of the Saiga Antelope Mortality

Thursday, February 9, 2017 (Continued)			
Time	**Speaker**	**Country**	**Project**
11:40 AM-12:30 PM	**Session 4: Viral Pathogens of Security Concern**		
	(b) (6)	Georgia	(Call) Assessing the Seroprevalence and Genetic Diversity of CCHFV and Hantaviruses in Georgia
		Cameroon	Epidemiology of Emerging Viruses
		South Africa	(BAA) Understanding Rift Valley Fever in South Africa.
		Malaysia	(BAA) Biosurveillance for Henipaviruses and Filoviruses at the Agricultural Animal-Human Interface in Malaysia
12:30 PM - 1:30 PM	**Lunch**		
1:30 PM - 2:20 PM	**Session 5: Community Outreach to Combat African Swine Fever**		
	(b) (6)	Implementer	ASF Public Outreach Project Overview
		Armenia	TAP-A1: Community Outreach to Support Understanding of ASF Ecology and Epidemiology in Eastern Europe: Training and Implementation for Methods and Strategies for Control and Prevention
		Georgia	TAP-9: Community Outreach to Support Understanding of ASF Ecology and Epidemiology in Eastern Europe: Training and Implementation for Methods and Strategies for Control and Prevention
		Ukraine	TAP-4: Community Outreach to Support Understanding of African Swine Fever (ASF) Ecology and Epidemiology in Eastern Europe

Thursday, February 9, 2017 (Continued)			
Time	**Speaker**	**Country**	**Project**
2:20 PM - 3:20 PM	**Session 6: Transboundary Animal Diseases**		
	(b) (6)	Vietnam	(Call) Foot-and-Mouth Disease Virus Surveillance and Ecology in Vietnam
		Kenya	(BAA) Whole Genome Sequencing of African Swine Fever Virus in Kenya.
		Ukraine	TAP-6: Analysis of the Threat of Spread of African Swine Fever and Classical Swine Fever in Wild Boar Populations in Ukraine: Improving Diagnosis, Surveillance, and Prevention
		Uganda	UG-2: Research and Development of Countermeasures to Support the Control of FMDV in Uganda
		Ukraine	(USDA ARS) African Swine Fever Threat Reduction Through Surveillance in Ukraine
3:20 PM - 3:40 PM	**Coffee Break**		
3:40 PM - 5:00 PM	**Session 7: Bacterial Pathogens of Security Concern - Group 1**		
	(b) (6)	Georgia & Azerbaijan	(BAA) Molecular Epidemiology and Ecology of *Yersinia* spp. in the Transboundary Plague Endemic Territory in Georgia and Azerbaijan
		Kazakhstan	KZ-31: Effect of *Rickettsia* spp. upon Fitness of *Yersinia pestis* in Fleas that Vector Plague in the Republic of Kazakhstan
		Ukraine	UP-2: Development of the Epidemiological Forecasting System for Zoonotic Diseases Employing GIS Technology
		Georgia	GG-23: Creation of Sustainable Immunodiagnostics
		Armenia	AM-1: Medical/Biological Mapping of Tularemia Natural Foci and Epidemiology using GIS in Armenia
5:00 PM - 5:10 PM	**Break**		

Thursday, February 9, 2017 (Continued)			
Time	**Speaker**	**Country**	**Project**
5:00 PM - 6:20 PM	**Session 8: Avian Transmissible Diseases**		
	(b) (6)	Ukraine	(USDA ARS) Genomic, Epidemiological, and Biological Characterization of Newcastle Disease Virus Isolates from Ukraine
		Kenya	(Call) Newcastle Disease: Surveillance, Molecular Epidemiology, and Control of NDV in Kenya
		Kazakhstan	TAP-11: Molecular Characterization and Complete Genome Sequence of Newcastle Disease Virus Isolated in Kazakhstan
		Ukraine	UP-4: Risk Assessment of Selected Especially Dangerous Pathogens Potentially Carried By Migratory Birds Over Ukraine
		India	(BAA) Detection and Molecular Epidemiologic Analysis of Especially Dangerous Pathogens in Backyard Poultry, Commercial Broilers and Waterfowl in India
6:20 PM - 6:30 PM	**Day 1 Wrap-up**		

Friday, February 10, 2017

Time	Speaker	Country	Project
8:30 AM - 8:40 AM	**Opening Remarks**		
8:40 AM - 10:20 AM	**Session 9: Bacterial Pathogens of Security Concern - Group 2**		
	(b) (6)	Georgia	(BAA) Characterization of NCDC Strain Repository by Next Generation Sequencing (NGS)
		Pakistan	(BAA) High Resolution Chemical Characterization of *Yersinia pestis* Cells Within Soil Matrices: Implications for Understanding Natural Foci and Telluric Reservoirs of Plague
		Kenya	(CDC IACRO) Estimating Incidence and Socio-economic Impact of Brucellosis in Humans and Animals in Kajiado County, Kenya
		Georgia	TAP-10: Molecular Epidemiology of *B. anthracis* and *Brucella* spp. in Turkey and Georgia
		Georgia	GG-27: Regional Study of the Ecology of Anthrax Foci in Georgia and Azerbaijan
		Azerbaijan	AJ TAP-11: Regional Study of the Ecology of Anthrax Foci in Georgia and Azerbaijan
		Lao PDR	Environmental Surveillance of *Bukholderia pseudomallei*, Pilot Study in Lao PDR
		Thailand	Melioidosis Regional Coordination Network
10:20 AM- 10:40 AM	**Coffee Break**		
10:40 AM - 12:00 PM	**Session 10: Emerging Infectious Diseases**		
	(b) (6)	Georgia	(Call) Enhancing Capacity for Case Detection and Diagnosis of Febrile Zoonotic-related Cutaneous Lesions in Georgia
		Georgia	(BAA) Enhancing Capacity for Case Detection and Diagnosis of Febrile Zoonotic-related Cutaneous Lesions in Georgia
		Malaysia	(Call) Multi-Year Prospective Cohort Study to Evaluation the Risk Potential of MERS-CoV
		Kazakhstan	KZ-33: Middle East Respiratory Syndrome Coronavirus (MERS-CoV): Surveillance for Distribution and Prevalence in Kazakhstan
		Tanzania	(BAA) Global Health, Emerging Infectious Diseases and Food Safety Implications of Bushmeat in Tanzania
		Malaysia	(BAA) Etiology of Severe Acute Respiratory Infections in Kuala Lumpur, Malaysia

Friday, February 10, 2017 (Continued)			
Time	**Speaker**	**Country**	**Project**
12:00 PM - 1:00 PM	**Lunch**		
1:00 PM - 2:40 PM	**Session 11: Epidemiology & Biosurveillance - Group 2**		
	[redacted]	Armenia	AM-4: One Health Surveillance for Brucellosis in Armenia
		Uganda	(BAA) Acute Febrile Illness in Uganda.
		Azerbaijan	(Call) Infectious Etiologies of Acute Febrile Illness Among Members of the Azerbaijan Military
		Georgia	GG-20: Prevalence, Epidemiological Surveillance, and Laboratory Analysis of *Coxiella burnetii* in Georgia
		Kazakhstan	KZ-32: Prevalence of *Brucella* Species and Bluetongue Virus Serotypes Among Domestic Livestock or Ruminants in Southern Kazakhstan
		Armenia	TAP-H1: Identification of Etiology, Clinical Outcomes, Incidence, and Epidemiological Patterns of Hospitalized Febrile Patients in Armenia
		Georgia	GG-21: Human Disease Epidemiology and Surveillance of Especially Dangerous Pathogens in Georgia
		Armenia	AM-3: The Epidemiological Status of African Swine Fever in Domestic Swine Herds in the Tavush Marz Region, Republic of Armenia
2:40 PM - 2:50 PM	**Break**		
2:50 PM - 3:30 PM	**Session 12: Arthropods and Arboviruses**		
	[redacted]	Azerbaijan	AJ TAP-13: Investigation of Mosquito and Tick-Borne Arboviruses in Southeastern Azerbaijan
		Georgia	TAP-12: Analysis of Previously Identified *Rickettsia* Positive Georgian Ticks by Multi-locus Sequence Typing
		Pakistan	(BAA) The Role of Arboviruses as a Cause of Undifferentiated Febrile Illness in Sind, Pakistan
3:30 PM - 3:50 PM	**Closing Remarks**		

Presentation Summaries

February 8, 2017

Session 1

Doing Science in Difficult Places

PRESENTERS: (b) (6) (CRDF)/ (b) (6) (CRDF)/ (b) (6) (CRDF)/ (b) (6) (Univ. of Michigan)/ (b) (6) (Univ. of Veterinary Medicine Hannover – Germany)

Since 1995, CRDF Global has worked in over 40 countries using science to address priority issues in support of U.S. and foreign government agencies, foundations, and private sector organizations. Many of these countries may be considered difficult places to work due to past or ongoing war or other conflicts, political or economic instability, resource constraints, or significant differences in research capabilities, scientific norms and practices.

Application of tailored programming approaches, genuine commitment to understanding the environments in which partner country scientists operate, and flexibility form the foundation for effective science collaboration in difficult places. Ultimately, scientists are eager to engage and work together in search of solutions to the world's problems.

In 2013-2016, CRDF Global's work with scientists and researchers from Iraq and Afghanistan in support of CBEP included a research grant competition, science fellowships and network building and exposure programs. CRDF Global's ability to meet program objectives required building trust and rapport with Iraqi and Afghan participants, regular communication, engagement of all necessary stakeholders, adaptability, flexibility, and creative solutions. The programs' success is, in large part, attributed to participation of scientists in the U.S., Germany, Italy, India, Australia, Switzerland, Jordan and Malaysia, who hosted Iraqi and Afghan researchers at their labs for trainings, lab tours, exposure visits and fellowships.

Presentation Summaries

February 9, 2017

Session 2: Chiroptera (Bats) – Important Reservoir Hosts of Emerging Viruses

Understanding the Risk of Bat-Borne Zoonotic Disease Emergence in Western Asia

PRESENTERS: (b) (6)

COUNTRY: United States/Georgia/Jordan

Bats are natural reservoir hosts to several emerging viruses with pandemic potential, including Ebola, Marburg, Nipah, and SARS and MERS-coronaviruses, but current research on the distribution of bats, diversity of their viruses, and potential for zoonotic disease emergence in Western Asia is severely limited. To fill this gap and contribute to biological threat reduction, we propose a hypothesis-driven One Health research project focused on characterizing bat coronavirus diversity and the risk of bat-borne zoonotic disease emergence. This will include extensive non-lethal field sampling of bats, screening and characterization of viruses from bat specimens with two regional partner laboratories currently operating within the region, the Lugar Center in Georgia and RSS in Jordan, and modeling emerging disease risk by combining viral data with host, geographic, and ecological data. Data for risk modeling will be collated across a larger region than our field sampling will allow through the creation of a collaborative Western Asia Bat Research Network (WAB-Net) – including key researchers and public health representatives from >12 countries. Research activities will be strengthened via laboratory exchanges and annual data sharing and capacity building workshops. This integrated, multi-disciplinary approach presents a coordinated strategy to advance scientific knowledge around transboundary zoonotic disease emergence risk in Western Asia to inform early detection, diagnosis, and response to support the Global Health Security Agenda and CBEP goals.

Investigating the Risk of Human Disease from Parasites of Small Mammals and Bats

PRESENTER: (b) (6)

COUNTRY: Cambodia

Emerging and re-emerging infectious diseases pose a significant public health challenge globally, with severe economic, social, and health consequences. It is estimated that the SARS outbreak alone cost over $50 billion dollars in lost global economic activity. The frequency of epidemics caused by newly emerging and re-emerging pathogens and the likelihood of rapid global spread have increased dramatically in recent decades, with Southeast Asia considered a hot spot for future emergence events. Small mammals and bats play an important role in the maintenance and transmission of select agents that infect humans such as *Brucella* species, coronaviruses, filoviruses, henipaviruses, hantaviruses/bunyaviruses, plague, rabies (lyssaviruses) and *Rickettsia* species. The global distribution of several species of small mammals and bats, in addition to the ever-increasing interface between humans and wildlife, ensures that cross-species transmission events will continue to occur, often with devastating effects. By proactively sampling animal populations in Cambodia to discern circulating parasitic genotypes and screening human sera for evidence of exposure, we can determine which parasites have human pathogenic potential. A standardized trapping regimen will allow us to understand which ecological and environmental variables are associated with host and parasite presence-absence, facilitating the creation of ecological niche maps and models to determine risk and inform future surveillance efforts across Southeast Asia.

Arthropod-Borne Viruses Associated with the Chiroptera of Uganda: Isolation and Characterization

PRESENTER: (b) (6)

COUNTRY: Uganda

This project aimed to achieve a better understanding of bats, their ecology and their potential roles in virus ecology. This has been done through graduate training and research, training in field techniques of capture and processing of bats for virus detection and characterization, and a compilation of reference calls of micro-chiropteran bats for Uganda. Field biosurveillance training was held with participants from NADDEC, UVRI and Makerere University at Zika forest. A graduate student was recruited and completed an ecological study on bats in the Kaptum cave. We conducted bat surveys from around Uganda and have collected voucher calls from 28 microchiropteran bats from over 10 localities in Uganda.

From our graduate student's research, we now know there are 6 bat species (*Nycteris thebaica, Rhinolophus landeri, Rhinolophus hildebrandtii, Hipposideros caffer, Hipposideros ruber,* and *Myotis bocagei*) in Kaptum cave. Although they may mix up, these seem to have preferred roosting corners in the cave defined by slight differences in temperature and relative humidity.

Besides Kaptum Cave, we have documented the existence of many other caves around the country with bats. These caves are frequently visited by local people for various reasons. This could inevitably expose such members of the local communities to aerosols in the caves. Collectively, this project has advanced our knowledge of bat ecology in Uganda and enhanced collaborative research between US and Ugandan institutions which will promote cooperation during future biosurveillance and outbreak events.

Bat Harvesting in India: Detection, Characterization and Mitigation of Emerging Infectious Diseases Risk

PRESENTER: (b) (6)

COUNTRY: India

Emerging infectious diseases pose a continual risk for humans, imparting major health and economic challenges. It is estimated that greater than 70% of these diseases originate in wildlife, which demonstrates the importance of understanding the diversity of parasites that circulate in animals. Bats are important reservoirs of several medically important viruses that have high case fatality rates, including rabies/lyssaviruses, the henipaviruses, SARS-like coronaviruses, and Ebola virus and the related filoviruses. India is one of the most biologically diverse countries in the world, however there are currently few reports of viruses detected in bats. Outbreaks will often begin from point source origins, as phylogenetic data from the recent Ebola outbreak in West Africa indicates. Therefore, studying specific interfaces where humans are exposed more frequently due to their proximity and heightened interactions with wildlife can provide critical information on exposure. Bat harvesting is a common practice across India, as concentrated efforts can yield high numbers of bats. This contact and processing of bushmeat is an opportune place for cross-species transmission to occur. By sampling the bat populations where harvesting occurs before, during and after the trapping, we can determine if bat harvests increase the shedding of viruses and what medically important viruses are circulating in these bat populations. This will allow us to generate risk models and understand which species may be natural virus reservoirs in India.

Evaluating Zoonotic Viral Sharing Among Bats, Primates, and People in a High-risk Transmission Interface in Southern Tanzania (VISHA Project)

PRESENTER: (b) (6)

COUNTRY: Tanzania

Through project partners, including Metabiota, University of California Davis, Sokoine University of Agriculture, and the Ifakara Health Institute, we are evaluating: 1) the risk of zoonotic virus transmission among bats, non-human primates (NHP), and people sharing a forest interface with high human-wildlife contact in Southern Tanzania; 2) the impact of bat and primate community composition on virus diversity; and 3) exposure to zoonotic viruses in high-risk human populations living at the forest interface. By investigating cross-species viral sharing at this high-risk interface, our team will increase the understanding of pathogen emergence risk and transmission between human and wildlife hosts, strengthen Tanzanian surveillance and diagnostic capacity for pathogens of pandemic and biosecurity concern, and identify key intervention points to reduce local viral spillover from wildlife into human populations. The VISHA project team is investigating the epidemiology of known and novel zoonotic viruses (including filoviruses) in bats, NHPs, and humans by: 1) characterizing forest field sites; 2) collecting bat and NHP specimens during wet and dry seasons; 3) collecting specimens from high-risk human groups near forest areas during wet and dry seasons; 4) testing wildlife and human specimens for potential zoonotic viral pathogens using virus family-level RT-PCR; 5) performing phylogenetic and other genomic analyses on all detected viruses; 6) identifying risk factors for human exposure to bat and NHP viral pathogens using spatial analyses and epidemiologic modeling; and 7) training Tanzanian scientists in surveillance, molecular diagnostics, genomic analyses, and field and laboratory biosecurity practices.

Session 3: Epidemiology & Biosurveillance – Group 1

Epidemiology and Ecology of Tularemia in Georgia

PRESENTER: (b) (6)

COUNTRY: Georgia

Our study investigated the epidemiology of human and animal tularemia in Georgia. The project included (1) the study of seroprevalence of tularemia among individuals living in geographic areas with tularemia, and healthy individuals; and (2) estimate risk factors for seropositive humans and animals. We also established active surveillance for human tularemia clinical cases with the goal of increasing the efficiency and tularemia diagnostic capability. Isolates from human cases (900 volunteers) for comparison to both current environmental and historical isolates (National Center for Disease Control and Public Health). From an environmental prevalence emphasis, we will establish active surveillance for *F. tularensis* in the environment, including a study of small rodents and associated vectors and identification of the sources of outbreaks among humans. Field sampling for active surveillance for *F. tularensis* in the environment included small rodents and associated vectors, linking with human cases and seroprevalence study among the population living in foci area. More than 60,000 vectors were collected and pooled into 6,000 collections. All strains were isolated, evaluated, and monitor patterns of antimicrobial resistance. A bacteriophage component of this project examined isolated *F. tularensis* strains by genomic sequencing, proteomics analysis (Ilia State University), and phage discovery (the Eliava Institute). Geographic information systems and genetic algorithm for rule-set production were used for pathogenic distribution. Ecological niche models were created for ectoparasite species and primary rodent vectors. Consistent differences were found by the expression of some proteins between the isolates. Real-Time PCR and Western immunoblotting further validated these differences.

Ecological and Epidemiological Study of *Yersinia pestis* and *Francisella tularensis* in the Northern Part of Azerbaijan Regions of Gusar and Khachmaz

PRESENTER: (b) (6)

COUNTRY: Azerbaijan

Robust surveillance of *Y. pestis* and *F. tularensis* was once carried out routinely, however since the collapse of the Soviet Union little funding has been allocated to such efforts, and thus almost no data are available on the current distribution of plague and tularemia foci and vectors in Azerbaijan.

TAP-10 project proposes to conduct surveillance of ectoparasite vectors of plague and tularemia in northern Azerbaijan, in an area with a historical presence of those diseases and located near known natural foci of plague and tularemia.

The project started in April 2015 and is being implemented by Khachmaz Anti-Plague Division (APD). Sample collection (arthropods: fleas and ticks) field activities were carried out over six months in spring, summer, and autumn of 2015. Sample collection was completed in total of 13 villages of Gusar and Khachmaz regions in September 2015. Collected ticks and fleas were sorted, counted, identified, pooled, homogenized and their nucleic acids were extracted. The extracted nucleic acid samples have been tested by PCR (Bio-Rad 96 instrument) for two targets for each of the pathogens. 8,216 ticks and 154 fleas have been counted, identified, and sorted. 1,269 tick pools and 55 flea pools have been created and homogenized and their DNA extracted. Primer and probe sets were optimized and testing is ongoing.

A One Health Approach to Brucellosis and Rift Valley Fever Surveillance in Tanzania

PRESENTER: (b) (6)

COUNTRY: Tanzania

Our One Health team, including partners from Metabiota, the University of California, Davis, Sokoine University of Agriculture, and Ifakara Health Institute is utilizing a transdisciplinary approach to investigate the epidemiology and genomic diversity of the zoonotic pathogens Rift Valley fever virus (RVFV) and *Brucella* in south-central Tanzania. The primary objectives of the project are to evaluate the influence of risk factors such as animal contact and climatic conditions on increased RVFV and *Brucella* infection among livestock, wildlife, and humans, and to enhance in-country capacity for RVF and brucellosis surveillance, prevention, and control. To attain these goals, the team is engaged in: 1) characterization of sites with historical RVFV and *Brucella* activity in humans and animals that represent diverse climatic and animal density variables; 2) concurrent pathogen surveillance for acute and convalescent infections among humans, livestock, conspecific wildlife, and RVFV mosquito vectors to elucidate key disease transmission pathways, 3) identification of climatic conditions and temporal patterns that increase disease risk among vector and host species; 4) characterization of the RVFV and *Brucella* spp. diversity detected in mosquitos, animals, and humans; 5) identification of potential cryptic wildlife maintenance hosts using serologic evidence of prior infection, or in the case of RVFV by using blood-meal analysis of recently fed virus-infected mosquitoes, and 6) the development of integrated models to predict geographic areas of increased pathogen maintenance and transmission to identify locations for targeted intervention strategies and enhanced disease surveillance.

Acute Febrile Illness Study among Patients in Nakhon Phanom and Tak Province, Thailand

PRESENTER: (b) (6)

COUNTRY: Thailand

There are many causes of acute febrile illness (AFI) including various emerging infectious diseases in Southeast Asia and Thailand. Such diseases can be difficult to differentiate by clinical signs and symptoms, leading to misdiagnosis and possibly serious consequences for patient care. Therefore, laboratory testing is necessary to establish an accurate diagnosis. The objectives of this study are to: 1) describe the etiologies for patients hospitalized with AFI, 2) estimate incidence of specific pathogens and monitor trends over time, 3) evaluate the performance of rapid diagnostic tests (RDTs), and 4) assess laboratory diagnostic accuracy based on specimen types and testing methods for disease surveillance and outbreak identification. The study is being conducted in two Thai border provinces, Nakhon Phanom in the northwest and Tak in the east. The project is divided into 3 phases: pilot, surveillance and research phase. In the pilot phase, demographic data, clinical information and routine laboratory results will be collected from eligible patients. During the surveillance phase, patients hospitalized with undifferentiated fever will be tested for bacteremia and dengue. If dengue is not diagnosed, samples will be tested for chikungunya, *Leptospira*, *Rickettsia* species, *O. tsutsugamushi*, and Zika virus. During the research phase, new RDTs for *B. pseudomallei*, *O. tsutsugamushi*, Zika and chikungunya viruses will be evaluated. Additional testing will be performed on an expanded range of bacterial and viral pathogens. This project will provide an increased spectrum of pathogen detection, improve the timeliness of pathogen characterization, and assess promising new advanced diagnostic tests for surveillance and clinical management in the region.

Differential Diagnostics Performed by Eliminating Especially Dangerous Pathogens and Simulation (EDP-DREAM) in Cases of Saiga Antelope Mortality

PRESNTER: (b) (6)

COUNTRY: Kazakhstan

Though several large die-offs in saiga antelope (*Saiga tatarica*) herds in Kazakhstan have been reported in the past decade, the intensity, and absolute threat to the species of the ongoing die-off in the Kostanay oblast of Kazakhstan is unprecedented. The loss of reproductive females and calves represents long-term impacts on herd recovery and may indicate an eminent threat to regional livestock health. Reports have suggested the saiga die-off may be related to pollution or plant toxins, yet it is unclear how such toxicity would result in 100% mortality of nursing calves. Such a rapid die-off could be explained by viral (or viral/bacterial) infection. Even though Foot and Mouth Disease Virus (FMDV) has been reported in saiga, relatively few viruses result in 100% mortality, particularly in calves. However, FMDV could potentially play a role in a multiple pathogen infection. This study will implement a differential diagnostic work flow to diagnose anthrax and other Especially Dangerous Pathogens (EDPs) that may be involved in the saiga die-off. The project will use high resolution GPS collar data from animals at-large during the die-off. The first field expedition began in September 2016, and experts from the lead KZ partner, the Research Institute for Biological Safety Problems (RIBSP), pursued saiga individuals of Ural population in West Kazakhstan and Atyrau Oblasts to take samples and fit them with collars. Fifty-four individuals were captured from the Ural population: fifty-two individuals in West Kazakhstan oblast, and two individuals in Atyrau Oblast. During the field expedition, the five available animal collars were placed on animals and found to provide a stable signal. All samples have been delivered to RIBSP and are being stored at the BSL-3 laboratory. Currently materials and reagents are bring procured. Dr. (b) (6) from University of Florida is the collaborator for the study.

Session 4: Viral Pathogens of Security Concern

Assessing the Seroprevalence and Genetic Diversity of CCHFV and Hantaviruses in Georgia

PRESENTER: (b) (6)

COUNTRY: Georgia

The proposed collaborative research project will assess the seroprevalence and genetic diversity of highly pathogenic bunyaviruses circulating in Georgia to include Crimean-Congo hemorrhagic fever virus (CCHFV) and hantaviruses causing hemorrhagic fever with renal syndrome (HFRS), such as Puumala (PUUV), Dobrava (DOBV), or Seoul (SEOV) viruses. Specific aims of the project are to: (1) Determine the prevalence and serological diversity of CCHFV and hantaviruses in patient populations using samples collected from previously funded CBEP projects, human samples (GG-21) and environmental samples (GG-19); (2) Establish multiplexed immunological and molecular diagnostic assays for detecting circulating strains of pathogenic bunyaviruses, and provide suitable training for sustainment of these assays and capabilities within Georgia; (3) Initiate active surveillance for CCHFV, and hantaviruses in the environment to include small rodents and ticks; (4) Identify and characterize the genetic diversity of CCHFV and hantaviruses detected in rodents and ticks; (5) Establish a DNA barcode reference library for local tick species, to facilitate future identification and incrimination of tick-borne disease in Georgia.

These studies will provide a baseline assessment of the potential for future outbreaks of CCHF and HFRS in this region. Rodent and tick samples will be tested by ELISA and RT-PCR for evidence of CCHFV or hantavirus infections. Selected samples will be sent to the USAMRIID for virus-specific neutralization tests in BSL-3 or BSL-4 containment laboratories.

Epidemiology of Emerging Viruses in Cameroon

PRESNTER: (b) (6)

COUNTRY: Cameroon

The geographical and socio-cultural diversity of Cameroon makes it vulnerable to many emerging tropical viral infections like, Ebola, Zika, Dengue, Lassa, etc. While some studies have been done on the epidemiology of some of these viruses, these dispersed studies remain insufficient as a basis for the implementation of effective public health measures in this regard. Our objective was to assess the circulation of certain emerging viruses in the Cameroonian population in order to make recommendations to strengthen their surveillance and reinforce the rapid detection of any eventual epidemic.

In this respect, different samples were collected from 6 sites of Cameroon in different regions. These samples were serum/whole blood from 1500 blood donors, blood/oral swabs from 500 febrile patients susceptible of carrying a potential viral infection, and samples from bats to look for potential viral reservoirs. Collection of samples is complete, but analysis is ongoing at French Arbovirus National Reference Centre (Marseille, France). Preliminary data are available from blood donors. All donor blood samples were analyzed by ELISA. Positive samples underwent seroneutralisation analysis, RT-PCR (for DENV) and serotyping and phylogeny subsequently. Blood donor sample preliminary ELISA positive results were as follows: WNV(61.6%),TBE(39,9%), DENV(56,9% with 7.6% positive on RT-PCR, serotyping in progress), TOSV(7%), RIFTV(0%), ZIKV(10,4%).

Our definitive findings hope to build a framework upon which public health decisions can be made to increase preparedness for an eventual epidemic of an emerging viral infection and improve laboratory detection capabilities at the national level.

Understanding Rift Valley Fever in the Republic of South Africa

PRESENTERS: (b) (6)

COUNTRY: South Africa

Rift Valley fever virus (RVFV) is a vector-borne pathogen causing significant livestock, wildlife and human morbidity and mortality, and results in significant economic damages and food security concerns. RVFV has spread from Africa to the Arabian Peninsula, has the potential to spread to the Americas, and is considered a potential bioweapon. In South Africa, we have a multi-disciplinary team working to correlate environmental factors with vector succession and abundance, understand the role herd immunity may play in the occurrence of outbreaks and characterize the risk to people working in high-risk occupations. Initial analyses indicate that there are several vegetation and soil characteristics associated with locations of animal cases of RVF during the 2010-2011 outbreak. The baseline seroprevalence (representing herd immunity) in livestock have been established and experiments are running to understand how this may change. In people, the seroprevalence against RVFV is 0.1. Each year we hold national-level and local stakeholders' meetings to disseminate the data to national and provincial departments of agriculture, health and wildlife, as well as to farmers and workers, animal production groups and the weather service. Through this collaborative, One Health approach the project has become more robust and has strong support locally. The resultant data represents a significant step toward improving prediction of outbreaks and understanding how RVFV might spread, what effect climate change may have on the virus, how vaccination strategies may affect the risk of an epizootic and the risk of translocation to naïve countries, such as the United States.

Biosurveillance for Henipaviruses and Filoviruses at the Agricultural Animal-Human Interface in Malaysia

PRESENTER: (b) (6)

COUNTRY: Malaysia

The henipaviruses and filoviruses include Hendra virus (HeV) and Nipah virus (NiV), and several species of Ebola (EBOV) and Marburg virus (MARV), respectively, which are highly pathogenic viruses and select agents capable of causing public health emergencies of international concern. Bats are recognized as reservoirs for both henipa- and filoviruses, and zoonotic transmission of these viruses from bats to humans via domestic animals has occurred in Southeast Asia. The full diversity of henipa- and filoviruses in bats and their potential to infect livestock and people is unknown. This project will enhance early detection and surveillance capacity in Malaysia by: 1) transferring Luminex-based technology with validated reagents to detect IgG antibodies against henipa- and filoviruses to Government of Malaysia partner labs in wildlife, livestock and human health sectors; 2) training laboratory personnel to develop and utilize Luminex-based assays to identify exposure to henipa- and filoviruses; 3) conduct biological surveillance in wildlife (esp. bats), livestock and people around indigenous communities that hunt wildlife and on farms in Peninsular Malaysia, where there are high levels of contact among people and animals. Based on building local capacity for hypothesis driven research and improved use of technology, this project will help characterize the distribution and spillover potential from bats of henipa- and filoviruses in Peninsular Malaysia. Activities will be coordinated with and complimentary to the USAID Emerging Pandemic Threats: PREDICT program and surveillance data will be shared with the Govt. of Malaysia (GoM). The proposed project is closely aligned with the aims of the Cooperative Biological Engagement Program in that it will support biosurveillance and capability building, engages partner-country scientists, and promotes a One-Health approach to threat reduction.

Session 5: Community Outreach to Combat African Swine Fever

ASF Public Outreach Project Overview

PRESENTER: (b) (6)

The Defense Threat Reduction Agency (DTRA) sponsored a four-country regional public outreach project to combat African Swine Fever (ASF). The primary objective of the project was to develop a comprehensive and sustainable regional network of knowledge, expertise, and know-how for the control and prevention of ASF emerging events through a joint-effort based Threat Agent Detection and Response (TADR) Activity Project (TAP) that combines four countries (Armenia, Georgia, Kazakhstan, and Ukraine) in a common activity-based Science plan. To achieve this goal, delegates from each country worked with project implementers to identify gaps in knowledge, legislative framework, and outbreak preparedness. As a pathogen of high bioterrorism potential, veterinary health importance, and/or responsible for major economic instability, it is of critical importance that ASF-threatened and ASF-affected countries have appropriate infrastructure to accurately and rapidly identify and report ASF activity and types to international veterinary health agencies, as well as provide follow-up concerning the spread of the virus. This project sought to identify key human resources and veterinary health systems in order to initiate implementation of solutions to address recognized gaps, thereby enhancing the capacity by which to mitigate the risk of ASF. The project used a “train-the-trainer” approach to successfully reach over 10,000 farmers, veterinarians, pig traders, rangers, and hunters among others, teaching the signs and symptoms to identify ASF along with the proper reporting measures. This project was the first four-country public outreach project sponsored by DTRA and is considered a monumental success due to the large numbers reached. The project also improved collaboration among regional partners with the hopes of additional similar outreach efforts on other pathogens and diseases of public health importance in the years to come.

Community Outreach to Support Understanding of ASF Ecology and Epidemiology in Eastern Europe: Training and Implementation for Methods and Strategies for Control and Prevention

PRESENTER: (b) (6)

COUNTRY: Armenia

African swine fever (ASF) is a highly contagious viral disease of swine populations that can have significant economic consequence. It was successfully eradicated from most of the Eurasian continent almost 30 years ago, but was re-introduced in Georgia in 2007. Since then ASF has spread widely affecting swine in Georgia, Azerbaijan, Armenia, Ukraine, and Russia.

The primary goal of this project was to develop a comprehensive and sustainable regional network of expertise for the control and prevention of ASF through a joint-effort-based project combining four countries: Armenia, Georgia, Kazakhstan, and Ukraine. The project aims to identify and then educate persons working in the pig production chain (e.g., pig keepers, butchers, community veterinarians) to recognize clinical and epidemiological patterns of ASF. The training will allow these workers to understand (1) common routes of exposure, (2) preventative measures, (3) how to recognize clinical signs, (4) the importance of reporting to veterinary authorities, and (5) how to respond to suspected ASF cases.

In Armenia, a Knowledge and Attitude survey was implemented through veterinary authorities to assess the knowledge of the chosen target groups in ASF. For the public outreach campaign ten inspectors and ten epidemiologists were selected to train target groups, but due to the short time just 301/603 veterinarians, 1000/2412 farmers and 100/2670 hunters have been trained to date (Map 1). An additional 1000 farmers were subsequently educated by community vets during their routine work after the public outreach campaign ended. Over the course of the project and its aftermath, 3000 booklets and 150 posters were distributed and 2401 total persons informed.

This project facilitated the development of a sustainable capacity to implement outreach campaigns for future disease outbreaks in the region. Countries receiving training were taught how to run an outreach campaign including how to identify the target audience, how to produce effective educational materials, and how to effectively distribute those materials.

Community Outreach to Support Understanding of ASF Ecology and Epidemiology in Eastern Europe: Training and Implementation for Methods and Strategies for Control and Prevention

PRESENTER: (b) (6)

COUNTRY: Georgia

In 2007, Georgia was affected by a nationwide outbreak of African swine fever (ASF), and outbreaks were simultaneously reported in three different locations across the country. Monitoring ASFV in Eastern European countries is a top priority for the U.S. Defense Threat Reduction Agency (DTRA). The primary objective of the project was to develop a comprehensive and sustainable regional network of expertise for the control and prevention of ASF events through a collaborative project that united four countries (Armenia, Georgia, Kazakhstan, and Ukraine). Gaps in knowledge, legislative framework, and outbreak preparedness in the partner countries were identified. Additionally, this project contributed to long-term sustainability by conducting outreach campaigns for future disease outbreaks. Georgian directors were taught to lead and implement outreach campaigns, including how to identify target audiences (e.g. local farmers, veterinarians, and members of the pork industry) and efficiently create and disseminate educational materials. Over 450 veterinarians and farmers were trained, and 20,000 educational materials were distributed. Pre and post ASF campaign tests were developed to track progress; post-test scores were 16% higher than pre-test scores, which indicates that the outreach program was successful. Regional and state veterinarians, along with government agencies will be responsible for measuring the long-term success of these programs through laboratory results, monthly disease reports, and veterinarian updates. The number of ASF outbreaks are an indicator of the long-term success of the outreach program.

Community Outreach to Support Understanding of African Swine Fever (ASF) Ecology and Epidemiology in Eastern Europe

PRESENTER: (b) (6)

COUNTRY: Armenia

In 2015, the State Scientific and Research Institute of Laboratory Diagnostics and Veterinary and Sanitary Expertise (SSRILDVSE) and Institute of Veterinary Medicine (IVM) of the National Academy of Agrarian Sciences took part in an ASF outreach activity implemented within the Defense Threat Reduction Agency (DTRA) Cooperative Biological Engagement Program (CBEP) in Ukraine. The project aimed at establishing a regional alliance between Armenia, Georgia, Kazakhstan, and Ukraine to exchange experience, raise awareness, and provide education on ASF. Specialists from SSRILDVSE and IVM were trained as trainers before implementing their own outreach program in Ukraine. During in-country workshops and 14 training sessions in 14 Oblasts of Ukraine, veterinarians from 307 rayons, 531 epizootologists of state regional veterinary administrations and 4482 veterinary doctors of district animal hospitals were educated in the country. Additionally, information materials (flyer and poster) were developed. They included information about the clinical and epidemiological patterns of ASF, common sources and routes of exposure, preventative measures, how to recognize symptoms, and how to respond to suspected ASF cases. 100,000 flyers were printed and distributed among farmers and populations, as well as 1,500 posters for veterinarians in 24 Oblasts. Due to these efforts, necessary information was brought to the attention of veterinary doctors at district animal hospitals who communicate directly with farmers and persons that work with swine. This project demonstrated an approach for conducting and applying a public outreach program in Ukraine that can be used to raise awareness and help mitigate future outbreaks of ASF and other diseases.

Session 6: Transboundary Animal Diseases

Foot-and-Mouth Disease Virus Surveillance and Ecology in Vietnam

PRESENTER: (b) (6)

COUNTRY: Vietnam

The purpose of this project is to improve understanding of the epidemiology of foot-and-mouth disease virus (FMDV) in the Vietnamese setting using state of the art tools for genetic characterization of the virus in this endemic context. The intended goal is to mitigate the impact of this disease on local agriculture and improve preparedness for potential disease incursions in the US homeland with awareness of emerging virus strains.

Foot-and-mouth disease (FMD) is a contagious viral disease of domestic and wild cloven-hoofed animals, most notably cattle, pigs, sheep, buffalo and goats. Despite recent successes in controlling the disease in Europe and some parts of South America, FMD remains one of the most important infectious diseases of livestock due to the potential impact of an outbreak on trade in animals and animal products.

The project is currently operating in the second year of the (expected) four year period of performance. Five of the seven defined project objectives are either completed or well-underway. Two objectives are still in the planning phase. Current and ongoing activities in the field consist of passive surveillance of all FMDV outbreaks reported to the Vietnam Department of Animal Health and active surveillance of healthy livestock in six provinces spanning northern, central and southern Vietnam.

This collaborative endeavor has already resulted in two published, peer-reviewed scientific papers and two more papers have been submitted for peer review. Specific accomplishments achieved within the project include 1) genetic characterization of over 160 novel strains of FMDV, 2) enhanced understanding of risk factors for FMDV infection, 3) unique analyses of movement of FMDV strains across regions of Vietnam, and 4) improved understanding of the role of asymptomatic carriers in FMD epidemiology in Vietnam.

Whole Genome Sequencing of African Swine Fever Virus in Kenya

PRESENTER: (b) (6)

COUNTRY: Kenya

African swine fever (ASF) is a lethal disease of domestic pigs caused by a large DNA virus. In endemic areas ASF virus (ASFV) circulates in asymptomatic wild pigs and ticks and can remain stable in pork products for several months. ASF is endemic to Africa and was restricted only to that continent until 1957. Since then it has spread to Europe, Latin America and Asia. It is currently endemic in Russia and the Caucasus. There is a real risk of accidental or deliberate introduction of ASF to the United States of America (US), which would be devastating to the US $1.25 billion/year pig industry. There are at present no control measures other than test and slaughter. The objective of the proposed research is to determine the genome sequences of at least 60 ASFV isolates, selected so as to include as many as possible of the major genotypes for which genome sequences are currently lacking. These will be made available to veterinary and biosecurity authorities in the US and endemic countries through GenBank. This knowledge will enable more precise identification of the source of future ASF outbreaks, both within and outside Africa. It will also underpin development of future ASF control tools including diagnostic products and vaccines. The project will build ASFV research and surveillance capacity in Africa to support future disease control efforts.

Analysis of the Threat of Spread of African Swine Fever and Classical Swine Fever in Wild Boar Populations in Ukraine: Improving Diagnosis, Surveillance, and Prevention

PRESENTER: (b) (6)

COUNTRY: Ukraine

Listed as high priority pathogens by the World Organization for Animal Health (OIE), African swine fever virus (ASFV) and classical swine fever virus (CSFV) cause epizootically and economically significant animal diseases. Since 2012, the number of reported ASF outbreaks in Ukraine has increased, with 148 confirmed ASF incidents registered in 18 Oblasts of Ukraine (households - 123; wild boar populations – 24; infected object - 1) including 91 in 2016 (84 in the households and 7 in wild boar population). In order to strengthen the accuracy and effectiveness of ASF/CSF diagnostics in the country and to provide Ukrainian scientists with the ability and infrastructure to quickly and accurately monitor ASF and CSF movement, DTRA supported TAP-6 project that commenced on 1 September 2016. The aims of this project are (1) to analyze the distribution of ASFV and CSFV among wild boar populations inhabiting regions of Ukraine, which border the Russian Federation, Belarus, and Poland, and (2) to evaluate the risk of transmission to domestic pigs in the country. In addition to ELISA and PCR, monitoring targeted wild boar populations in Ukraine for ASF and CSF will be accomplished by using genomic-based biosurveillance methods and trainings on real-time PCR, molecular analysis, phylogenetic analysis, and complex sequence data analysis. This will provide an improved scientific basis to optimize current interventions and develop new tools and strategies to reduce the risk of ASFV transmission to domestic pigs. These biosurveillance efforts will facilitate development of ASF and CSF control strategies, which will contribute to limiting the spread of both infectious agents.

Research and Development of Countermeasures to Support the Control of Foot and Mouth Disease Virus (FMDV) in Uganda

PRESENTER: (b) (6)

COUNTRY: Uganda

Foot and Mouth Disease (FMD) virus causes an acute and the most contagious vesicular disease of livestock. The causative agent is a virus of the *Aphthovirus* genus in the *Picornaviridae* family. This disease in endemic in Uganda. Here, we report on a cross-sectional surveillance study designed to monitor and isolate FMDV serotype(s) circulating in the country divided into four regions: Northern, Western, Central and Eastern. A total of 38 representative districts from all the regions of Uganda were selected where 10,321 cattle sera and 1,300 oral-pharyngeal fluid samples were collected. All sera were analysed by the presence of antibodies directed against the virus non-structural proteins (NSP) using commercially-available kits at Makerere University. In addition, all oral-pharyngeal fluid samples were tested by rRT-PCR and virus isolation (VI) tissue culture followed by virus capsid sequencing to determine the FMDV serotypes by ARS, USDA at Plum Island Animal Disease Center. From samples collected during 2014-2015, thirty two percent of the serum samples were positive towards NSP antibodies. FMDV serotype O was isolated from Northern and Eastern regions while serotype SAT 2 was isolated from Western region of Uganda during samples collected in 2014. However, FMDV serotype SAT 1 (from the same region) and O were isolated in oral-pharyngeal fluid samples collected in 2015. The phylogenetic analysis of the P1 sequences for the viruses isolated in relation to geographical distribution of FMDV serotypes isolated during 2014-2015 in Uganda will be discussed. This information is of great importance for the improvement of disease control strategies and for vaccine strain selection for Uganda in the future.

African Swine Fever Threat Reduction Through Surveillance in Ukraine: Surveillance of Potential Arthropod Vectors

PRESENTER: (b) (6)

COUNTRY: Ukraine

African swine fever (ASF) is a high-consequence viral disease threatening the pig industry in Western Europe. In its native range, ASF virus (ASFV) is transmitted to pigs and maintained in Nature by soft ticks. Eastern European nations, including Ukraine, are considered to be endemic with ASF outbreaks occurring in pigs and wild boar. However, the factors underlying ASF westward expansion and reoccurrence on the affected territories remain to be fully understood.

Because certain soft tick species of the genus *Ornithodoros* can serve as biological vectors and reservoirs of ASFV, our research group focused on the development and implementation of vector surveillance in selected regions of Ukraine to establish methods that could be scaled up to the national level. The surveys conducted updated known soft tick distribution patterns. Field samples were identified morphologically as *Ornithodoros verrucosus*, and live specimens used to establish a laboratory colony of this suspected ASFV vector at the NSC IECVM. The *O. verrucosus* colony is a valuable resource to assess the risk of soft tick involvement in the epidemiology of ASF in Eastern Europe.

Ukrainian scientists developed research capacity in soft tick biology, collection methods, rearing and colonization techniques, and vector-host-pathogen interactions at USDA-ARS locations, and collaborating universities in Texas. Results of the project were presented at five scientific national and international conferences, summarized in a book published in Ukrainian and English, and two research papers published in international peer-reviewed journals.

Molecular Epidemiology and Ecology of *Yersinia* spp in the Transboundary Plague Endemic Territory in Georgia and Azerbaijan

PRESENTER: (b) (6)

COUNTRY: United States, Georgia, and Azerbaijan

Plague has been known in Caucasus region for many centuries, with the reports in Georgia dated back to the XI century and in Azerbaijan to VII century. The absence of recent reports can be partially explained by reduced surveillance, but also by the possibility of existence of atypical strains of *Y. pestis*, which can be missed by the testing laboratory due to presence of non-culturable strains as well as an absence of some genetic loci which would render the use of common molecular markers ineffective. The investigations of natural foci of plague in both Georgia and Azerbaijan have resulted in numerous cases of isolation of *Yersinia* species other than *Y. pestis*. The exchange of genetic material between *Yersinia* strains can challenge the detection of the strains of *Y. pestis*. We hypothesize that: 1) diverse *Yersinia* species, including *Y. pestis* and *Y. pseudotuberculosis* coexist; 2) *Yersinia* diversification is driven by host ecology; 3) *Yersinia* diversification is driven by exchange of genes within rodent populations; and 4) novel genetic methodology can improve identification of *Yersinia* species. To test these hypotheses, we propose: 1) ecological surveys targeting rodent populations in the transboundary focus located in both Georgia and Azerbaijan; 2) development of culturing and PCR-based procedures for detection of multiple strains of *Yersinia*; 3) molecular screening of the collected rodent and ectoparasite samples; 4) comparative analysis of genomes of obtained strains of *Yersinia spp*; 5) spatial analysis of distribution of strains of *Yersinia spp.* This project will result in enhancement of the plague biosurveillance capacities in the endemic regions of Georgia and Azerbaijan. This will be the first comprehensive project investigating and modeling plague foci in the South Caucasian region, which will foster improvements in national and international public health, surveillance, and biodefense efforts. This project will not overlap with national surveillance efforts.

Effect of *Rickettsia* spp. upon Fitness of *Yersinia pestis* in Fleas that Vector Plague in the Republic of Kazakhstan

PRESENTER: (b) (6)

COUNTRY: Kazakhstan

The goal of this study is to strengthen surveillance of important flea-borne human infectious diseases (plague and rickettsioses) in the context of vertebrate reservoirs, invertebrate vectors (e.g. ectoparasites), and infectious disease agents. Plague and certain rickettsiae are flea-borne diseases that share similar characteristics in disease symptoms, case definitions, and association with co-infections. These data will be very important to health authorities and government agencies in Kazakhstan and the US, where they will support important DTRA objectives including: enabling a partner country to detect/identify/report disease outbreak (naturally occurring or intentional) and providing a system that is sustainable by that partner country's budget and infrastructure. From a public health standpoint, it is important to know which vertebrate species are reservoirs for particular infectious agent(s) and if they are infested with ectoparasites. The Kazakh Science Center for Quarantine & Zoonotic Diseases (KSCQZD) is the lead institute in the implementation of the project, while the anti-plague stations will be involved in providing samples for research. This project will study the effect of flea-borne rickettsial infections upon the fitness of *Yersinia pestis* (causative agent of plague) within plague vector fleas obtained from small mammals in various environmental settings of Kazakhstan. Specifically, the study hypothesizes that in austere environments, rickettsiae and *Y. pestis* will compete for limited resources within the invertebrate host, allowing one species to out compete the other.

Development of the Epidemiological Forecasting System for Zoonotic Diseases Employing GIS Technology

PRESENTER: (b) (6)

COUNTRY: Ukraine

The project was a multi-year study focused on surveillance, mapping, and modeling the spatio-temporal and ecological patterns of *Francisella tularensis* and *Bacillus anthracis* in Ukraine. Collaborative efforts of researchers from the institutes of the Ministry of Health of Ukraine and National Academy of Agrarian Sciences of Ukraine included:

- analyses of historical tularemia and anthrax data sets;
- active surveillance for *Francisella tularensis* in small mammals and ticks and validated integration of these collections with appropriate culture and PCR-based analyses at the laboratory;
- surveillance and environmental sampling for *Bacillus anthracis*, with the integration of appropriate PCR-based detection assays in the laboratory;
- serological (ELISA) tests of sera sampled from wild boars;
- forecast pathogen outbreak using advanced spatial analyses, analyses with Geographic Information Systems (GIS) and Remote Sensing (RS) approaches to define the geographic extent of the pathogens and landscape dynamics that effect those distributions.

Throughout the period of performance, databases related to the epidemiological situation of tularemia and anthrax in Ukraine and geospatial data were developed and analyzed and areas historically at risk were determined for each disease. Ukrainians obtained extensive training in a variety of GIS and spatial analytical techniques, as well as research support at the sites and at the University of Florida. GIS and laboratory capacity at UCDCM and IVM were established. The GIS sector at IVM is now capable of sustaining basic GIS data development. Much of the work from the UP-2 project has been or will be published in the peer-reviewed literature.

Creation of Sustainable Immunodiagnostics

PRESENTER: (b) (6)

COUNTRY: Georgia

The primary goal of this project was to provide Georgian scientists with critical educational tools that will help detect biological threats using immunological assays developed within their own laboratories. This included training on developing and validating immunoassays for proteins and antibodies (monoclonal [mAbs] and polyclonal). The project also provided the tools necessary to develop novel diagnostic measures against new or re-emerging biological threats. Antigenic material from *Francisella tularensis* and *Brucella* species (both endemic in Georgia) were used for the development of antibodies and subsequently for immunoassay development and testing. Collaborators from the Naval Medical Research Center (NMRC), helped develop and validate antigen production, antibody production and purification, ELISA development, optimization, and validation. Polyclonal antibodies and mAbs were raised against *F. tularensis* and *Brucella* spp. whole cell extracts. Antibodies of interest were selected by affinity, and were purified; corresponding ELISA procedures were optimized and validated. In addition, the antibodies were characterized by Western immunoblotting. Antibodies protein targets were examined on polyacrylamide gels, in-gel digestion, and liquid chromatography-mass spectrometry. Using this approach, chaperone protein DnaK and GroEL/ES from *F. tularensis* were identified as a candidate targets of MAB 3.2 and MAB 8.2. As a result, this project yielded a comprehensive suite of protocols that can be used by Georgian scientists to develop assays and scale up production of reagents for commercial production and distribution.

Medical/Biological Mapping of Tularemia Natural Foci Cases, Reservoirs, and Vectors Using GIS in Armenia

PRESENTER: (b) (6)

COUNTRY: Armenia

Background: Past studies have reported over 95% of Armenia is endemic for tularemia. The development of a Geographic Information System (GIS) database and mapping system will provide a tool for tularemia data management and introduce a sustainable technology for continued surveillance in Armenia.

Methods: A retrospective study using the archives of NCDCP centers across Armenia. For GIS data processing we used the ArcGIS 10.1. The extensions Spatial Analyst and Geostatistical Analyst from ArcView GIS were utilized to process data.

Results: In the period 1981-2012, tularemia epizootics were recorded in 27 of the 38 administrative regions of Armenia. These cases involved five species of rodents, one type of insectivore, ticks of the *Ixodidae* and *Gamasidae* genera, and three species of flea. The optimal habitat was characterized by middle mountain steppe landscape zone at 2,065 to 2,407 meters above sea level, receiving 600-800 mm yearly precipitation, and moderate climates with short cool summer and cold winters or dry warm summers with cold winters. A total of 266 human cases of tularemia were recorded in Armenia from 1996-2012. GIS mapping showed that 199 tularemia human cases were registered in the steppe vegetation zone, 224 cases- in a zone with moderate, relatively dry warm summers and cold winters (1400-2300m), 206 cases in a middle mountain steppe zone.

Discussion: The developed model showed an association ($p < 0.05$) between number of human cases and the number of epizootic sites, number of isolates, and percent of samples with positive cultures. The analysis conducted using GIS methodology delineated the risk zones with a high probability of tularemia occurrence. Identification of high-risk areas will serve public health officials in focusing surveillance efforts.

Session 8: Avian Transmissible Diseases

Genomic, Epidemiological, and Biological Characterization of Newcastle Disease Virus Isolates from Ukraine

PRESENTER:(b) (6)

COUNTRY: Ukraine

The genetic diversity of Newcastle Disease virus (NDV) in Ukraine was studied for the first time. Newcastle disease (ND) is caused by the virulent forms of this virus that affects a wide range of wild and domestic bird species. At least 15 genotypes cause periodic large-scale outbreaks with extensive morbidity and mortality in avian species worldwide.

We conducted passive surveillance in poultry and active surveillance in wild birds in the Southern-Eastern territories of Ukraine and bordering regions with Russia. These studies characterized viral distribution and identification of predominant circulating strains from 1967 to 2016, including the characterization of repository viruses. Genotype identification and pathotype of NDV were determined by direct sequencing of the complete F-gene and full genome using next-generation sequencing. Based on the sequence of the fusion protein cleavage site a more precise pathotyping in eggs and chickens was conducted using isolates that represent the most typical isolates of each of the geno-groups.

Migratory birds in Ukraine are part of east-to-west and north-to-south migration routes. The phylogenetic study allowed to determine the relationship of Ukrainian isolates with other viruses from Eastern Europe, Asia and Africa and a better understanding the transcontinental movement of NDV.

In addition, training programs on standard operation procedures for BSL3 laboratories, biosecurity and biosafety practices were implemented for Ukrainian scientists. Results from this collaboration were presented at three conferences and summarized in six publications in an international journal.

Newcastle Disease: Surveillance, Molecular Epidemiology, and Control of NDV in Kenya

PRESENTER: (b) (6)

COUNTRY: Kenya

Although Newcastle disease (ND) is a notifiable disease in Kenya, the disease is under-reported. Current ND control measures include vaccinations after hatching and outbreaks. Currently, there is no active surveillance for ND. Project objectives are to: (1) improve ND surveillance, detection, and diagnosis and reporting of pandemics and (re-) emerging pathogens; (2) facilitate & improve collaborative research amongst Kenyan institutions employing modern methods; and (3) characterize NDV strains, assess ND socio-economic impacts and identify risk factors contributing to NDV spread in Kenya. The project hypothesis is that virulent NDV reservoirs are asymptomatically maintained in wild birds and pet avian species that reside in the proximity of domestic poultry. Samples will be collected in four agro-ecological zones: (1) Zone II (tropical highlands) with poultry markets and where poultry is culturally significant; (2) Zone III (Kenyan food basket with crop-livestock farming system); (3) Zone IV (part of seasonal wild bird migratory and poultry trade routes); and (4) Zone V (free-range small-scale poultry farming). Sampling will be mainly on case-based and passive and active response surveillance. Positive samples identified by antigen detection, hemagglutination of allantoic fluids, or RT-PCR will be used to characterize and define viral strains circulating in Kenya. Ten-year (2005-2015) historical data on ND incidence and outbreaks have been analyzed and used to validate the project's sampling sites. Some of the sites have been visited and samples collected. Four MSc students, the key players in transfer of acquired technologies, have been recruited.

Molecular Characterization and Complete Genome Sequence of Newcastle Disease Virus Isolated in Kazakhstan

PRESENTER: (b) (6)

COUNTRY: Kazakhstan

Newcastle disease virus (NDV) is a highly contagious viral infection of birds, characterized by pneumonia, encephalitis, multi-site hemorrhages and destruction of internal organs. It is considered one of the two most devastating diseases in poultry and wild birds, similar to avian influenza. Since Kazakhstan is on the main pathways of transcontinental migratory routes of many wild birds, genomic analysis of circulating NDV strains in Kazakhstan can potentially provide insights to its genetic evolution and add insight to pathogenic characteristics. Only limited studies on the genetic variability of NDV strains in Kazakhstan have been conducted. The project aims to perform full genome sequencing of five (5) isolates, conduct phylogenic analysis, and develop correlation between genotypic features and pathogenicity of five isolates from chicken flocks of previous outbreak during a one-year period. The team will retrieve full genome sequence of representative isolates from open source genomic sequence repository (GenBank), design primer sets for sequencing, and conduct sequencing using the ABI 3130xl platform at the Research Institute for Biological Safety Problems (RIBSP). Bioinformatic analysis will be performed to determine phylogenetic placement of these isolates among those in Genbank, and analyze the likelihood of introduction to and spread from Kazakhstan. The sequence data will be deposited to the National Center for Biotechnology Information (NCBI) database.

Risk Assessment of Selected Especially Dangerous Pathogens Potentially Carried By Migratory Birds over Ukraine

PRESENTER: (b) (6)

COUNTRY: Ukraine

Highly pathogenic avian influenza (HPAI) and Newcastle disease viruses (NDV) cause serious diseases in domestic and wild birds, and also pose additional risk due to the potential for spillover into human populations. Monitoring of orthomyxoviruses and paramyxoviruses conducted by NSC IECVM in 2010-2015 within the USDA/ARS projects revealed 168 avian influenza virus (AIV) and ND viruses, 23 antigenic combinations, and new serotypes (APMV-13). The extreme variability of AIV can lead to new genetic variants of the virus with pandemic potential that could damage people. The geographical position, natural conditions, large amount of birds and several transcontinental migration routes contribute to the re-emergence of those pathogens that was confirmed by outbreaks of HPAIV H5N1 and NDV in 2005-2008 and HPAIV H5N8 in poultry in 2016-2017. Commencing on December 1, 2016 the project aims to survey areas of Ukraine for AIV and NDV that may be harbored by wild birds associated with major northern and southern migratory flyways in the country. The project will assess the ecologic, epizootic, and epidemiologic risk of disease transmission. Bird observations, viral detection data, and Geographic Information System will be used to analyze and predict the anthropogenic impact on viral prevalence and type. The project will support local capacity building through enhancement of diagnostic capability, data management and analysis, and reporting. The project supports a One Health approach through linkage of researchers from the Institutes of the Ministry of Health, National Academy of Agrarian Sciences, and State Service for Food Safety and Consumer Protection of Ukraine.

Detection and Molecular Epidemiologic Analysis of Especially Dangerous Pathogens in Backyard Poultry, Commercial Broilers and Waterfowl in India

PRESENTER: (b) (6)

COUNTRY: India

Emerging and re-emerging respiratory diseases in poultry, especially velogenic viscerotropic Newcastle disease (vvNDV) and highly pathogenic avian influenza (HPAI), present a major threat to animal and public health worldwide, particularly in rapidly developing nations such as India. The core viral pathogens of human and animal concern involved in Respiratory Disease Complex of poultry have not been studied. To fill this knowledge gap, we propose to test three hypotheses. **Hypothesis 1.** The respiratory tract viromes from commercial and backyard flocks (herein referred to as domestic poultry) and waterfowl in Haryana, Odisha and Kerala are a potential source of especially dangerous pathogens (EDPs); **Hypothesis 2.** Molecular genotyping approaches will identify host- and region-specific fingerprint profiles of select agents including vvNDV and HPAI; **Hypothesis 3.** Next generation sequencing will help identify nucleic acid signatures of novel / emerging viruses in domestic poultry and waterfowl. The overall goal of this project is to discover novel viruses and enable the development of genomics-based strain-typing capability of EDPs and emerging viral pathogens from avian sources in academic research settings in India. Molecular epidemiologic analysis and comparative metagenomics investigation of respiratory viromes of domestic poultry and migratory birds, using a comprehensive and statistically robust stratified random sampling approach, will provide a strong foundation for the development of evidence-based approaches for implementing sustainable measures to control EDP. This investigation will also help generate data and tools, build sustainable capabilities, and enhance domestic and international academic partnerships critical to characterizing the zoonotic pathogens associated with domestic poultry and waterfowl in India.

Presentation Summaries

February 10, 2017

Session 9: Bacterial Pathogens of Security Concern – Group 2

Characterization of NCDC Strain Repository by Next Generation Sequencing

PRESENTER: (b) (6)

COUNTRY: Georgia

The three year project was launched in March 2016. It is a collaborative effort of the Los Alamos National Laboratory (LANL), NM, USA and National Center for Disease Control and Public Health of Georgia (NCDC), Tbilisi, Georgia. LANL has been working alongside the Georgia National Center for Disease Control and Public Health (NCDC) Genome Center Facility at the R. G. Lugar Center for Public Heath Research in developing Next Generation Sequencing (NGS) and analytic capabilities. The proposed project leverages the technical capabilities at both institutes to complete sequencing and characterization of the especially dangerous pathogens stored in the freezer archive at the NCDC. Up to 100 isolates of *Yersinia pestis, Bacillus anthracis, Brucella spp*., and *Francisella tularensis* from the NCDC pathogens collections will be selected for draft sequencing on the Illumina MiSeq. The draft genomes will be comparatively analyzed against strains from worldwide databases, SNPs will be discovered and subjected to phylogenetic analysis.

During the past year, ten *Brucella spp*. and twelve *F. tularensis* were have been sequenced. *F. tularensis* strains were processed for genome assembly and phylogenetic analysis on CLC-Bio, EDGE and PHAME software. Two draft genome announcement manuscripts have been drafted. A two week training for one bioinformatician from the NCDC team was held at LANL in November 2016. The sequencing of the rest of the samples is in process at NCDC. Ten isolates will be chosen to forward to LANL for PacBio sequencing. NCDC staff, as well as students involved in the project, will exercise their sequencing and bioinformatics skills on the samples and data from this project. Additional skills will be acquired through advanced training on data generated by this project. Completion of this research project will provide novel genomic characterization of the NCDC's extensive pathogen archive, cement the collaborative network between the NCDC and US collaborators, and ensure the NCDC's ability to utilize advanced sequencing technologies as independent researchers.

High Resolution Chemical Characterization of *Yersinia pestis* Cells within Soil Matrices: Implications for Understanding Natural Foci and Telluric Reservoirs of Plague

PRESENTER: (b) (6)

COUNTRY: Pakistan

The persistence of *Yersinia pestis* in soil matrices suggests a novel, yet completely uncharacterized, environmental reservoir for plague organisms. This has critical implications for understanding natural plague foci,non-traditional transmission routes between hostsand its changing risks for humans, and finally, the detection of *Y. pestis* in environmental samples.To address this challenge, we have constructed a multidisciplinary project to examine the chemical and physical response of *Y. pestis* cells after exposure to soil habitats. The project includes high-resolution, single cell analytics to characterize the metabolism, surface chemistry, and structural changes in individual *Y. pestis* cells within this unique growth environment. Results from this work will increase basic understanding of pathogen ecology and the molecular mechanisms by which*Y. pestis* functions in environmental reservoirs outside of traditional transmission vectors.

This project is a collaboration between Virginia Commonwealth University (Richmond, VA, USA) and the HEJ Research Institute of Chemistry at the University of Karachi (Karachi, Pakistan). VCU is leading culturing efforts and surface characterization of *Y. pestis* cells using a range of high resolution microscopy and mass spectrometry techniques and the University of Karachi is developing novel techniques for chemical analysis of cell surfaces and in situ assays with nanoparticle-based probes. Training in Biosafety practices and microbiological characterization will facilitate collaborative pathogen research and build new capabilities at the University of Karachi that will complement its existing analytical strengths and create a new center in Pakistan capable of identifying unknown bacterial samples and conducting basic research on endemic pathogens.

Estimating Incidence and Socio-economic Impact of Brucellosis in Humans and Animals in Kajiado County, Kenya

PRESENTER: (b) (6)

COUNTRY: Kenya

Brucellosis is a common bacterial zoonotic infection, but there is limited data on burden of the disease in Kenya for humans and animals. To generate data to inform prevention and control strategies, we conducted a longitudinal study to determine incidence of brucellosis in humans and animals and estimate its socio-economic impact at the household level among a pastoralist community in Kajiado.

All households and their livestock in four sub-locations in Kajiado County were enrolled for follow-up for 12 months from 2015 to 2016. A subset of their livestock were recruited in to the study after screening for *Brucella* using RBPT, and sera was collected every four months and tested for *Brucella* IgG antibodies using ELISA. Incidence in humans was calculated from the number who were determined to be acutely ill with brucellosis from among those who presented at study health facilities, while incidence in livestock was the number of livestock that sero-converted for brucella antibodies between two sampling points. To calculate economic losses, direct losses in livestock were calculated using parameters derived from the incidence study and literature, and the results were analyzed in an analytical economic model.

801 households with a total of 4,729 humans and their 5,746 livestock were recruited in the study. Of the household members enrolled, 52% (n=2475) were males. The mean household (HH) size was 6 persons (range 1 – 19). Average HH income over a 3 months period was $ 820 (range $100 - $ 16,000). Overall incidence rate of brucellosis in livestock on ELISA sero-positivity between the first and the second visit was 0.0076 (8/1,000) cases per animal; 3 months at-risk equivalent to 0.0304 (30 animals per 1,000 animal-year at risk). Total direct losses due to brucellosis in livestock was estimated to be KES 6.6 Million (USD 66,000). The losses due to abortion accounted for 54% of the total losses in livestock. Estimated out of pocket expenditures incurred by HHs was $12.2 per human case (range $2 -$100). This study reports a high burden of brucellosis in humans in a pastoral community in Kajiado with substantial economic losses arising from brucella infection in livestock at the household level.

Lugar Center Regional Integration: Kafkas University Partnering for Molecular Epidemiology *of Bacillus anthracis* and *Brucella* species in Turkey

PRESENTER: (b) (6)

COUNTRY: Georgia

It is vital that effective collaborative links are built between the National Center for Disease Control and Public Health (NCDC), Richard G. Lugar Center for Public Health and institutions in neighboring countries to tackle shared common bacterial threats. Kafkas University's veterinary school, located in the northeastern Turkish city of Kars, serves a primarily agricultural region adjacent to the Georgian/Turkish border; diseases including anthrax and brucellosis, are endemic in these regions. Molecular typing tools were outdated at Kafkas. A study using the high-resolution molecular tools available at NCDC Lugar Center on common bacterial strains (*Bacillus anthracis* and *Brucella* spp.) on both sides of the border was developed. To achieve this aim, a team at the Lugar Center supported by WRAIR provided training to researchers from Kafkas University in the use of the state-of-the-art capabilities available at the Center. The project consisted of three main technical elements: bacterial DNA extraction; molecular analysis of *B. anthracis*; and molecular analysis of *Brucella* spp. Overall, 60 bacterial isolates including *B. anthracis* (n=30) and *Brucella* spp. (n=30) from Turkey were analyzed at the Lugar Center using multiple locus variable number tandem repeat analysis (MLVA) and single nucleotide polymorphisms SNP. MLVA and SNP typing results show genetic homogeneity of Georgian and Turkish *B. anthracis* strains, which may be caused by migration of the pathogens across the Georgia-Turkey border over time. This study also resulted in successful capacity-building efforts for pathogen typing for the Turkish researchers from Kafkas University.

Regional Study of the Ecology of Anthrax Foci in Georgia and Azerbaijan

PRESENTER: (b) (6)

COUNTRY: Azerbaijan

Anthrax is a livestock-borne zoonotic disease that is endemic in the South Caucasus region. It is hypothesized that both human and environmental factors affect the migration of *Bacillus anthracis* strains across borders and throughout regions. This study evaluates the following: (1) identify regional foci and spatial risk factors of anthrax to improve surveillance and documentation of the disease; (2) assess the genetic relationships of environmental *B. anthracis* isolates to better understand the organism's ability to persist in the environment and to allow for improved epidemiologic "trace-back" of human and livestock infections; and 3) collaborate on regional issues related to the control and management of a re-emerging infectious disease by sharing local/regional predictions of risk and molecular profiles of the pathogen. Collaborators from both Georgia and Azerbaijan have collected and tested soil samples for the presence of *B anthracis.* Positive samples were molecularly characterized and were examined using multilocus variable number tandem repeat analysis (MLVA), as well as global and regional single nucleotide polymorphisms (SNPs), including a well-established Georgian SNP typing panel. Spatial genetic pattern analysis and risk factor mapping were conducted using geographic information system. In turn, this regional collaboration will provide the potential for the cooperative development of strategies for the control and trans-boundary management of this disease.

Regional Study of the Ecology of Anthrax Foci in Georgia and Azerbaijan

PRESENTER: (b) (6)

COUNTRY: Azerbaijan

Anthrax is a livestock-borne zoonotic disease that is endemic in the South Caucasus region. Not much is known about the regional epidemiology of this pathogen nor the transboundary factors related to its persistence. The aim of this collaborative (Azerbaijan and Georgia) effort was to ascertain both human and environmental factors that may affect migration of *Bacillus anthracis* strains across borders and through regions. There are seven Azerbaijan rayons (Gazakh, Agstafa, Tovuz, Samukh, Gakh, Zagatala, and Balakan) bordering Georgia with recurring cases of both human and animal anthrax; from those, 30 villages were selected for sample collection. Samples were to be collected from known animal burial sites and areas of previous contamination, as evidenced by livestock outbreaks or human case records. Organisms isolated from the collected soil samples were to be characterized by biochemical methods (Gram stain, motility, Trypticase Soya Broth) and then by gamma phage. The nucleic acids extracted from presumptive positive samples were to be tested by PCR using Amplisense kits on BioRad.

A total of 804 soil samples have been collected from five rayons Nov 2015 - Sept 2016. From the 768 processed, over 200 samples exhibited colony morphology suggestive of *B. anthracis*; 10 were deemed positive by gamma phage. DNA extractions from 60 presumptives (by biochemistry and gamma phage) have been sent to Georgia Lugar Center for genotyping in April 2016 and were negative for *B. anthracis*. The samples should be retested by Tetracore PCR kit.

Environmental Surveillance of *Burkholderia pseudomallei,* Pilot Study in Lao PDR

PRESENTER: (b) (6)

COUNTRY: Lao PDR

The environmental bacterium *Burkholderia pseudomallei* (*Bp)* is the causative agent of melioidosis and a Tier 1 Select Agent. *Bp* is endemic in tropical soils and has been found in surface waters. To investigate the distribution of this pathogen in Lao PDR, its occurrence in rivers, and associated environmental factors, we studied 23 rivers (including the Mekong) in the South, Center and North of the country, applying culture-based methods and a specific quantitative real-time PCR assay to water filters and streambed sediments. Geochemical measurements included turbidity, a proxy for suspended sediment load which was measured on-site using a turbidity meter and confirmed in the laboratory by dry weight measurements. *Bp* was present in 9% of the rivers in the dry season. In contrast, we found *Bp* in the water of 57% of the rivers in the rainy season, 35% of them with associated *Bp*-positive sediments. Turbidity correlated positively with *Bp* presence (p=0.01). All *Bp* positive rivers were situated in the South and Center of the country. Our preliminary results provide evidence for a heterogeneous spatial and temporal distribution of *Bp* in Lao PDR. The seasonal dynamics and predominant occurrence of *Bp* in particle-rich water and the lower yield in sediments suggest that *Bp* is washed out with eroded soil during periods of heavy rainfall and transported by rivers. These findings will contribute to improved *Bp* risk modelling and health management strategies. Further laboratory and statistical analyses, including additional geochemical factors, and land-cover data analyses using geographic information systems, are in progress.

Melioidosis Research Coordination Network

PRESENTER: (b) (6)

COUNTRY: Thailand

Melioidosis is caused by *Burkholderia pseudomallei,* a Tier 1 select agent and an environmental bacterium commonly found in tropical countries. It is estimated that 89,000 deaths were caused by melioidosis per year worldwide. However, melioidosis remains under-reported due to its diverse clinical manifestation, incapacity of bacterial isolation and identification in low-resource settings, and limitation of national notifiable diseases surveillance systems in low and middle-income countries (LMICs). The unawareness of the disease becomes problematic for policy makers, clinicians and researchers in many LMICs because melioidosis is not perceived as a threat.

To solve the problems, the Melioidosis Research Coordination Network (RCN) was developed. The RCN aims (1) to make data of total number of culture-confirmed melioidosis cases and deaths worldwide openly available for policy makers, clinicians, funders and researchers worldwide, and (2) to support connections and communications among those stakeholders.

The RCN working group will call for information from clinicians and researchers at any hospitals or institutions worldwide for the total number of culture-confirmed melioidosis cases and deaths they observed yearly from 2012 to 2016. We will curate and make those data openly available on www.melioidosis.info. The system will be quite similar to PROMED but better curation and better supports from funders and melioidosis research community. The RCN will make the information of data contributors clearly available so that funders and other researchers know where they can fund melioidosis research and find collaborators, respectively. The RCN will also make information about potential support from contributors available, including bacterial identification and biosafety support.

Session 10: Emerging Infectious Diseases

Enhancing Capacity for Case Detection and Diagnosis of Febrile Zoonotic-related Cutaneous Lesions in Georgia

PRESENTER: (b) (6)

COUNTRY: Georgia

In the summer of 2013 an outbreak of febrile rash illness in herders in the Akhmeta region of Georgia was linked to a novel *Orthopoxvirus* (OPXV). The unique genetic profile of this emerging zoonotic disease made it difficult to identify using the existing molecular assays for OPXV species. Two other OPXV isolates have been identified in Georgia. One was found in 1986 in a rodent in the southeastern part of the country; the other was recently identified by the National Center for Disease Control and Public Health of Georgia (NCDC) and the Centers for Disease Control and Prevention (CDC) Poxvirus laboratory in a retrospective analysis of a suspected anthrax case that occurred in 2010. Activities undertaken by CDC Headquarters in conjunction with the CDC South Caucasus Office are part of a collaborative effort comprising three complementary proposals. The goal of these combined efforts is to: 1) identify the geographic distribution and natural hosts of the known virus, 2) determine if any other such viruses occur in this region, 3) investigate the impact of OPXV infection on dairy production and to at-risk individuals, and 4) increase in-country capacity to detect, identify, and respond to future outbreaks. Activities are approached through a One Health framework, promoting timely, actionable communication among in-country counterparts representing the animal and human health sectors.

Enhancing Capacity for Case Detection and Diagnosis of Febrile Zoonotic-related Cutaneous Lesions in Georgia

PRESENTER: (b) (6)

COUNTRY: Georgia

The discovery of a new *Orthopoxvirus* demonstrates the need for poxvirus detection and diagnosis capacity in Georgia (country). Human illness caused by this virus has implications for differential diagnosis of cutaneous lesion-producing zoonotic infections, principally anthrax. Simultaneously, animal infection may impact agricultural productivity and food safety. Therefore, accurate detection and case diagnosis is important for both humans and domestic animals. In collaboration with the CDC Atlanta, we are working to enhance capacity to detect, diagnose and report *Orthopoxvirus* infections. Health-care workers will be trained to recognize zoonotic-related cutaneous lesions to detect human cases; NCDC personnel will be trained to collect clinical specimens from patients with such lesions and from the rodents; NFA and regional veterinarians will be trained to recognize lesions associated with orthopoxviruses among domestic and wild animals as well as field collection of pertinent animal specimens. Emphasis will be placed on field and laboratory biosafety. Additionally, CDC Atlanta Poxvirus Team members will provide training for animal (LMA) and human sample (NCDC) processing with rapid qPCR-based diagnostic tests and serologic assays for case diagnosis. Retrospective case diagnosis is needed as testing by rapid qPCR on anthrax-negative samples has already identified an orthopox positive sample, thus warranting further investigation on stored samples. New assays will be developed and assessed for detecting any new orthopoxvirus variants in humans, rodents, domestic and wild animals found in Georgia. Activities will result in improved capacity for efficient identification of emerging orthopoxviruses, as well as biosurveillance capacity for orthopoxviruses in human and animal populations.

Multi-Year Prospective Cohort Study to Evaluate the Risk Potential of MERS-CoV

PRESENTER: (b) (6)

COUNTRY: Malaysia

Since its first appearance in 2012, the Middle East respiratory syndrome coronavirus (MERS-CoV) has emerged as a serious public health threat of global concern. As of December 2016, the World Health Organization has been notified of 1,879 laboratory confirmed cases and the case fatality rate is estimated at 35%. Beyond its high fatality rate, significant concern lies in the potential for MERS-CoV to spread beyond the Middle East, as was recently witnessed in the South Korea. In collaboration with the Ministry of Health Malaysia (MoHM) and the Malaysia Hajj Pilgrims Fund Board (MHPFB), a multi-year cohort of pilgrims departing for Hajj from Malaysia has been established to assess the risk that MERS-CoV infection poses to travellers to the Middle East. Pre- and post-pilgrimage blood specimens were collected for serologic analysis to estimate MERS-CoV exposure rates, while survey data will be used to stratify the risk of exposure by factors such as age, gender, geographic regions visited during the pilgrimage. For the 2016 Hajj cohort, a total of 568 participants were enrolled and 367 (65%) submitted to post-pilgrimage blood draws and data collection upon their return from the Hajj. To date, paired blood samples (pre- and post- pilgrimage) from 318 participants have been analyzed by ELISA for the presence of IgG antibodies to MERS-CoV. Preliminary findings suggest at least three individuals showed increases in MERS-CoV specific IgG titers post-Hajj. Confirmatory microneutralization assay is ongoing and planning has started for the 2017 Hajj cohort studies.

Middle East Respiratory Syndrome Coronavirus (MERS-CoV): Surveillance for Distribution and Prevalence in Kazakhstan

PRESENTER: (b) (6)

COUNTRY: Kazakhstan

Middle East Respiratory Syndrome (MERS) is a viral respiratory illness caused by a recently identified zoonotic coronavirus, MERS-CoV, which is transmitted from camels to humans. The southern border regions of Kazakhstan contain more than 150,000 camels, which creates the potential for endemic circulation of the virus. Bats have also been associated with presence of the MERS-CoV, and there are 24 species of bats which may carry related coronaviruses. Due to camel trade with MERS-CoV endemic areas, the project hypothesizes that MERS-CoV is likely present in the Kazakhstani camel population. One of the objectives is to determine the sero-prevalence and epidemiology of MERS-CoV in adult and juvenile camels in seven regions of Kazakhstan to verify the prediction that camel populations in Kazakhstan have been exposed to and generated antibodies against MERS-CoV. Also, the MERS-CoV genetic diversity in camels across Kazakhstan will be determined as will the identity of coronaviruses in Kazakh bats. The project has been approved by DTRA and kick-off was held on 14 October 2016. The lead institute for the study, KZ's Research Institute for Biological Safety Problems (RIBSP), has submitted paperwork requesting permit from Ministry of Agriculture for sample collection for the study. RIBSP also reported that they have a small group of pre-existing samples from camels and bats in storage that may be of use to the project. RIBSP is currently seeking to identify the location of camel farms that have interaction with camels from the Middle East. Drs. (b) (6) and (b) (6) from Duke-NUS Medical School are collaborators for the study. Currently materials and reagents are being procured.

Global Health, Emerging Infectious Diseases, and Food Safety Implications of Bushmeat Consumption In Tanzania

PRESENTER: (b) (6)

COUNTRY: Tanzania

“Bushmeat”, the meat and organs derived from wildlife species, is a major source of protein in many parts of Africa, and is often hunted illegally and transported to markets in unsanitary conditions, thereby representing an important conduit for the transmission of zoonotic pathogens. Despite considerable evidence that select agents, including *Bacillus anthracis*, *Brucella* and *Coxiella,* are frequently found in animals harvested for consumption in Tanzania, their distribution in bushmeat and the related human health risks are not known. To fill this critical knowledge gap, we have initiated a program to map the distribution of especially dangerous pathogens in bushmeat from three major ecosystems in Tanzania (Serengeti, Ruaha-Rungwa, Selous-Mikumi) during wet and dry seasons to capture spatial and seasonal variation in pathogen prevalence.

Preliminary PCR based-analyses of more than 500 fresh and dried bushmeat samples collected from 25 villages in the Western Serengeti revealed nucleic acid signatures of *Bacillus anthracis* and *Brucella* species in bushmeat. Further, host species identification with PCR sequencing of the *cytochrome B* gene suggests initial misclassification by bushmeat traders in a significant fraction (~ 40%) of the samples. Together with 16S rDNA based microbiome profiling, our preliminary investigations reveal the presence of major zoonotic pathogens, in bushmeat in Tanzania, and provide an opportunity to discover novel emerging pathogens. In the long-term, our research is positioned to provide a rational basis for defining and mitigating the public health risk associated with the harvesting, trade, and consumption of bushmeat in Tanzania.

Etiology of Severe Acute Respiratory Infections in Kuala Lumpur, Malaysia

PRESENTER: (b) (6)

COUNTRY: Malaysia

The epidemiology of severe acute respiratory infections (SARI) in adults in Asia is relatively understudied, but of critical global importance. This is because of the potential emergence from this region of rare/new pathogens posing a pandemic threat or a danger as potential weapons of mass destruction. Malaysia, situated in Southeast Asia, a known hotspot for emerging diseases, has had previous imported human cases of SARS-CoV, MERS-CoV, and H7N9, and H5N1 in birds. Melioidosis, caused by *Burkholderia pseudomallei*, is also endemic in Malaysia. Kuala Lumpur, the capital, is a major travel hub for immigrants and tourists. We hypothesize that potential biological threats do cause SARI in Malaysia, but that these remain largely undiagnosed due to lack of awareness and laboratory diagnostic capacity. Therefore, we propose to study the etiology of SARI in adults admitted to a teaching hospital in Kuala Lumpur over 3 years. We will establish a comprehensive panel of molecular assays to detect respiratory viruses and bacterial select agents, supplementing existing diagnostics for bacteria and mycology. This will enable our centre to detect sporadic cases and outbreaks of respiratory agents which are potential global threats. We propose a workshop for biosafety practices in diagnostic microbiology laboratories, and a practical workshop to teach these newly established assays to other laboratories in the country, thereby enhancing national capacity to safely diagnose these agents, and contribute to threat reduction. In addition, a number of samples testing negative to known viral agents will be analysed by next-generation sequencing for potential viral pathogen discovery.

One Health Surveillance for Brucellosis in Armenia

PRESENTER: (b) (6)

COUNTRY: Armenia

Brucellosis is a highly infectious zoonotic disease caused by bacteria of the genus *Brucella*. It is reported to be the most common zoonotic disease worldwide. The disease causes abortion, infertility and reduction in milk production in animals and a serious, recurring febrile condition in man which may become chronic and may affect any organ of the body. Successful treatment of the chronic form is very difficult. No effective treatment for brucellosis in animals has been described. The incidence of brucellosis in humans in unknown, but is suspected to be between 10 and 25 times larger than indicated by the number of cases reported to public health agencies.

Prevalence of brucellosis in humans and animals in Armenia and neighboring countries is known to be significant. However, due to the fact that comprehensive surveillance for the disease is lacking in both the human and animal populations, and the lack of shared data between human and animal health authorities, little can be said with certainty about how widespread the disease is in either population.

A “One Health Surveillance for Brucellosis in Armenia” project has just been initiated whose objective is to strengthen coordination and collaboration between the Ministry of Health and the Ministry of Agriculture during epidemiological and outbreak investigations of brucellosis, and increase the understanding of the disease burden in Armenia. A project outcome will be a draft document outlining a comprehensive surveillance and control system for brucellosis in Armenia.

Acute Febrile Illness in Uganda

PRESENTER: (b) (6)

COUNTRY: Uganda

Acute febrile illness (AFI) causes significant morbidity and mortality in the tropical countries including Uganda. While part of it is caused by malaria and other common treatable infections, a significant part is caused by unknown agents. VHFs have particularly occurred recently with more frequency in Uganda and the region. This study is undertaking systematic investigations to determine the occurrence of the select agents and other previously unknown highly pathogenic pathogens with potential for causing pandemic threats. The work leverages upon existing field and laboratory research capabilities within Makerere University Walter Reed Project (MUWRP) in the country. The project does sampling from already existing sentinel surveillance sites. We collect and test at least 26 samples from AFI patients per month from at least five geographically diverse hospital sentinel sites (Gulu, Mulago, Jinja, Bwera and Bombo Hospitals). In addition, disease vector distribution and mapping is being undertaken through vector collection and identification and climate data analysis for ecological niche modeling and risk assessment. The study is expected to generate data that contributes to create in-country capabilities for more robust systems for early detection and prediction of outbreaks and rapid response and to define risk factors and vector distributions important for long-term surveillance, early detection and rapid response, and intervention strategies in contribution to global biological threat reduction.

Infectious Etiologies of Acute Febrile Illness in the Azerbaijan Military

PRESENTER: (b) (6)

COUNTRY: Azerbaijan

Mortality rate attributable to infectious and parasitic diseases in Azerbaijan is 16.4 deaths per 100,000 population. After the collapse of Soviet Union, healthcare and public health deterioration created severe gaps in accessibility and allowed for emergence/re-emergence of infectious diseases. Epidemiological information about infectious syndromes in Azerbaijan is very limited, compounded by the lack of appropriate diagnostic assays and febrile illness knowledge, precipitating unconfirmed clinical diagnoses for most febrile illnesses. Infectious etiologies of acute febrile illness (AFI) among Azerbaijan military members have not been studied, thus causative etiologies are unknown. In the proposed study serum samples from patients with undifferentiated febrile syndrome will be tested for *Brucella* spp., Dengue, WNV, *F. tularensis*, rickettsia, Q fever, hantaviruses, CCHF and TBE using standard ELISA assays. Positive or indeterminate ELISA results will be confirmed using PCR or IFA. All laboratory analyses will be done at the Epidemiological Monitoring Station of the Ministry of Defense in Baku City. Most etiologies to be targeted are considered potential biological weapon agents. Identification of these along with incidence and probable transmission pathway will significantly increase preparedness of the Azerbaijan military medical system, develop sustainable biological agent detection capabilities, and contribute health surveillance data for Ministry of Defense (MoD) force health protection policy. This study will significantly improve laboratory skills for diagnosis to improve clinical management of AFI. In addition, findings will improve the capability to differentiate between infections with similar nonspecific clinical syndromes that are not currently diagnosed or are presumably underreported among military personnel in Azerbaijan.

Prevalence, Epidemiological Surveillance, and Laboratory Analysis of *Coxiella burnetii* in Georgia

PRESENTER: (b) (6)

COUNTRY: Georgia

Q fever is a zoonotic bacterial disease resulting from infection by the bacteria *Coxiella burnetii*. The goal of this study is to (1) investigate the seroprevalence of *C. burnetii* among exposed cattle and small ruminants across Georgia, (2) establish active surveillance and detection of cases of Q fever across Georgia, (3) collect isolates from veterinary cases, (4) implement and evaluate new diagnostic methods, and (5) study *C. burnetii* using GIS and DNA sequencing and genotyping. Samples were collected from nine regions by the National Food Agency (NFA) of the Ministry of Agriculture (MoA): 16,343 blood, 15,269 serum, 3,970 milk, and 5,156 swab samples were collected from these animals. Samples were tested by immunofluorescent assay (IFA), polymerase chain reaction (PCR), and bacteriology to detect *C. burnetii*. For IFA, 8,688 samples were tested: 218 were Phase I positive and 42 Phase I susceptive samples; and for Phase II 40 samples were positive, and 34 susceptive. In total, 1,311 samples were tested on ELISA: 1,218 were negatives, 76 positives, and 17 susceptive. We have tested 10,310 PCR samples and seven were positive. Bacteriological tests were performed on serologically positive/ susceptive samples, and PCR positive samples (n=340). Currently, we have isolated two cultures (confirmed by PCR). Due to the amount of sample processing, the project has been extended, and research is going.

Prevalence of *Brucella* Species and Bluetongue Virus Serotypes among Domestic Livestock or Ruminants in Southern Kazakhstan

PRESENTER: (b) (6)

COUNTRY: Kazakhstan

Brucellosis and Bluetongue (BT) are high consequence infectious diseases that affect domestic animals in Kazakhstan and worldwide. *Brucella* spp. and an array of Bluetongue virus (BTV) serotypes are considered endemic in Southern Kazakhstan where the largest concentration of cattle, sheep and goat herds in the country are located. Southern Kazakhstan is thought to be at high risk for these two diseases. The project will determine the prevalence of circulating *Brucella* spp. and BTV serotypes over a period of two years in domestic cattle, sheep and goat holdings in Southern Kazakhstan. A disease survey is the first step to link distribution of brucellosis and bluetongue with potential risk factors. The project has been approved by DTRA and kick-off was held on 14 October 2016. The lead KZ partner, the Research Institute for Biological Safety Problems (RIBSP), has submitted paperwork requesting permit from Ministry of Agriculture for sample collection for the study. RIBSP has reported the BSL-3 laboratory has been validated and BSL-3 staff and KZ-32 participants received BSL-3 facility training. RIBSP has developed 13 SOPs focused on sample collection and transport which are currently being reviewed by the collaborators. RIBSP is working closely with collaborators and CBEP's BTRIC (CH2M) to prioritize items for procurement of laboratory supplies. Dr. (b) (6) (University of Connecticut), Drs. (b) (6) and (b) (6) (Louisiana State University Agricultural Center) are collaborators for the study.

Identification of Etiology, Clinical Outcomes, Incidence, and Epidemiological Patterns of Hospitalized Febrile Patients in Armenia

PRESENTER: (b) (6)

COUNTRY: Armenia

Background: Hospitalized patients with fever represent a diagnostic challenge for physicians. Scant information is available on identification of etiology, clinical outcomes, and epidemiological patterns of hospitalized patients with fever in “Nork” Infectious Clinical Hospital. The goal of this study was to describe the most common causes, with a focus on zoonotic and arboviral infections.

Methods: Medical records of hospitalized patients with fever were retrospectively reviewed in 2014. Data were abstracted from medical charts of adults (≥ 18 years) with a fever (≥ 38°C), who were hospitalized (for ≥ 24 hours) in 2010–2012.

Results: Of the 600 patients whose charts were analyzed, 76% were from Yerevan and 51% were male; the mean age was 35.5 (± 16) years. Livestock exposure was recorded in 5% of charts. Consumption of undercooked meat and unpasteurized dairy products were reported in 11% and 8% of charts, respectively. The most common signs or symptoms reported were fatigue (97%), diarrhea (56%), nausea/vomiting (54%), shaking (52%), and abdominal pain (46%). The mean duration of hospitalization was 5.5 days. The most common physical examination findings reported were: pallor (64%), abdominal tenderness (52%), pharyngeal injection (43%), and lymphadenopathy (35%). Twenty-four percent of patients received antibiotics prior to hospital admission. Intestinal infections of known (30%) and unknown (21%) etiology were the most frequently reported final medical diagnoses, followed by diseases of the respiratory system (11%), infectious mononucleosis (9.5%), chickenpox (8.3%), brucellosis (8.3%), viral hepatitis (3.2%), and erysipelas (1.5%).

Conclusions: Half of the patients were diagnosed with enteric infections, nearly half of these had no clear etiologic agent. Brucellosis was the most frequently reported zoonotic disease. Solitary cases of anthrax, leptospirosis, FUO, imported malaria, rickettsiosis, and rat-bite fever were also reported. Further prospective studies are required to identify risk factors associated with febrile illnesses, as well as to estimate the burden of selected arthropod-borne and zoonotic infections.

Human Disease Epidemiology and Surveillance of Especially Dangerous Pathogens in Georgia

PRESENTER: (b) (6)

COUNTRY: Georgia

Especially Dangerous Pathogens (EDPs), or select agents, represent a major concern for global public health. These highly pathogenic agents have the potential to be weaponized. Our project was designed to expand on the successes of the Cooperative Biological Engagement Program Collaborative Biological Research and TADR surveillance efforts, and examine the human disease incidence and prevalence of pathogens of public health and biodefense in Georgia. This project has three aims: (1) to study the epidemiology and clinical manifestations of selected pathogens among patients with undifferentiated fever and hemorrhagic fever/septic shock; (2) study the seroprevalence of selected pathogens in humans in Georgia; and (3) implement and evaluate diagnostic methods for selected pathogens and monitor patterns of antimicrobial resistance in identified bacterial infections. Laboratory surveillance for acute undifferentiated febrile illness (AUFI) was established in three major Georgian hospitals by the National Center for Disease Control and Public Health in collaboration with the U.S. Navy Medical Research Unit-3 and USAMRIID. Pathogens that are studied in the seroprevalence protocol include *Bacillus anthracis*, *Brucella* species, Crimean-Congo hemorrhagic fever virus, *Coxiella burnetii*, *Francisella tularensis*, Hantavirus, *Rickettsia* species, and tick-borne encephalitis virus. Furthermore, several other pathogens were tested to develop a comprehensive differential diagnostic algorithm and antibody prevalence of similar diseases. In patients treated at participating hospitals with an undifferentiated febrile illness were tested and involved in the developing process for a comprehensive diagnostic algorithm. Currently, 98.9% of seroprevalence tests and 69.4% of febrile studies are completed. These are preliminary results of screening trial; confirmatory testing continues.

The Epidemiological Status of African Swine Fever in Domestic Swine Herds in the Tavush Marz Region, Republic of Armenia

PRESENTER: (b) (6)

COUNTRY: Armenia

The factors associated with the spread and persistence of African Swine Fever (ASF) in the Caucasus Region remain to be fully identified. It is assumed that large naïve domestic, free-ranging, and wild pig populations are critical to disease transmission and maintenance. Nonetheless, nine years since its epidemic introduction in the region in 2007, the virus that causes ASF is still circulating suggesting that an endemic cycle is established where contact between free ranging domestic pigs, wild pigs, and probably native *Ornithodoros* ticks serve as reservoirs. Thus, research is required to gather information on the epidemiological status of ASF in the Caucasus Region focusing on understanding modes of ASFV spread and persistence in the area

An active surveillance program was established in Armenia to determine the epidemiological status of ASF focusing on an area at high risk, Tavush marz. This region was the first to report the presence of ASF in Armenia in 2007 and 2010-2011. It is the main terrestrial point of entry for traffic into Armenia and it shares a border with Georgia where the disease was introduced. Most pigs in Tavush are kept in backyard operations and allowed to free-forage, providing contact with wild pigs and ticks.

A total of 1,506 pigs were sampled from small-scale farms clustered in 30 communities across the marz. Samples were taken from the sera, complete blood, and nasal swabs tested by ELISA, IPA, and qPCR. Fifty nine ticks were collected, but the *Ornithodoros* was not found among them. All samples were negative for ASFV and ASFV antibodies suggesting that AFSV is not circulating in the sampled population.

Since sporadic ASFV outbreaks in domestic pigs have continued to occur in the Caucasus, the capacity for long-term environmental survival should be investigated. Further research is required on the epidemiological status of ASF in ticks and swine populations deemed at high risk for ASF.

Investigation of Mosquito and Tick-Borne Arboviruses in Southeastern Azerbaijan

PRESENTER: (b) (6)

COUNTRY: Azerbaijan

Twelve different arboviruses were identified between 1967 and 1980 in humans and animals in Azerbaijan. Despite the known presence of arboviruses, very little vector-borne pathogen research has been conducted within Azerbaijan since then. TAP-13 project is designed to identify selected arboviruses and their arthropod vectors in southeastern Azerbaijan (Lankaran, Masalli and Gizil-Aghaj State Reserve). The aim of the project was to facilitate the development of effective strategies for the biosurveillance, control, and mitigation of four arboviral pathogens (CCHF, TBE, Sindbis and WNV) and their arthropod vectors.

The project was initiated in August 2016 and is being implemented at Lankaran Anti-Plague Division (APD) with mentorship of the Republican Anti-Plague Station (RAPS). The UK Public Health collaborators provided two PCR trainings in Lankaran APD (November and April, 2016). Arthropods were collected from three regions in October 2015, April and May, 2016 using cloth dragging and livestock collection of ticks, as well as mosquito collection using CDC-light and BG-sentinel traps. Coordinates were recorded via GPS.

Out of 1,777 collected ticks, 1,606 ticks have been counted, identified, and sorted. 590 tick pools have been prepared; 46 pools have been homogenized, extracted, and tested for TBE, Tamdy and West Nile viruses, and all were negative. Out of 5,217 collected mosquitoes, 1,152 have been counted, identified, and sorted. 96 pools have been prepared. 37 mosquito pools were tested for West Nile and Sindbis viruses, and all were negative.

The project was temporarily halted, but will resume February 2017.

Analysis of Previously Identified *Rickettsia* Positive Georgian Ticks by Multi-locus Sequence Typing

PRESENTER: (b) (6)

COUNTRY: Georgia

Preliminary studies have shown the presence of three spotted fever group *rickettsiae* (SFGR) species among ticks in Georgia: *R. aeschlimanii, R. raoultii*, and *R. slovaca*. The identity and prevalence of other *Rickettsia* species in ticks have not been determined. The overall goal of this project was to improve the surveillance of tick-borne pathogens in Georgia in the context of rickettsial diseases. This project used the Rickettsia-positive tick samples collected in Georgia from GG TAP-4 project. All tick samples were previously entered into a database, and pertinent sample information and GPS location data were recorded. The tick DNA preparations from the tick samples were tested using multi-locus sequence typing (MLST) to identify which Rickettsia species were present. Tick samples were not collected from the field; only tick DNA preparations already tested for Rickettsia were utilized. Overall, nine species were found among 12 different tick species from five different genera: *Ixodes, Hyalomma, Haemaphysalis, Dermacentor,* and *Rhipicephalus*. Geographical distribution maps of *Rickettsia* –infected ticks that were developed in eight regions eastern and western Georgia showed that the most common *Rickettsia* species were: *R. raoultii*, *R. slovaca*-also, *R. aeschlimannii*, and *R. monacensis*. For the first time the SFGR species *R. massiliae, R. monacensis*, *R. conorii* subsp. *conorii*, *R. hoogstraalii, R. helvetica* and *Ca. R. barbariae* were detected in ticks from Georgia. High prevalence and wide distribution of *Rickettsia* species among ticks make rickettsiosis a potential public health problem in Georgia.

The Role of Arboviruses as a Cause of Undifferentiated Febrile Illness in Sindh, Pakistan

PRESENTER: (b) (6)

COUNTRY: Pakistan

The objective of this study was to develop training in arboviral diagnostics and surveillance, to determine the burden of mosquito-borne viruses that can be used as biological agents of warfare. The Pakistani collaborators then trained other public health practitioners in Pakistan to expand arboviral diagnostics and surveillance capabilities. We recorded the presence of several co-circulating arboviruses in Pakistan. Our data show that a significant percentage of patients presenting with undifferentiated fever are afflicted with at least one arbovirus with 14.2% of patients infected with dengue or West Nile virus, 3% with chikungunya virus, and 10.7% with Japanese encephalitis virus. Diagnostic techniques developed through this project helped in the early diagnosis of Chikungunya during the Nov-Dec 2016 outbreak in Karachi. We published one paper disseminating preliminary project data and are currently drafting several more papers for publication in the coming year. Additionally, based on data from this project, a team member was awarded a 12-month Fellowship in One-Health by the National Academy of Science for 2016-2017.

During the next reporting period, we will complete validation via PRNT for all samples. In light of the global outbreak of Zika virus, we will screen our samples for Zika virus. We will present data at several conferences this year.

Event Logistics and Local Information

Venue Information

2017 Science Program Review Meeting Site

Hilton Alexandria Mark Center
5000 Seminary Road
Alexandria, Virginia 22311
(703) 845-1010

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Embassy Contact Information

Embassy of Armenia
2225 R St NW
Washington, DC 20008
Phone: (202) 319-1976

Embassy of Azerbaijan
2741 34th St NW
Washington, DC 20008
Phone: (202) 337-3500

Embassy of Cambodia
4530 16th St NW
Washington, DC 20011
Phone: (202) 726-7742

Embassy of Cameroon
3400 International Dr NW
Washington, DC 20008
Phone: (202) 244-4911

Embassy of Georgia
2209 Massachusetts Ave NW
Washington, DC 20008
Phone: (202) 387-2390

Embassy of India
2107 Massachusetts Ave NW
Washington, DC 20008
Phone: (202) 939-7000

Embassy of Jordan
3504 International Dr NW
Washington, DC 20008
Phone: (202) 966-1009

Embassy of Kazakhstan
1401 16th St NW
Washington, DC 20036
Phone: (202) 232-5488

Embassy of Kenya
2249 R St NW
Washington, DC 20036
Phone: (202) 387-6101

Embassy of Lao PDR
2222 S St NW
Washington, DC 20008
Phone: (202) 332-6416

Embassy of Malaysia
3516 International Ct NW
Washington, DC 20008
Phone: (202) 572-9700

Embassy of Pakistan
3517 International Ct NW
Washington, DC 20008
Phone: (202) 243-6500

Embassy of South Africa
3051 Massachusetts Ave NW
Washington, DC 20008
Phone: (202) 232-4400

Embassy of Tanzania
1232 22nd St NW
Washington, DC 20037
Phone: (202) 939-612

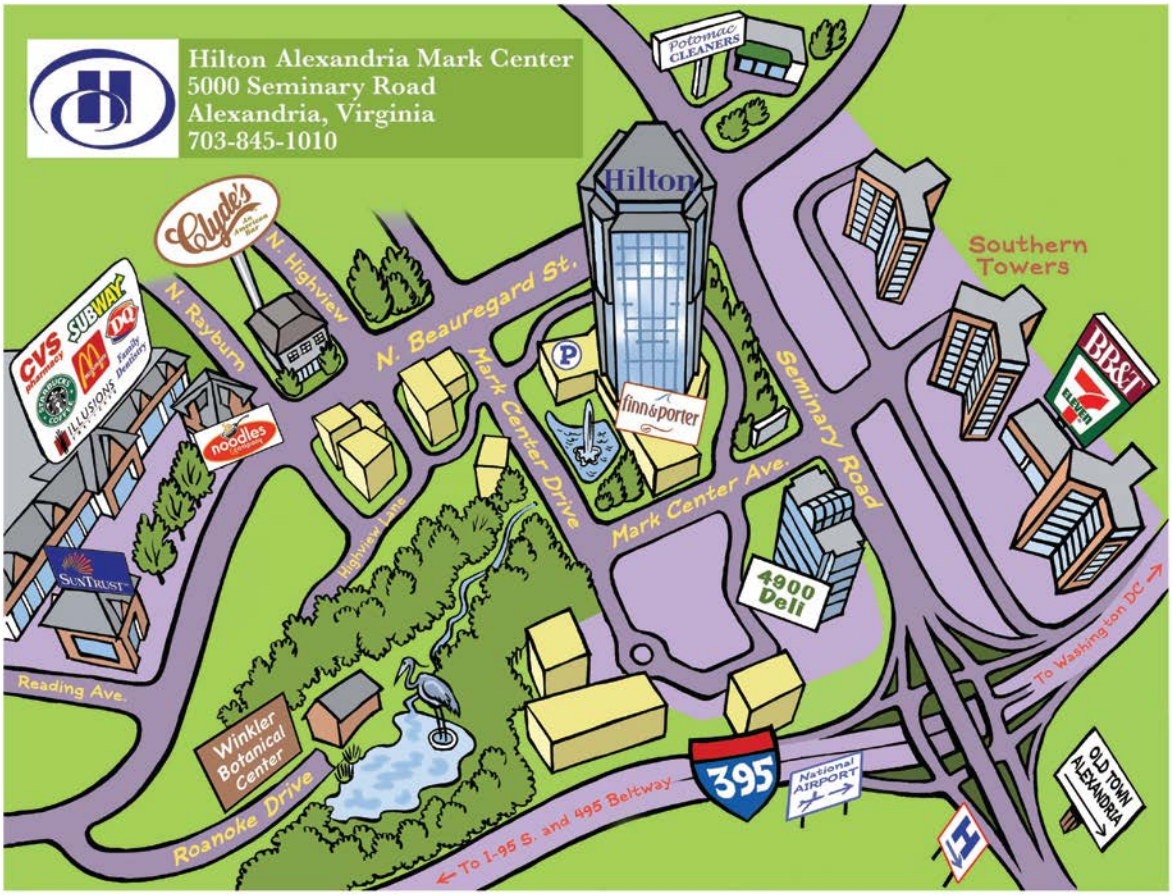
Embassy of Thailand
1024 Wisconsin Ave NW, Ste 401
Washington, DC 20007
Phone: (202) 944-3600

Embassy of Uganda
5911 16th St NW
Washington, DC 20011
Phone: (202) 726-7100

Embassy of Ukraine
3350 M St NW
Washington, DC 20007
Phone: (202) 333-0600

Embassy of Vietnam
1233 20th St NW, Ste 400
Washington, DC 20036
Phone: (202) 861-0737

Local Information



Presenter Index

Country	Project	Speaker	Session	Day/Time	Summary Page Number
Eastern Europe Regional	ASF Public Outreach Session Overview	(b) (6)	Session 5: Community Outreach to Combat African Swine Fever	Thursday 1:30 PM	28
Armenia	TAP-A1: Community outreach to support understanding of ASF ecology and epidemiology in Eastern Europe: Training and implementation for methods and strategies for control and prevention		Session 5: Community Outreach to Combat African Swine Fever	Thursday 1:30 PM	29
Armenia	AM-1: Medical/Biological Mapping of Tularemia Natural Foci and Epidemiology using GIS in Armenia		Session 7: Bacterial Pathogens of Security Concern - Group 1	Thursday 3:40 PM	41
Armenia	AM-4: One Health Surveillance for Brucellosis in Armenia		Session 11: Epidemiology & Biosurveillance - Group 2	Friday 1:00 PM	61
Armenia	TAP-H1: Identification of Etiology, Clinical Outcomes, Incidence, and Epidemiological Patterns of Hospitalized Febrile Patients in Armenia		Session 11: Epidemiology & Biosurveillance - Group 2	Friday 1:00 PM	66
Armenia	AM-3: The Epidemiological Status of African Swine Fever in Domestic Swine Herds in the Tavush Marz Region, Republic of Armenia		Session 11: Epidemiology & Biosurveillance - Group 2	Friday 1:00 PM	68
Azerbaijan	TAP-10: Ecological and epidemiological study of *Yersinia pestis* and *Francisella tularensis* in the Northern part of Azerbaijan regions of Gusar and Khachmaz		Session 3: Epidemiology & Biosurveillance - Group 1	Thursday 10:30 AM	20
Azerbaijan	Molecular Epidemiology and Ecology of *Yersinia* spp. in the Transboundary Plague Endemic Territory in Georgia and Azerbaijan (BAA)		Session 7: Bacterial Pathogens of Security Concern - Group 1	Thursday 3:40 PM	37
Azerbaijan	AJ TAP-11: Regional Study of the Ecology of Anthrax Foci in Georgia and Azerbaijan		Session 9: Bacterial Pathogens of Security Concern - Group 2	Friday 8:40 AM	52

Country	Project	Speaker	Session	Day/Time	Summary Page Number
Azerbaijan	Infectious Etiologies of Acute Febrile Illness Among Members of the Azerbaijan Military (Call)	(b) (6)	Session 11: Epidemiology & Biosurveillance - Group 2	Friday 1:00 PM	63
Azerbaijan	AJ TAP-13: Investigation of Mosquito and Tick-Borne Arboviruses in Southeastern Azerbaijan		Session 12: Arthropods and Arboviruses	Friday 2:50 PM	69
Cambodia	Investigating the Risk of Human Disease from Parasites of Small Mammals and Bats (BAA)		Session 2: Chiroptera (Bats) - Important Reservoirs Hosts of Emerging Viruses	Thursday 8:50 AM	15
Cameroon	Epidemiology of Emerging Viruses		Session 4: Viral Pathogens of Security Concern	Thursday 11:40 AM	25
Georgia	Understanding the Risk of Bat-Borne Zoonotic Disease Emergence in Western Asia (BAA)		Session 2: Chiroptera (Bats) - Important Reservoirs Hosts of Emerging Viruses	Thursday 8:50 AM	14
Georgia	GG-19: Epidemiology of Human Tularemia in Georgia		Session 3: Epidemiology & Biosurveillance - Group 1	Thursday 10:30 AM	19
Georgia	Assessing the Seroprevalence and Genetic Diversity of CCHFV and Hantaviruses in Georgia (Call)		Session 4: Viral Pathogens of Security Concern	Thursday 11:40 AM	24
Georgia	GG TAP-9: Community Outreach to Support Understanding of ASF Ecology and Epidemiology in Eastern Europe: Training and Implementation for Methods and Strategies for Control and Prevention		Session 5: Community Outreach to Combat African Swine Fever	Thursday 1:30 PM	30
Georgia	Molecular Epidemiology and Ecology of *Yersinia* spp. in the Transboundary Plague Endemic Territory in Georgia and Azerbaijan (BAA)		Session 7: Bacterial Pathogens of Security Concern - Group 1	Thursday 3:40 PM	37
Georgia	GG-23: Creation of Sustainable Immunodiagnostics		Session 7: Bacterial Pathogens of Security Concern - Group 1	Thursday 3:40 PM	40
Georgia	Characterization of NCDC Strain Repository by Next Generation Sequencing (BAA)		Session 9: Bacterial Pathogens of Security Concern - Group 2	Friday 8:40 AM	47

Country	Project	Speaker	Session	Day/Time	Summary Page Number
Georgia	GG-27: Regional Study of the Ecology of Anthrax Foci in Georgia and Azerbaijan	(b) (6)	Session 9: Bacterial Pathogens of Security Concern - Group 2	Friday 8:40 AM	51
Georgia	TAP-10: Molecular Epidemiology of *B. anthracis* and *Brucella* spp. in Turkey (GG and Turkey)		Session 9: Bacterial Pathogens of Security Concern - Group 2	Friday 8:40 AM	50
Georgia	Enhancing Capacity for Case Detection and Diagnosis of Febrile Zoonotic-related Cutaneous Lesions in Georgia (BAA)		Session 10: Emerging Infectious Diseases	Friday 10:40 AM	56
Georgia	Enhancing Capacity for Case Detection and Diagnosis of Febrile Zoonotic-related Cutaneous Lesions in Georgia (Call & BAA)		Session 10: Emerging Infectious Diseases	Friday 10:40 AM	55
Georgia	GG-20: Prevalence, Epidemiological Surveillance, and Laboratory Analysis of *Coxiella burnetii* in Georgia		Session 11: Epidemiology & Biosurveillance - Group 2	Friday 1:00 PM	64
Georgia	GG-21: Human Disease Epidemiology and Surveillance of Especially Dangerous Pathogens in Georgia		Session 11: Epidemiology & Biosurveillance - Group 2	Friday 1:00 PM	67
Georgia	TAP-12: Analysis of Previously Identified Rickettsia Positive Georgian Ticks by Multi-locus Sequence Typing		Session 12: Arthropods and Arboviruses	Friday 2:50 PM	70
India	Bat Harvesting in India: Detection, Characterization and Mitigation of Emerging Infectious Diseases Risk (BAA)		Session 2: Chiroptera (Bats) - Important Reservoirs Hosts of Emerging Viruses	Thursday 8:50 AM	17
India	Detection and Molecular Epidemiologic Analysis of Especially Dangerous Pathogens in Backyard Poultry, Commercial Broilers and Waterfowl in India (BAA)		Session 8: Avian Transmissible Diseases	Thursday 5:00 PM	46
Jordan	Understanding the Risk of Bat-Borne Zoonotic Disease Emergence in Western Asia (BAA)		Session 2: Chiroptera (Bats) - Important Reservoirs Hosts of Emerging Viruses	Thursday 8:50 AM	14
Kazakhstan	TAP-8: Especially Dangerous Pathogen Differential/Rule-Out Elimination Assays & Modeling (EDP-DREAM) of the Saiga Antelope Mortality		Session 3: Epidemiology & Biosurveillance - Group 1	Thursday 10:30 AM	23

Country	Project	Speaker	Session	Day/Time	Summary Page Number
Kazakhstan	KZ-31: Effect of *Rickettsia* spp. upon fitness of *Yersinia pestis* in fleas that vector plague in the Republic of Kazakhstan	(b) (6)	Session 7: Bacterial Pathogens of Security Concern - Group 1	Thursday 3:40 PM	38
Kazakhstan	TAP-11: Molecular Characterization and Complete Genome Sequence of Newcastle Disease Virus Isolated in Kazakhstan		Session 8: Avian Transmissible Diseases	Thursday 5:00 PM	44
Kazakhstan	KZ-33: Middle East Respiratory Syndrome Coronavirus (MERS-CoV): Surveillance for Distribution and Prevalence in Kazakhstan		Session 10: Emerging Infectious Diseases	Friday 10:40 AM	58
Kazakhstan	KZ-32: Prevalence of *Brucella* Species and Bluetongue Virus Serotypes Among Domestic Livestock or Ruminants in Southern Kazakhstan		Session 11: Epidemiology & Biosurveillance - Group 2	Friday 1:00 PM	65
Kenya	Whole Genome Sequencing of African Swine Fever Virus in Kenya (BAA)		Session 6: Transboundary Animal Diseases	Thursday 2:20 PM	33
Kenya	Newcastle Disease: Surveillance, Molecular Epidemiology, and Control of NDV in Kenya (Call)		Session 8: Avian Transmissible Diseases	Thursday 5:00 PM	43
Kenya	Estimating Incidence and Socio-economic Impact of Brucellosis in Humans and Animals in Kajiado County, Kenya		Session 9: Bacterial Pathogens of Security Concern - Group 2	Friday 8:40 AM	49
Lao PDR	Environmental Surveillance of *Burkholderia pseudomallei*, Pilot Study in Lao PDR		Session 9: Bacterial Pathogens of Security Concern - Group 2	Friday 8:40 AM	53
Malaysia	Biosurveillance for Henipaviruses and Filoviruses at the Agricultural Animal-Human Interface in Malaysia (BAA)		Session 4: Viral Pathogens of Security Concern	Thursday 11:40 AM	27
Malaysia	Multi-Year Prospective Cohort Study to Evaluation the Risk Potential of MERS-CoV (Call)		Session 10: Emerging Infectious Diseases	Friday 10:40 AM	57
Malaysia	Etiology of Severe Acute Respiratory Infections in Kuala Lumpur, Malaysia (BAA)		Session 10: Emerging Infectious Diseases	Friday 10:40 AM	60

Country	Project	Speaker	Session	Day/Time	Summary Page Number
Pakistan	High Resolution Chemical Characterization of *Yersinia pestis* Cells within Soil Matrices: Implications for Understanding Natural Foci and Telluric Reservoirs of Plague (BAA)	(b) (6)	Session 9: Bacterial Pathogens of Security Concern - Group 2	Friday 8:40 AM	48
Pakistan	The Role of Arboviruses as a Cause of Undifferentiated Febrile Illness in Sind, Pakistan (BAA)		Session 12: Arthropods and Arboviruses	Friday 2:50 PM	71
South Africa	Understanding Rift Valley Fever in the Republic of South Africa (BAA)		Session 4: Viral Pathogens of Security Concern	Thursday 11:40 AM	26
Tanzania	Evaluating Zoonotic Viral Sharing Among Bats, Primates and People in High Risk Transmission Interface in Southern Tanzania (BAA)		Session 2: Chiroptera (Bats) - Important Reservoirs Hosts of Emerging Viruses	Thursday 8:50 AM	18
Tanzania	A One Health Approach to Brucellosis and Rift Valley Fever Surveillance in Tanzania (BAA)		Session 3: Epidemiology & Biosurveillance - Group 1	Thursday 10:30 AM	21
Tanzania	Global Health, Emerging Infectious Diseases and Food Safety Implications of Bushmeat in Tanzania (BAA)		Session 10: Emerging Infectious Diseases	Friday 10:40 AM	59
Thailand	Acute Febrile Illness Study Among Patients in Nakhon Phanom and Tak Province, Thailand (Call)		Session 3: Epidemiology & Biosurveillance - Group 1	Thursday 10:30 AM	22
Thailand	Melioidosis Research Coordination Network		Session 9: Bacterial Pathogens of Security Concern - Group 2	Friday 8:40 AM	54
Uganda	Arthropod-borne Viruses Associated with the Chiroptera of Uganda: Isolation and Characterization (Call)		Session 2: Chiroptera (Bats) - Important Reservoirs Hosts of Emerging Viruses	Thursday 8:50 AM	16
Uganda	UG-2: Research and Development of Countermeasures to Support the Control of FMDV in Uganda		Session 6: Transboundary Animal Diseases	Thursday 2:20 PM	35
Uganda	Acute Febrile Illness in Uganda (BAA)		Session 11: Epidemiology & Biosurveillance - Group 2	Friday 1:00 PM	62

Country	Project	Speaker	Session	Day/Time	Summary Page Number
Ukraine	TAP-4: Community Outreach to Support Understanding of African Swine Fever (ASF) Ecology and Epidemiology in Eastern Europe (EE)	(b) (6)	Session 5: Community Outreach to Combat African Swine Fever	Thursday 1:30 PM	31
Ukraine	TAP-6: Analysis of the Threat of Spread of African Swine Fever and Classical Swine Fever in Wild Boar Populations in Ukraine: Improving Diagnosis, Surveillance, and Prevention		Session 6: Transboundary Animal Diseases	Thursday 2:20 PM	34
Ukraine	African Swine Fever Threat Reduction Through Surveillance in Ukraine (USDA ARS)		Session 6: Transboundary Animal Diseases	Thursday 2:20 PM	36
Ukraine	UP-2: Development of the Epidemiological Forecasting System for Zoonotic Diseases Employing GIS Technology		Session 7: Bacterial Pathogens of Security Concern - Group 1	Thursday 3:40 PM	39
Ukraine	Genomic, Epidemiological, and Biological Characterization of Newcastle Disease Virus Isolates from Ukraine (USDA ARS)		Session 8: Avian Transmissible Diseases	Thursday 5:00 PM	42
Ukraine	UP-4: Risk Assessment of Selected Especially Dangerous Pathogens Potentially Carried By Migratory Birds Over Ukraine		Session 8: Avian Transmissible Diseases	Thursday 5:00 PM	45
Vietnam	Foot-and-Mouth Disease Virus Surveillance and Ecology in Vietnam (Call)		Session 6: Transboundary Animal Diseases	Thursday 2:20 PM	32

CURRENT ENGAGEMENTS

Africa (USAFRICOM)

- Cameroon
- Ethiopia
- Gabon
- Guinea
- Kenya
- Liberia
- Senegal
- Sierra Leone
- South Africa
- Tanzania
- Uganda

Europe (USEUCOM)

- Armenia
- Azerbaijan
- Georgia
- Ukraine

Middle East South Asia (USCENTCOM)

- Jordan
- Kazakhstan
- Iraq
- Pakistan
- Turkey
- Uzbekistan

Southeast Asia (USPACOM)

- Cambodia
- India
- Laos
- Malaysia
- Philippines
- Thailand

FOR MORE INFORMATION

(b) (6)

Defense Threat Reduction Agency
USSTRATCOM Center for Combating WMD
8725 John J. Kingman Road, MSC 6201
Fort Belvoir, VA 22060

COOPERATIVE BIOLOGICAL ENGAGEMENT PROGRAM

(b) (6)

PROGRAM MISSION

The Cooperative Biological Engagement Program (CBEP) recognizes the danger to U.S. and global health security posed by the risk of outbreaks of dangerous infectious diseases. Whether natural or manmade, disease outbreaks pose a risk to the global community. CBEP strives to address this risk by promoting best practices in biological safety and security, improving partner countries' capacities to safely and rapidly detect and report dangerous infections, and establishing and enhancing international research partnerships.

PROGRAM OBJECTIVES

1. *Secure and consolidate collections* of especially dangerous pathogens (EDPs) and their associated research at a minimum number of secure facilities
2. Enhance partner country/region's capability to prevent the sale, theft, diversion, or accidental release of biological weapons (BW)-related materials, technology, and expertise *by improving biological safety and security standards*
3. Enhance partner country/region's capability to *detect, diagnose, and report* endemic and epidemic, man-made or natural EDPs, bio-terror attacks, and potential pandemics
4. Ensure the developed capabilities are designed to be *sustainable*
5. Facilitate engagement of partner country/region's scientific and technical personnel in *research areas of interest* to both the partner country/region and the United States
6. *Eliminate any BW-related infrastructure* and technologies encountered

CORE PROGRAM AREAS

Two core program areas contribute to CBEP mission success: Biosafety and Biosecurity, and Biosurveillance.

BIOSAFETY AND BIOSECURITY

Strengthen biosafety and biosecurity standards and practices. CBEP activities improve the capability of global partners to handle, store, and account for EDPs safely and securely in accordance with U.S. and international standards, guidelines, and best practices.

Objectives

- Assess existing biosafety and biosecurity capabilities for facilities, procedures, and personnel involved with EDPs
- Provide sustainable solutions to increase biosafety and consolidate and secure pathogens, to include: facility upgrades, engineering controls, training and mentorship, and personal protective equipment
- Facilitate collaborative development of country-specific biosafety and biosecurity standards and guidelines that are sustainable and promote international scientific cooperation

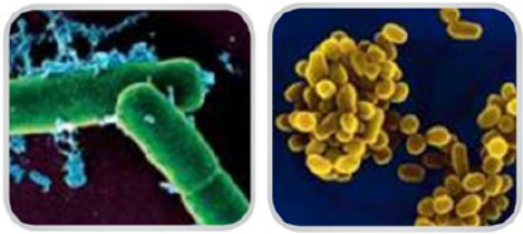
BIOSURVEILLANCE

Enhance Country Disease Surveillance, Detection, Diagnostics, and Reporting Capacity. CBEP works with partner countries across human and clinical, veterinary,

epidemiological, and laboratory communities to enhance partner country disease surveillance, detection, diagnostics, and reporting capabilities related to EDPs.

Objectives

- Develop disease surveillance and diagnostic laboratory networks at the national, regional, and district levels to facilitate near real-time reporting of outbreak data to national authorities
- Survey suspicious disease outbreaks, analyze epidemics, and collect disease reports from veterinarians, clinicians, and epidemiologists
- Aid partner capability to comply with the World Health Organization's International Health Regulations and World Organization for Animal Health's reporting guidelines



PROGRAM ENABLERS

CBEP-sponsored research is an important enabling tool. CBEP funds and conducts research that involves partner-country scientists and directly advances strategic policy objectives.

RESEARCH

Facilitate strategic research partnerships. CBEP establishes international research partnerships and engages scientists to enhance epidemiological and diagnostic capacities related to EDPs. Engagement focuses on research areas of interest to both the partner country and the United States.

Objectives

- Prevent the proliferation of dual-use expertise
- Increase transparency and encourage high standards of openness, ethics, and conduct
- Integrate scientists and institutes into the international scientific community
- Improve partner's understanding of endemic and emerging diseases
- Share unique pathogen strains/data with the scientific community for joint research

CURRENT ENGAGEMENTS

Africa (USAFRICOM)

- Cameroon
- Ethiopia
- Guinea
- Kenya
- Liberia
- Senegal
- Sierra Leone
- South Africa
- Tanzania
- Uganda

Middle East South Asia (USCENTCOM)

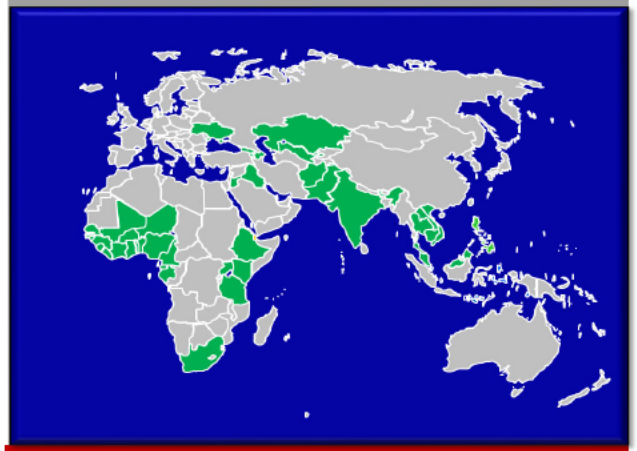
- Jordan
- Kazakhstan
- Iraq
- Pakistan
- Turkey
- Uzbekistan

Southeast Asia (USPACOM)

- Cambodia
- India
- Laos
- Malaysia
- Philippines
- Thailand
- Vietnam

Europe (USEUCOM)

- Armenia
- Azerbaijan
- Georgia
- Ukraine



FOR MORE INFORMATION

(b) (6)

Defense Threat Reduction Agency
8725 John J. Kingman Road, MSC 6201
Fort Belvoir, VA 22060

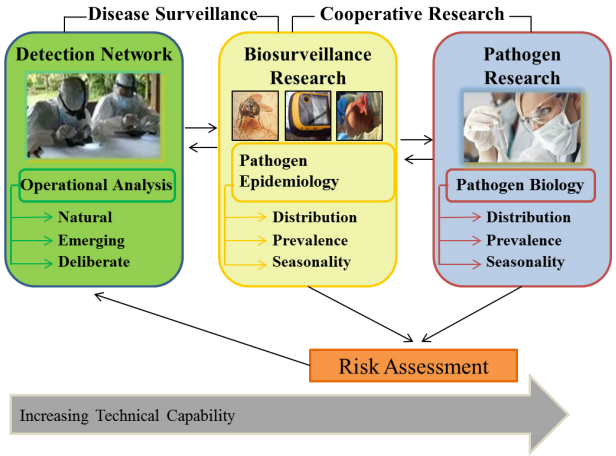
Applying for Research Funding from the Cooperative Biological Engagement Program

RESEARCH PROGRAM MISSION AND VISION:

The Cooperative Biological Engagement Program (CBEP) seeks to use cooperative international research partnerships to support its mission to reduce the threat to the U.S. and to global health security from the spread of pathogens of security concern, which includes U.S. Biological Select Agents and pathogens of pandemic potential, emerging, and reemerging infectious diseases. CBEP aims to establish and maintain these partnerships to inform and enhance operational biosurveillance systems, enhance global health security, and foster safe, secure, and sustainable bioscience capability with partner countries.

COOPERATIVE BIOLOGICAL RESEARCH

The CBEP uses Cooperative Biological Research (CBR) projects to support, enhance, and inform biosurveillance (BSV) and biosafety and biosecurity (BS&S) capabilities that reduce the threat of pathogens of security concern, while sustainably addressing infectious disease priorities of the CBEP and its partner countries.



RESEARCH FIELDS AND PRIORITIES

CBR supports and informs operational biosurveillance through an improved understanding of pathogens and their risk to global health security. In order to remain relevant, agile, and sustainable, the scope of the CBEP's research priorities include:

1. Understanding the ecology and epidemiology of pathogens of security concern
2. Differentiating pathogens of security concern

RESEARCH GOALS

The CBEP is committed to fair and open competition of research topics that enhance biosurveillance, mutually benefit the U.S. and global partner institutes, and achieve the goals outlined below:

1. Support biosurveillance and biosafety and security (BS&S) capability building efforts
2. Engage partner country scientists in ethical hypothesis-driven research resulting in high-quality data and active participation in professional societies
3. Foster sustainable partnerships with key national and international stakeholders, as well as advance partner country sustainment of global health security and one-health initiatives.

PROJECT SCOPE

The CBEP is most likely to fund projects demonstrating:

- Clear relationships to pathogens of security concern
- Links to the CBEP's threat reduction mission
- Support of BS&S and BSV capabilities
- Alignment with the CBEP and partner country priorities
- Use of sustainable techniques for partner countries in appropriate facilities
- Ethical, Hypothesis-Driven research aims

Projects focusing on or likely to result in Dual Use Research of Concern (DURC) will not be supported. Additionally, the CBEP research objectives do not include diagnostic assay development, medical countermeasures, or research focused on non-infectious diseases.

APPLYING FOR FUNDING

Research projects supported by the CBEP must align with the CBEP's mission and vision and are expected to produce results suitable for scientific publication. The CBEP welcomes research funding applications from the following domestic and foreign entities:

- Academic institutions, NGOs, industry, foreign laboratories, and private sector members through the Broad Agency Announcement (BAA)
- U.S. government partners and federally funded Research and Development Centers through the Government Service Call (Call).

ROADMAP FOR APPLICATIONS

Submitting your proposal to the BAA or the Call involves three key steps: Pre-coordination, Phase I, and Phase II. Note, each step has its own rules and requirements. A summary of each step is provided as follows:

1. Pre-coordination: This phase involves discussion and coordination between proposal offerors and the CBEP Country/Regional Manager and/or a Science Manager to ensure the proposed work is within the CBEP scope and meets CBEP priorities. Abstracts are to be emailed to the appropriate administrative email listed below. The abstract must be reviewed favorably by the CBEP prior to continuing on to a Phase I white paper. Prior to the official white paper request from CBEP, direct contact and communication is allowed. Following an invitation to submit a Phase 1 white paper, all communications must be conducted through the appropriate administrative email address listed below.
2. Phase I: Upload the Phase 1 submission with application package to www.grants.gov (BAA) or www.dtrasubmission.net (Call). Following favorable review by the CBEP, a Phase II full proposal may be requested.
3. Phase II: If invited to submit a full proposal, develop the proposal incorporating any comments from the Phase I debrief summary. Submit completed Phase II information and attachments to www.grants.gov or www.dtrasubmission.net as appropriate. Applicants must complete additional appropriate documents for BS&S review as well as documentation if research involves human or animal use.

More detailed instructions and requirements can be found through the solicitation links at www.grants.gov.

WHERE TO APPLY

For abstract submission to the BAA (HDTRA1-14-24-FRCWMD-BAA) or Service Call (HDTRA1-12-17-FRCWMD-Call) please use: HDTRA1-FRCWMD-TA6@mail.mil

For administrative questions for the BAA, please use: HDTRA1-FRCWMD-A@mail.mil

For administrative questions for the Service Call , please use: HDTRA1-FRCWMD-C@mail.mil

Navigating the Defense Threat Reduction Agency and United States Strategic Command Center for Combating Weapons of Mass Destruction Cooperative Biological Engagement Program Proposal Submission Process

Thank you for your interest in working with the Defense Threat Reduction Agency and United States Strategic Command Center for Combating Weapons of Mass Destruction's (DTRA/SCC-WMD) Cooperative Biological Engagement Program (CBEP). CBEP often works with universities, U.S. Government agencies, non-profit organizations, foreign laboratory equivalent entities, and many other organizations to implement the biological threat reduction mission through research projects.

This correspondence is intended to assist offerors in applying for DTRA/SCC-WMD CBEP funding. Information contained herein is intended to supplement, not replace, official documents including:

- DTRA/SCC-WMD's Broad Agency Announcement (BAA): HDTRA1-14-24-FRCWMD-BAA (*BAA*)
- DTRA/SCC-WMD's Government Service Call (Call): HDTRA1-12-17-FRCWMD-Call (*Call*)

These documents can be found through the solicitation links at www.dtrasubmission.net/portal/.

CBEP recommends all offerors read the applicable official document, but frequent references and citations are provided below in red to facilitate understanding of the process. Information here will help offerors answer specific questions and ease the initial stages of project/proposal development and application submission.

Frequently Asked Questions:

Question One: *Is my project appropriate for the BAA or the Call?*

The BAA and the Call both enable the same types of projects; the differentiating factor is the applying entity.

Eligible applicants under the BAA include: accredited degree-granting colleges, universities, and academic institutions; industrial and commercial entities, including small businesses with a portfolio predominantly in research; non-government organizations; not-for-profit entities with a portfolio predominantly in research; and foreign government laboratories. *BAA Section 3, pp 12- 13.*

Eligible applicants under the Call include: Federal laboratories to include Department of Defense (DoD), Department of Energy (National Labs), Department of Homeland Security (National Biodefense Analysis and Countermeasures Center, Plum Island Animal Disease Center), Health and Human Services (Centers for Disease Control and Prevention, National Institutes of Health), and U.S. Department of Agriculture (Agriculture Research Service, Animal Plant and Health Inspection Service); and DoD sponsored Federally Funded Research and Development Centers as specified in

Defense Federal Acquisition Regulation Supplement 235.017-1 and Federal Acquisition Regulation 35.017-1. *Call Section 4, pp 9.*

Question Two: *Is my project appropriate for Thrust Area 6?*

Thrust Area 6 (TA6) is intended to receive exploratory basic and applied research project abstracts, white papers, and proposals. Projects that are hypothesis driven and involve data generation in a laboratory or field environment are considered research projects and are applicable to TA6. This also includes projects that plan to display or present an analysis of data in a peer-reviewed publication or presentation. *BAA Section 1.5.6, pp 7-9; Call Section 2.1.6, pp 5-7.*

Question Three: *How can I ensure my proposal is aligned with CBEP's mission?*

At its highest level, CBEP strives to address the risk of outbreaks of dangerous infectious diseases by promoting biological safety and security, improving partner country capacity to detect and report dangerous diseases, and establishing and enhancing international research partnerships. The contribution to threat reduction is critically important to a successful white paper/proposal. Applicants have the opportunity, and are encouraged, to reach out to CBEP and coordinate project scope through submission of an abstract or concept note before applying to the BAA or Call. *BAA Section 1.5.6, pp 7-9, Section 4.2.1, pp 14; Call Section 2.1.6, pp 5-7, Section 9.2, pp 27.*

Understanding the process:

Question Four: *How long should I expect the review and award process to take?*

Receiving an award through the BAA or Service Call requires a number of key steps. Each step involves a number of factors that can influence the timeline from initial submission to award. Generally, the average length of time between an official white paper submission and project award is 12 months for both BAA and Service Call submissions. Factors influencing the process include: internal review processes, external interagency review processes, offeror submission of revised proposal documents, and contract/grant award processes. The specific timelines and deadlines for submission of documents related to each application phase will be provided in the official correspondence during the submission process.

Question Five: *What is the application process?*

Submitting your proposal to the BAA or the Service Call involves three key steps: Pre-coordination, Phase I, and Phase II. Note each step has its own rules and requirements. A summary of each step is provided in the chart on the following page.

	Thrust Area 6
Pre-Coordination	• Initiate direct communication with the Regional/Country Lead and Science Manager to determine project viability and align proposed research scope with CBEP's objectives. o *BAA Section 1.5.6, pp 7-9* o *Call Section 2.1.6, pp 5-7* • Develop an abstract of the research project and submit in the body of an email to HDTRA1-FRCWMD-TA6@mail.mil. o *BAA Section 4.2.1, pp 14; Section 7, pg 37* o *Call Section 5.1, pp 9; Section 9.2, pg 27* • Abstract must be reviewed favorably by CBEP prior to the submission of the Phase I white paper. Applicant must receive an invitation to continue on to Phase I white paper. o *BAA Section 4.2.1, pp 14-15* o *Call Section 5.1, pp 9*
Phase I – Pre-Application White Paper	• Please note, following an invitation to submit a Phase I pre-application white paper (white paper), communications must be conducted via the appropriate administrative email address: o *BAA: HDTRA1-FRCWMD-A@mail.mil* o *Call: HDTRA1-FRCWMD-C@mail.mil* • Develop a Phase I white paper. o *BAA Section 4.2.4, pp 14-16* o *Call Section 5.1.4, pp 10; 5.3, pp 11-12* • BAA only o Register at www.grants.gov. o Submit a completed application to www.grants.gov. o *BAA Section 4.2.4, pp 14-16* • Call only o Ensure to register with the DTRA/SCC-WMD submission website www.dtrasubmission.net. o Submit the completed application to www.dtrasubmission.net. o *Call Section 5.1-5.2, pp 9-11*
Phase II – Full Proposal	• Upon receipt of an invitation for a Phase II full proposal, develop a proposal and incorporate any comments from DTRA/SCC-WMD's Phase I debrief summary. o *BAA Section 4.2.6., pp 16-22* o *Call Section 5.5, pp 12-18* • All proposals involving infectious or potentially infectious materials must include a completed and signed Protocol Risk Assessment Tool (PRAT). The purpose of this form is to ensure laboratory work sponsored by CBEP is conducted safely, securely, and responsibly. The PRAT should be completed and signed by the Principal Investigator. A blank form is available via the www.dtrasubmission.net Document and Template library. • All TA6 research Phase II proposals (BAA and Call) that involve human or animal use must provide Institutional Review Board (IRB) and/or Institutional Animal Care and Use Committee (IACUC) protocols and provide provisional protocol numbers as well as IRB/IACUC point of contact information. No human/animal is permitted until BOTH the institutional review and U.S. Department of Defense approval authorities grant approval. DTRA/SCC-WMD sends all proposals through its internal Research Oversight Board and an interagency review to ensure use of best practices and a safe and secure research environment for all participants. Awardees will be notified via email when permission to begin human/animal work has been granted. This process may increase the timeline between Phase II review, funding approval, and project start. • Submit completed Phase II information including all attachments to www.grants.gov (BAA), or www.dtrasubmission.net (Call), as applicable and instructed in BAA and Call instructions. Note that Protocol Risk Assessment Tool attachments must be emailed to HDTRA1-FRCWMD-A@mail.mil (BAA) or HDTRA1-FRCWMD-C@mail.mil (Call) and NOT attached to the www.grants.gov (BAA) or www.dtrasubmission.net (Call) submission. o *BAA Section 4.2.6, pp 22* o *Call Section 5.5.3, pp 17*

CBEP Points of Contact

For clarifications or concerns, please reach out to the following CBEP representatives:

From: (b) (6)
To: (b) (6)

Cc: Christopher Broder; Laing, Eric D CTR (US); (b) (6)
Subject: RE: [Non-DoD Source] USU and biothreat reduction
Date: Thursday, January 4, 2018 8:18:09 AM
Attachments: 2017 CBEP SPR Program Book_Final.pdf
CBEP_Trifold_Brochure-PAO_Approved.pdf
CBEP Research Trifold Dec 2017.pdf
CBEP RD-BA Roadmap_OCT2016_PAO approved.pdf

Hi (b) (6),
Thanks for arranging everything and taking the time to meet with us and provide an overview of the work you've been doing.

Attached are the updated tri-fold brochures that I handed out yesterday. I'm also attaching the Roadmap document (b) (6) mentioned and the book of abstracts from the 2017 CBEP Science Program Review. The abstracts will give you an idea of the kinds of projects we currently have underway.

If you or any of your colleagues have questions, feel free to give any of us a shout and we can get the inquiry sent on to the right folks.

Cheers,
(b) (6)

**NOTICE: Nothing in this email is intended to constitute contractual direction or impact currently negotiated cost, price, or schedule contained within the contract. If the contractor believes there is an impact, the contractor must disregard that portion of the communication and contact the contracting officer for direction.

-----Original Message-----
From: (b) (6)
Sent: Wednesday, January 3, 2018 4:10 PM
To: (b) (6)
Cc: Christopher Broder (b) (6) ; Laing, Eric D CTR (US) (b) (6)
Subject: [Non-DoD Source] USU and biothreat reduction

All active links contained in this email were disabled. Please verify the identity of the sender, and confirm the authenticity of all links contained within the message prior to copying and pasting the address to a Web browser.

Many thanks for making the drive and your time! I attach a PDF of the slide set I used today. Best wishes

(b) (6)

[redacted]

[redacted]

[redacted]

From: (b) (6)
To: (b) (6) ; Broder, Christopher; (b) (6)
Cc: (b) (6)
Subject: Nipah Conference Summary - v3
Date: Tuesday, April 14, 2020 4:50:19 PM
Attachments: Summary for mSphere_v3.docx
image004.png
image006.png
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image010.png
image012.png
image016.png

Dear All,

Thanks again to those of you who have sent contributions to earlier versions on the summary from the Nipah Conference.

Please see the third version of the document here. Co-authors are those who have provided specific feedback, comments and content.

This version will now go for internal review at CEPI prior to submission to mSphere.

I wish to acknowledge the remaining IOC members who are not able to contribute to authorship this time. I understand all of us are swamped with the current pandemic situation, and not all of you will be available to comment or contribute this time.

Best wishes,

(b) (6)

[redacted]

CEPI New vaccines for a safer world

[redacted]

[redacted]

[redacted]

From: (b) (6)
Sent: torsdag 2. april 2020 23:44
To: (b) (6)
Broder, Christopher
(b) (6)
Cc: (b) (6)
Subject: RE: Proceedings from Nipah Virus International Conference

Dear All,

We would like to thank those of you who have sent your comments and suggestions. Please find the second draft of the Nipah@20 summary paper for *mSphere*. This incorporates comments received thus far into a more refined version in which the table has been removed.

Kindly send any comments/edits back to us **before April 10th**. We will only include as authors those of you who have expressed a wish to co-write this summary. We will include the remaining IOC members in the acknowledgements section.

Dear (b) (6) and All – please hang in there and receive our solidarity in these difficult times.

Best wishes,
(b) (6)

CEPI New vaccines for a safer world

(b) (6)

From: (b) (6)
Sent: tirsdag 31. mars 2020 09:43
To: (b) (6)
Cc: (b) (6) Broder, Christopher (b) (6)
Subject: RE: Proceedings from Nipah Virus International Conference

Dear (b) (6)

Wow! How confronting. It is one thing to see a clip on the news, but another to hear it from someone in the hot zone. I am so sorry that you, and the rest of your colleagues, are experiencing this. Modern medicine wasn't supposed to be like this. This really is a tsunami.

Please don't hesitate to reach out if I can help you in anyway.

Thinking of you,
Kindest regards
(b) (6)

Sent from Mail for Windows 10

From: (b) (6)
Sent: Tuesday, 31 March 2020 4:43 PM
To: (b) (6)
Cc: (b) (6) ; Broder, Christopher; (b) (6)

(b) (6) [redacted]

Subject: Re: Proceedings from Nipah Virus International Conference

(b) (6) no longer remembers what day it is anymore. (b) (6), you'll have to remind me.

As of yesterday we have 1,249 COVID-19 positive patients in our hospitals. That includes 233 patients in our ICUs. We only had ~130 ICU beds . . now we walk by this in our lobby (everything is public so no confidentiality breached here) as every square inch gets gradually taken up by temp beds for COVID-19 patients. If New York was a country, we would be No. 5 in the world in no. of COVID-19 cases. By the time I wake up tomorrow, NY would have surpass the maximal number of cases in Hubei province.

We are trying innovative ways of re-using N95s and/or impregnating them with virucides. Innovative ways of trying to consent patients for longitudinal studies, developing serology testing to identify sero-converters for both human plasma therapy and de-risk front-line responders . . .our dept works as one now. On staggered shifts as we all work only on COVID-19 related research while still maintaining social distancing

I know this is not related to our Nipah conference but thought you all might like to know what it is being in the middle of the world's hottest zone. Being spat on by strangers while taking the subway to work does not make it easier. I no longer take public transport.

#BeSafe #FlattenTheCurve #SocialDistancingWorks

(b) (6) [redacted]

[redacted]

On Mar 30, 2020, at 12:28 AM, (b) (6) [redacted] wrote:

USE CAUTION: External Message.

Hi there

I'm not sure if others have contributed to the *mSphere* article as requested by (b) (6) (email below), but please find my contribution.
Others are welcome to build on this, if they have time. It would be great to see a few

more comments to round out the summary of this amazing (and somewhat prophetic!) conference.

I think (b) (6) wanted this by 30th March – so I'm cutting this a bit fine – sorry.

Thanks everyone
Kind regards
(b) (6)

From: (b) (6)
Sent: Thursday, 19 March 2020 9:57 PM
To: (b) (6) Broder, Christopher
(b) (6)
Cc: (b) (6)
Subject: [WARNING : MESSAGE ENCRYPTED]Proceedings from Nipah Virus International Conference

Dear International Organizing Committee (IOC) members for the Nipah Virus International Conference,

Please find a ***draft*** (pdf) with the Proceedings from the Nipah Virus International Conference, which took place in Singapore last December. The password for this document is (b) (6). Please consider this document under 'embargo' until cleared by the the respective Communications departments from CEPI and its Conference organizing partners (WHO, NIAID, Duke-NUS).

We hope this email finds you well. We apologise for not sharing the Proceedings with you sooner, but we hope you will understand all of us have been swamped with the present COVID-19 situation. Although we do appreciate the Nipah Conference may be perceived as less of a global priority now comparted to COVID-19, we believe the Proceedings contain relevant insights that should be shared with the global community in a pandemic situation.

Our plan is to upload a refined and cleared version of the Proceedings into a suitable online platform for distribution with our partners, ideally within 4 months of the Conference having taken place (i.e. before April 11th).

In addition, and thanks to (b) (6), the Editor-in-Chief for *mSphere* has also agreed

to publish a *summary*version of this and we have started working on that. Please also find the *word* document with a *draft* summary of Conference. We would like to invite you to co-author this summary and edit/add comments to this **by Monday, March 30**. If you do not wish to co-author the summary, please let us know.

Again, we do recognise that this may be perceived as less of a priority now, but we hope the insights from the Conference can shed light in some of the scientific issues being discussed now. We would like to thank you again for your valuable contributions to this Conference, and we look forward to your participation in this process.

Best wishes,
(b) (6)

[redacted]

[redacted]

[redacted]

[redacted]

[redacted]

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<Summary and Outline for mSphere_v1_(b) (6).docx>

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Received: 26 May 2021 | Accepted: 30 May 2021

DOI: 10.1111/irv.12882

LETTER TO THE EDITOR

WILEY

Susceptibility of different cell lines to the novel canine coronavirus CCoV-HuPn-2018

Dear Editor,

Over the past few decades, we have witnessed the emergence of numerous novel viruses within the family Coronaviridae. These have included the swine acute diarrhea syndrome coronavirus (SADS-CoV), the canine respiratory coronavirus (CRCoV), the feline coronavirus serotype II (FCoV-II), and the latest severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2).[1–5] Coronaviruses have increased opportunities for mutation and spill-over due to the frequent recombination and mutation events during replication, which helps them generate new viral threats. In fact, it is understood that all currently recognized human coronaviruses, HCoV-229E, HCoV-OC43, HCoV-NL63, HCoV-HKU1, SARS-CoV, MERS-CoV, and SARS-CoV-2, are zoonotic in origin.[6,7] However, evidence for canine and feline coronaviruses spilling over to humans has been sparse.

In a recent study evaluating a molecular diagnostic assay for coronaviruses, our team found evidence of canine coronavirus (CCoV) in eight patients hospitalized with pneumonia in Sarawak, Malaysia between 2017 and 2018.[8] Further analysis and viral isolation were then conducted in canine fibroblast tumor cells (A72). Among the eight samples, one specimen yielded a viral isolate, which was characterized by complete genome sequencing. The identified virus was a novel canine–feline recombinant alphacoronavirus (genotype II) that was named CCoV-HuPn-2018.[9]

We sought to assess the receptivity of different animal and human cell lines to the novel canine coronavirus CCoV-HuPn-2018 in comparison to another canine coronavirus, CCoV-UCD1 and a seasonal human coronavirus, HCoV-229E. The studied cell lines included adenocarcinomic human alveolar basal epithelial cells (A549), the human lung fibroblast cell line (MRC-5), Madin–Darby canine kidney (MDCK) cells, African green monkey kidney epithelial cells (VeroE6), pig testis cells (ST), and mink lung epithelial cells (Mv1Lu). A72 cells were used as a positive control for the CCoVs.

In 24-well plates, monolayers of MDCK, ST, A549, MRC.5, and A72 cells were inoculated with the two canine coronaviruses, CCoV-HuPn-2018 and CCoV-UCD1. CCoV-HuPn-2018 was also inoculated in Mv1Lu and VeroE6 cells. The human coronavirus HCoV-229E was inoculated in monolayers of MDCK, ST, A549 and MRC.5 cells. Median tissue culture infectious dose (TCID50) was calculated for each virus using the Reed–Muench method,[10] and inoculations were conducted at a multiplicity of infection (MOI) of 0.1. Cells were then incubated for 1 h at 37°C and 5% CO_2, except A72 cells which were incubated without CO_2. Following the incubation, virus was removed, and cells were washed once with phosphate-buffered saline (PBS), then fresh infection media containing 2% fetal bovine serum was added. Cells were monitored for cytopathic effect (CPE) every 24 h. Cells and supernatant were harvested at 0-, 40-, 72-, and 192-h postinoculation. RNA was extracted using the QIAamp Viral RNA Mini Kit (QIAGEN, Inc., Valencia, CA) and screened with a real-time reverse transcription polymerase chain reaction (qRT-PCR) assay specific for the virus.[9,11] Virus culture was considered positive when the cycle threshold (Ct)[7] value was at least 2 points below the 0-h inoculum and CPE was present.

CPE was observed 40-h postinoculation in A72 cells inoculated with CCoV-HuPn-2018 and CCoV-UCD1 and confirmed with qRT-PCR (Table 1). No increase in the viral replication was observed in MDCK, ST, A549, MRC.5, Mv1Lu, and VeroE6 cells even after 192-h postinoculation, suggesting that these cell lines are not permissive for CCoV-HuPn-2018 and CCoV-UCD1 (Figure S1).

CPE was observed in MRC5 cells inoculated with HCoV-229E beginning at 72-h postinoculation. This observation was also confirmed by qRT-PCR as Ct values were significantly lower than the original result. MDCK, ST, and A549 cells were monitored up to 192-h postinoculation, and no CPE was observed in these cells nor were positive qRT-PCR results detected.

The ability of the CCoVs to form CPE in A72 cells and the HCoV-229E to infect MRC.5 cells has been previously described.[9,12,13] Our experiments suggest that the studied human lung cells are not receptive for CCoV-HuPn-2018 infection and replication, despite their expression of APN receptors. However, previous studies have suggested that some coronaviruses are resistant to cell culture.[14,15] Additionally, permissiveness of various cell lines to coronavirus infection in vitro does not always recapitulate the in vivo tissue and host.[16] In vitro infection of this novel CCoV in human cell lines is challenging and requires further understanding of the virus pathogenesis and infection initiation in the human respiratory system.

TABLE 1 Susceptibility of cells to CCoV-HuPn-2018 as assessed by cytopathic effect and qRT-PCR

Cell line	Species	Cell type	CPE	Quantitative PCR Ct (hours 0, 72, 192)
A549	Human	Lung carcinoma epithelium	–	24.7, 27, 29.9
MRC-5	Human	Fetal lung fibroblast	–	24.0, 30.7, 33.4
MDCK	Canine	Kidney epithelium	–	24.6, 29.6, 33.1
A72[a]	Canine	Tumor fibroblast	+	25.4, 17.7, 16.4
Vero E6	African green Monkey	Kidney epithelium	–	25.3, 28.7, 31.3
ST	Swine	Fetal testes	–	24.2, 28.5, 31.7
Mv1Lu	Mink	Lung epithelium	–	25.2, 28.1, 31.0

Abbreviations: CPE, cytopathic effect; Ct, cycle threshold; qRT-PCR, quantitative reverse transcription polymerase chain reaction.
[a]CPE was observed 40-h postinoculation in this cell line.

KEYWORDS

alphacoronavirus, canine coronavirus, CCoV-HuPn-2018, cell lines, receptivity

ACKNOWLEDGEMENT

This study was supported by Professor Gray's discretionary funding.

CONFLICT OF INTEREST

None declared.

AUTHOR CONTRIBUTIONS

Gregory Gray: Conceptualization; funding acquisition; supervision. **Anfal Abdelgadir:** Investigation. **Anastasia Vlasova:** Conceptualization; investigation; methodology; supervision.

Anfal Abdelgadir[1,2]
Anastasia N. Vlasova[3]
Gregory C. Gray[1,2,4,5]

[1]*Division of Infectious Diseases, School of Medicine, Duke University, Durham, NC, USA*
[2]*Duke Global Health Institute, Duke University, Durham, NC, USA*
[3]*Food Animal Health Research Program, Ohio Agricultural Research and Development Center, College of Food, Agricultural and Environmental Sciences, Department of Veterinary Preventive Medicine, The Ohio State University, Wooster, OH, USA*
[4]*Global Health Research Center, Duke Kunshan University, Kunshan, China*
[5]*Program in Emerging Infectious Diseases, Duke-NUS Medical School, Singapore*

Correspondence
Gregory C. Gray, Division of Infectious Diseases, School of Medicine, Duke University, DUMC Box 102359, Durham, NC 27710, USA.
Email: gregory.gray@duke.edu

ORCID

Anfal Abdelgadir https://orcid.org/0000-0001-5345-9972
Anastasia N. Vlasova https://orcid.org/0000-0002-1447-6283
Gregory C. Gray https://orcid.org/0000-0002-4628-5908

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

Clinical Infectious Diseases

BRIEF REPORT

Isolation of a Novel Recombinant Canine Coronavirus From a Visitor to Haiti: Further Evidence of Transmission of Coronaviruses of Zoonotic Origin to Humans

John A. Lednicky,[1,2,a] Massimiliano S. Tagliamonte,[1,3,a] Sarah K. White,[1,2] Gabriela M. Blohm,[1,2] Md. Mahbubul Alam,[1,2] Nicole M. Iovine,[1,4] Marco Salemi,[1,3] Carla Mavian,[1,3] and J. Glenn Morris Jr[1,4]

[1]Emerging Pathogens Institute, University of Florida, Gainesville, Florida, USA; [2]Department of Environmental and Global Health, College of Public Health and Health Professions, University of Florida, Gainesville, Florida, USA; [3]Department of Pathology, Immunology, and Laboratory Medicine, College of Medicine, University of Florida, Gainesville, Florida, USA; and [4]Division of Infectious Diseases, Department of Medicine, College of Medicine, University of Florida, Gainesville, Florida, USA.

We isolated a novel coronavirus from a medical team member presenting with fever and malaise after travel to Haiti. The virus showed 99.4% similarity with a recombinant canine coronavirus recently identified in a pneumonia patient in Malaysia, suggesting that infection with this virus and/or recombinant variants occurs in multiple locations.

Keywords. coronavirus; coronavirus: canine; coronavirus: zoonotic; coronavirus: recombinant; human coronavirus infection.

In March 2017, members of a medical team from University of Florida who had recently returned from a "mission trip" to Haiti presented with mild fever and malaise. Zika virus (ZIKV) was circulating in Haiti at the time, and because of concerns that their illnesses might represent ZIKV infection, freshly collected urine samples were obtained from team members and screened for ZIKV, in keeping with previously described methods [1]. All samples tested negative by reverse transcription polymerase chain reaction (RT-PCR) for ZIKV. However, at that time our routine procedure included efforts to isolate ZIKV from all diagnostic specimens, and consequently deidentified aliquots of the urine samples were subsequently inoculated onto Vero E6 and LLC-MK2 cells, which are susceptible and permissive for ZIKV.

Twenty urine samples from team members were screened. Samples from six patients produced cytopathic effects (CPE) in cell culture within 14 days of inoculation of cell lines; an example is shown for LLC-MK2 cells inoculated with urine sample Z19 (Figure 1). When aliquots of cell culture media from Vero E6 cells at 16 days post-inoculation were inoculated onto MDCK cells, CPE were noted the following day (Supplementary Figure 1). To determine whether the viruses causing CPE were of possible respiratory origin, cell culture media was tested using a GenMark eSensor XT-8 RVP system (eSensor RVP; GenMark Diagnostics, Inc., Carlsbad, California, USA) instrument [2]. Unexpectedly, the 6 samples tested showed mixed low signals for 3 of the 4 seasonal endemic human coronaviruses (threshold signal [nA] value above 3, the generally accepted positive cutoff, seen for Betacoronavirus OC43 [4 of 6 samples tested] and Alphacoronaviruses 229E [4 of 6 samples tested] and NL65 [2 of 6 samples]) (Supplementary Table 1). After follow-up RT-PCR tests of the cell culture media using species-specific coronavirus RT-PCR tests failed to establish an identity, an unbiased amplification and sequencing approach was attempted [3, 4].

As material extracted from Madin-Darby canine kidney (MDCK) cells culture media corresponding to sample Z19 appeared to have the highest virus yield based on the extent of CPE formed, RNA from this sample was purified and subjected to Sanger sequencing. Initial sequence analyses of a 2558 bp amplicon (Supplementary Figure 2) generated using an unbiased RT-PCR amplification method [5] indicated 97% (2475/2561) nucleotide (nt) identity to a porcine coronavirus, transmissible gastroenteritis virus (TGEV) strain Purdue P115 (Genbank Accession no. DQ811788.1), leading to the assumption that the virus was TGEV. However, primers based on TGEV did not effectively amplify or failed to amplify other sections of the virus' genome, suggesting that it was a different coronavirus. After the publication of Vlasova et al [6], primers that targeted parts of the RdRp gene and spike protein on the genome sequence they discovered (canine coronavirus isolate CCoV-HuPn-2018, GenBank MW591993.2) were tested and were found to produce PCR amplicons. This prompted us to focus efforts on amplifying the virus sequence of our isolate using canine coronavirus primers.

Ultimately, 39 primer pairs covering the whole virus genome were designed for complete genome sequencing (Supplementary Table 2). Three additional primers for 5′ and 3′ Rapid Amplification of cDNA Ends (RACE) were also designed for this work; that work was accomplished using the RACE System (Invitrogen) used according to the manufacturer's

Received 30 June 2021; editorial decision 20 October 2021; published online 28 October 2021.

[a]J. A. L. and M. S. T. share first authorship.

Correspondence: G. Morris, Emerging Pathogens Institute, PO Box 100009, Gainesville, FL32610-0009 (jgmorris@epi.ufl.edu).

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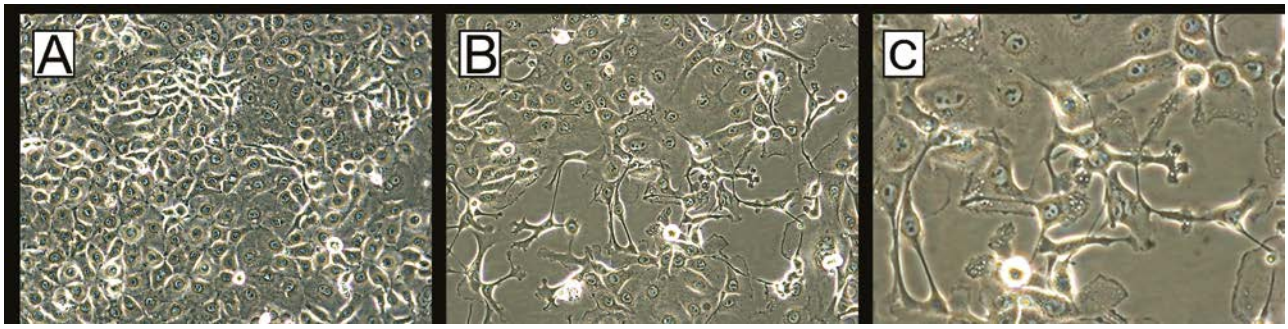

Figure 1. Virus-induced cytopathic effects in LLC-MK2 cells 16 days post-inoculation with urine specimen Z19. *A*, Mock-inoculated cells, original magnification 200×. *B*, Cells inoculated with urine, original magnification 200×. *C*, Detail from image *B*, original magnification 400×.

manual. By using the primers of Supplementary Table 2, virus genomic RNA (vgRNA) was reverse-transcribed into cDNA using an AccuScript High fidelity 1st Strand cDNA Synthesis Kit (Agilent, Santa Clara, California, USA), and PCR performed with Q5 high-fidelity DNA polymerase (New England Biolabs). Sanger sequencing was performed using a BigDye Terminator version 3.1 cycle sequencing kit (Applied Biosystems, USA). The strain has been designated HuCCoV_Z19Haiti; GenBank accession number is MZ420153. The five other samples showing CPE effects (Z03, Z04, Z11, Z12, and Z14) were only partly characterized (for verification purposes) after Z19 was fully sequenced. Primers 36F and 36R (Supplementary Table 2) were used to RT-PCR amplify 850 nt amplicons from the other 5, and after sequencing these amplicons were found to be identical to the sequence of Z19. The remaining 14 patient urine samples were negative on screening with multiple primers. Neither this virus—or other canine coronaviruses—were present in our laboratory prior to this study.

Further recombination and phylogenetic analyses were conducted as previously described in Lednicky et al [4]; details of methods are provided in Supplementary Materials. A maximum likelihood (ML) tree inferred on the full genome alignment (Figure 2A), regardless of potential recombinant genomic fragments, confirmed the close relationship between HuCCoV_Z19Haiti and CCoV-HuPn-2018, with 99.4% identity between the 2 virus strains. The second half of the HuCCoV_Z19Haiti genome, starting from gene E, showed greater divergence from CCoV-HuPn-2018 (similarity plot, Supplementary Figure 3, Supplementary Table 3). Furthermore, the HuCCoV_Z19Haiti isolate did not have the 36 nt deletion in gene N and the 228 nt deletion in ORF7b, both characteristic of the Malaysian strain. A further Blast search [7] on the NCBI database, conducted only with the genes E, M, N, and the ORF7 segment, did show a match with the Chinese canine coronavirus strain CCoV B639_ZJ_2019 [8] (Supplementary Figure 3). Fragmenting the genome of HuCCoV_Z19Haiti alignment by gene, as was done by Vlaslova et al [6], further confirmed the chimeric nature of the virus isolated in Haiti (Supplementary Figure 4). Both Spike S1 and S2 ML trees clustered HuCCoV_Z19Haiti with CCoV-HuPn-2018, although in the gene M ML tree the closest relative was the Chinese CCoV B639_ZI_2019. In the gene N phylogeny, the Haitian strain clusters with TGEV, although the bootstrap values might be too low to make a strong inference.

We identified the same pattern of recombination events reported by Vlasova et al [6] in the spike and ORF1 of the Haitian genome (Supplementary Table 4) suggesting that recombination occurred ancestrally to CCoV-HuPn-2018 and HuCCoV_Z19Haiti. The Haitian isolate, however, further diverged from the Malaysian strain through additional and multiple recombination events across the genome, notably affecting the gene E – ORF7 segment, which closely relates to CCoV B639_ZJ_2019. Further recombination events with other CCoVs overlapped to the segment originated from CCoV B639_ZJ_2019. To corroborate the recombination analysis, 5 subsets of genomic fragments were analyzed: the larger one constituted by most of the genome, minus the recombinant fragments, and 4 smaller ones constituted by the segments involved in the inferred recombination events involving HuCCoV_Z19Haiti. Recombinant segments common to CCoV-HuPn-2018 and HuCCoV_Z19Haiti were removed, as they were considered to have occurred prior to divergence; other recombinant segments, involving taxa other than the Haitian strain, were also removed from recombinant sequences. Following assessment of phylogenetic signal (Supplementary Figure 5), ML trees for each nonrecombinant fragment (Figure 2B–F) confirmed CCoV-HuPn-2018 as the major parent of the Haitian strain, and the chimeric nature of the other fragments, involving other CCoVs, as well as possibly unsampled ancestors of TGEVs.

COMMENT

Coronaviruses are known to infect a wide range of mammalian and bird species [9]. They have also long been recognized as one of the causes of the "common cold" in humans, associated with what have been termed the seasonal endemic human coronaviruses: HCoV 229E and NL63 in the genus *Alphacoronavirus*; and HCoV HKU1 and OC43 in the genus *Betacoronavirus* [10].

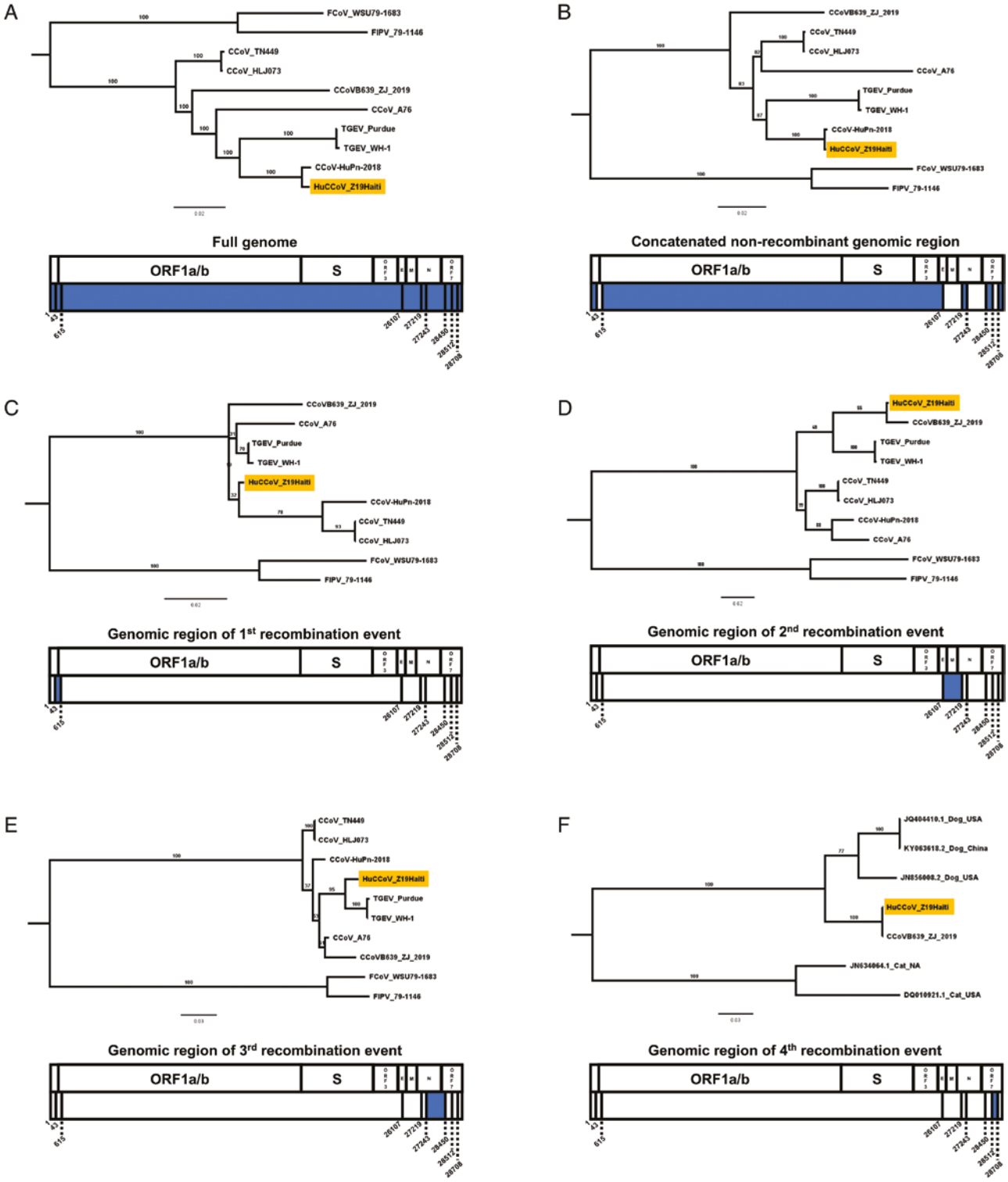

Figure 2. Maximum likelihood (ML) tree of alphacoronavirus strains. ML trees were inferred from 10 genome sequences, including CCoV-HuPn-2018 and CCoVB639_ZJ_2019, using the best fitting nucleotide substitution models as detected by Bayesian information criterion. Branches are scaled in number of nucleotide substitutions per site according to the bar below each tree. Nonparametric bootstrap values (1000 replicates) are indicated along supported branches. Haitian strain HuCCoV Z19 is highlighted. *A*, ML tree calculated using full genomes, prior to any recombination analysis. Panels *B–F* show trees inferred based on non-recombinant genomic fragments, indicated in blue in the schematic genome below each tree for clarity. Genome coordinates are based on HuCCoV Z19. *B*, ML tree calculated using non-recombinant segments of the genome. *C*, ML tree calculated using HuCCoV Z19 recombinant segment 43-615. *D*, ML tree calculated using HuCCoV Z19 recombinant segment 26107-27219. *E*, ML tree calculated using HuCCoV Z19 recombinant segment 27243-28450. *F*, ML tree calculated using HuCCoV Z19 recombinant segment 28512-28708. Segments involved in recombination events of genomes other than HuCCoV Z19 and CCoV-HuPn-2018 were replaced by gaps in the affected sequences in trees *B–F*.

However, over the past 2 decades we have seen the emergence of three coronavirus species that are highly pathogenic for humans, and which appear in each instance to have arisen from a zoonotic origin: severe acute respiratory syndrome coronavirus (SARS-CoV-1), Middle East respiratory syndrome coronavirus (MERS-CoV), and severe acute respiratory syndrome 2 (SARS-CoV-2), all in the genus *Betacoronavirus*.

Our group has recently reported isolation of a porcine deltacoronavirus (PDCoV) from children in Haiti presenting with fever and gastrointestinal complaints, with genomic and evolutionary analyses suggesting that human infections were the result of at least 2 independent zoonoses of distinct viral lineages that acquired a common mutational signature in the *nsp15* and the *spike* glycoprotein genes by convergent evolution [4]. As noted above, Vlasova et al reported isolation of an *Alphacoronavirus* of apparent canine origin, with evidence of recombination with a feline coronavirus, from patients with pneumonia in Malaysia [6]. We report here identification of a coronavirus of canine origin which is closely related to the Malaysian virus reported by Vlasova et al, albeit isolated in this instance from a visitor to Haiti, and with a further recombinational history. Samples were deidentified after initial screening by RT-PCR for Zika, limiting our ability to obtain detailed clinical and epidemiological information on specific infected individuals; however, all members of the group reported mild fever and malaise, and all recovered uneventfully. Our data highlight the potential among coronaviruses for rapid evolution combined with frequent recombination events, leading to periodic emergence of strains capable of crossing species barriers into human populations. In many instances such strains would appear to be of low virulence for humans, as reflected in our work with PDCoV and now CCoV-Haiti; however, the potential for such strains to carry or acquire genes capable of causing severe disease in humans remains of clear concern.

Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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Potential conflicts of interest. All authors: No reported conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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Clinical Infectious Diseases

MAJOR ARTICLE

Novel Canine Coronavirus Isolated from a Hospitalized Patient With Pneumonia in East Malaysia

Anastasia N. Vlasova,[1,a] Annika Diaz,[1,a] Debasu Damtie,[2,3] Leshan Xiu,[4,5,6,©] Teck-Hock Toh,[7,8] Jeffrey Soon-Yit Lee,[7,8] Linda J. Saif,[1] and Gregory C. Gray[4,5,9,10]

[1]Food Animal Health Research Program, Ohio Agricultural Research and Development Center, College of Food, Agricultural and Environmental Sciences, Department of Veterinary Preventive Medicine, The Ohio State University, Wooster, Ohio, USA; [2]Department of Immunology and Molecular Biology, School of Biomedical and Laboratory Sciences, College of Medicine and Health Sciences, University of Gondar, Gondar, Ethiopia; [3]The Ohio State University Global One Health LLC, Eastern Africa Regional Office, Addis Ababa, Ethiopia; [4]Division of Infectious Diseases, Duke University School of Medicine, Durham, North Carolina, USA; [5]Duke Global Health Institute, Duke University, Durham, North Carolina, USA; [6]NHC Key Laboratory of Systems Biology of Pathogens, Institute of Pathogen Biology, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing, China; [7]Clinical Research Center, Sibu Hospital, Ministry of Health Malaysia, Sibu, Sarawak, Malaysia; [8]Faculty of Medicine, SEGi University, Kota Damansara, Selangor, Malaysia; [9]Global Health Research Center, Duke Kunshan University, Kunshan, China; and [10]Program in Emerging Infectious Diseases, Duke-NUS Medical School, Singapore

Background. During the validation of a highly sensitive panspecies coronavirus (CoV) seminested reverse-transcription polymerase chain reaction (RT-PCR) assay, we found canine CoV (CCoV) RNA in nasopharyngeal swab samples from 8 of 301 patients (2.5%) hospitalized with pneumonia during 2017–2018 in Sarawak, Malaysia. Most patients were children living in rural areas with frequent exposure to domesticated animals and wildlife.

Methods. Specimens were further studied with universal and species-specific CoV and CCoV 1-step RT-PCR assays, and viral isolation was performed in A72 canine cells. Complete genome sequencing was conducted using the Sanger method.

Results. Two of 8 specimens contained sufficient amounts of CCoVs as confirmed by less-sensitive single-step RT-PCR assays, and 1 specimen demonstrated cytopathic effects in A72 cells. Complete genome sequencing of the virus causing cytopathic effects identified it as a novel canine-feline recombinant alphacoronavirus (genotype II) that we named CCoV–human pneumonia (HuPn)–2018. Most of the CCoV-HuPn-2018 genome is more closely related to a CCoV TN-449, while its S gene shared significantly higher sequence identity with CCoV-UCD-1 (S1 domain) and a feline CoV WSU 79-1683 (S2 domain). CCoV-HuPn-2018 is unique for a 36-nucleotide (12–amino acid) deletion in the N protein and the presence of full-length and truncated 7b nonstructural protein, which may have clinical relevance.

Conclusions. This is the first report of a novel canine-feline recombinant alphacoronavirus isolated from a human patient with pneumonia. If confirmed as a pathogen, it may represent the eighth unique coronavirus known to cause disease in humans. Our findings underscore the public health threat of animal CoVs and a need to conduct better surveillance for them.

Keywords. canine coronavirus; novel alphacoronavirus; pneumonia: zoonotic disease; East Malaysia.

Human coronaviruses (HCoVs) associated with common colds (HCoV-229E and HCoV-OC43) were initially identified in the mid-1960s, and 2 more, HCoV-NL63 and HCoV-HKU1, were described in 2004 and 2005, respectively [1–3]. The emergence of severe acute respiratory syndrome (SARS) coronavirus (CoV) in 2002–2003 and Middle East respiratory syndrome CoV in 2012 demonstrated that CoVs can cause severe to fatal disease [4]. Evidence suggests that bats are likely to be the original source of SARS-CoV and Middle East respiratory syndrome CoV [5, 6]. The most recent and notable CoV-related threat is represented by the coronavirus disease 2019 pandemic caused by SARS-CoV-2 [7]. While the origin of SARS-CoV-2 is still debated [8], it is thought to have emerged via a spillover event originating at a Chinese wet market. Thus, zoonotic CoVs pose a major threat to human health, with different animals serving as natural reservoirs or intermediate hosts to CoVs transmittable to humans [9, 10]. However, the potential threat represented by cats and dogs or their CoVs has been sparsely studied.

Different genotypes (I, II) of canine CoVs (CCoVs) of *Alphacoronavirus 1* species cause moderate-to-severe enteric disease in dogs [11]. CCoV-II circulation has been confirmed in dogs since 1971, and CCoV-I was discovered about 3 decades later [12, 13]. Transmissible gastroenteritis virus (TGEV), CCoV-II, and feline CoV (FCoV) II have reportedly originated from CCoV-I and FCoV-I through gene loss and recombination [14]. Similarly to FCoVs, CCoV-I strains do not grow or grow poorly in cell culture and their cellular receptor is unknown, while CCoV-II strains grow readily in culture using aminopeptidase N as a cellular receptor [15]. This emphasizes the complex evolution of CCoVs/*Alphacoronavirus 1* species and their ability to infect different hosts, inducing variable clinical disease. It has

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[a]A. N. V. and A. D. contributed equally to this work.

Correspondence: G. C. Gray, Division of Infectious Diseases, DUMC Box 102359, Duke University Durham, NC 27710 (gregory.gray@duke.edu).

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been demonstrated that another CoV, using aminopeptidase N as a cellular receptor, porcine deltacoronavirus, can infect cells of unusually broad species origin, including human and chicken [16].

Previous studies documenting CCoV in human patients with pneumonia in Sarawak [17] and FCoV-like CoVs in human patients with acute respiratory symptoms in Arkansas [18] represent the only evidence that *Alphacoronavirus 1* species may infect and be associated with a clinical disease in humans. Here we report isolation, complete genome sequencing and molecular analysis of a CCoV virus from one of the patients with pneumonia.

METHODS

Sample Source, Screening, and Cell Culture Isolation

Eight of 301 nasopharyngeal swab (NPS) specimens from hospitalized patients with pneumonia (2017–2018 at Sibu and Kapit Hospitals, Sarawak, Malaysia) were previously confirmed to contain CCoV using a seminested reverse-transcription polymerase chain reaction (RT-PCR) assay and Sanger sequencing (Table 1 and Supplementary Table 1) [17]. The 8 patients with pneumonia all came from Sibu Hospital (Table 1). Seven (87.5%) were aged <5 years, 4 were infants, and most were from Sarawak's indigenous ethnic groups, who typically live in rural or suburban longhouses or villages. Seven of the patients (87.5%) had evidence of a viral coinfection (Table 1). All bacterial blood cultures were negative, and all patients were hospitalized for 4–6 days and recovered.

RNA Extraction and RT-PCR

RNA was extracted from suspended NPS samples using the 5X MagMAX Viral Isolation Kit (Applied Biosystems). Because 1-step RT-PCR is less sensitive than nested or seminested RT-PCR, further characterization was conducted using 1-step RT-PCR assays to ensure no contamination. A Qiagen 1-step RT-PCR kit was used (primers and cycling protocols provided in Supplementary Table 2). Amplicons generated with CCoV-N-F/CCoV-N-R primers were gel extracted using the QIAquick Gel Extraction Kit (Qiagen) and sequenced using the Sanger method at the Molecular and Cellular Imaging Center (MCIC) at the Ohio Agricultural Research and Development Center, The Ohio State University, Wooster.

Virus Isolation in A72 Cell Culture and Transmission Electron Microscopy

Canine fibroblast tumor (A72) cells (received from Alfonso Torres, Cornell College of Veterinary Medicine) were maintained and used for sample inoculation, as described elsewhere [22]. Serially diluted NPS fluids (1:10–1:10 000) were used to inoculate the A72 monolayers. After 72 hours the infected cells and medium were harvested and used for RNA extraction with the RNEasy Mini Kit (Qiagen). Immune transmission electron microscopy (I-TEM) was conducted as described elsewhere,

Table 1. Demographic and Clinical Characteristics of 8 Patients With Molecular Evidence of Canine Coronavirus in Nasopharyngeal Swab Specimens

ID	Sex/Age/Ethnicity	Town/Housing Type (No. of Cohabitants at Home)	Underlying Condition/Medication	Known Exposure to Animals	Highest Oxygen Support During Admission	Duration of Hospital Stay	Other Concomitant Pathogens Detected[a]
1090	Male/13.5 m/Iban	Sibu/unknown (2)	Preschool wheeze/inhaled budesonide	No	No information	No information	Adenovirus
1116	Male/9.5 m/Iban	Sibu/longhouse (9)	Glucose-6-phosphate dehydrogenase deficiency/oral amoxycillin	No	Nasal prong oxygen (1 L/min)	5 Complete days	Adenovirus
1126	Female/2.5 y/Iban	Bintulu/longhouse (9)	None	No	No information	No information	Parainfluenza virus 3
1128	Female/11 m/Iban	Sibu/longhouse (7)	None	Yes (cats)	Nasal prong oxygen (1 L/min)	5 Complete days	Parainfluenza virus 3
1131	Female/4.5 y/Chinese	Sibu/townhouse (4)	None	Yes (cats and dogs)	No information	No information	Influenza A
1153[b]	Male/5.5 m/Melanau	Daro/village (10)	None/oral ampicillin and cloxacillin	No	No information	No information	Rhinovirus C
1157	Female/10 m/Bidayuh	Julau/longhouse (4)	Preschool wheeze/inhaled fluticasone	No	Nasal prong oxygen (1 L/min)	6 Complete days	Adenovirus
2062	Female/375 y/Iban	Sibu/staff quarter (15)	Bronchial asthma/inhaled fluticasone	Yes (dogs)	Nasal prong oxygen (3 L/min)	4 Complete days	None

Abbreviation: ID, patient identifier.

[a]Patients' nasopharyngeal swab specimens were studied with molecular assays for adenovirus, human enterovirus, influenza A, B, C, and D, respiratory syncytial virus A and B, parainfluenza viruses 1, 2, 3, and 4, and rhinovirus [19–21].

[b]Canine coronavirus–human pneumonia–2018 was isolated from sample 1153.

using polyclonal anti-CCoV guinea pig serum (BEI Resources; NR-2727); the I-TEM images were captured at the MCIC [23].

Complete Genome Sequencing With the Sanger Method

The viral RNA was converted into complementary DNA (cDNA) using a SuperScript III cDNA synthesis kit (Invitrogen). Forty-two primer pairs (Supplementary Table 3) covering the whole genome were designed based on the sequence of CCoV, strain TN-449, the most closely related strain, as determined by The Basic Local Alignment Search Tool (BLAST) nucleotide (BLASTn) analysis of the partial N gene sequence of the newly identified CCoV for which the complete genome was available. Using these primers and Platinum Taq (Invitrogen), 12 amplicons (1.7–3.6 kb) were generated and purified using the QIAquick Gel Extraction Kit and sequenced with 3× coverage, using the Sanger dideoxy method with a BigDye Terminator version 3.1 cycle sequencing kit (Applied Biosystems), at the MCIC and at the James Comprehensive Cancer Center Shared Genomics Core, The Ohio State University, Columbus. After the initial analysis/sequence assembly, 7 additional primer pairs were designed, based on the newly generated sequences, to close the remaining gaps (Supplementary Table 3). The fragments were amplified and sequenced as described above. The 5' and 3' genomic ends were amplified using the 5' and 3′ RACE System for Rapid Amplification of cDNA Ends (Invitrogen), according to the manufacturer's instructions.

Sequence Assembly and Analysis

Raw sequences were trimmed to remove low-quality reads and amplicon-primer linkers. Each open reading frame (ORF) was analyzed using Viral Genome ORF Reader (VIGOR4) to predict viral protein sequences. The annotated CCoV genome was submitted to GenBank (accession no. MW591993). The alignments were further analyzed using the Sequence Manipulation Suite (SMS; version 2) (https://www.bioinformatics.org/sms2/) to determine nucleotide identities between the reference and newly generated sequences. Sequence alignment and phylogenetic analysis were performed using the ClustalW method and the maximum-likelihood method with the general time-reversible nucleotide substitution model and bootstrap tests of 1000 replicates with MEGA X software. The CoV genomes for reference strains from GenBank used in the phylogenetic analyses are listed in (Table 2). The Recombinant Identification Program (RIP; http://www.hiv.lanl.gov/content/sequence/RIP/RIP.html) was used to identify recombination points within the CCoV–human pneumonia (HuPn)–2018 genome, with a window size of 400 and a confidence threshold of 90%. Glycosylation prediction was conducted using the NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/).

RESULTS

RT-PCR and Partial Sequencing of CCoV

Samples from 2 of the 8 patients from whom CCoV was earlier detected were positive in universal and CCoV-specific 1-step RT-PCR assays (Supplementary Table 2). This result could be due to differences in the quantity or integrity of CCoV in samples collected at variable time points after infection. According to the BLASTn search, the sequences obtained for both samples using CCoV-N-F/CCoV-N-R primers shared the highest nucleotide identity (96.31%) with several CCoV strains, including TN-449 and HLJ-073 (listed in Table 2). We selected the TN-449 sequence to design sequencing primers covering the complete genome (Supplementary Table 3).

CCoV Replication in A72 Canine Cells

While 8 CCoV-positive NPS samples were inoculated into A72 cells, only 1 sample (sample 1153; Table 1) produced cytopathic effects in the cells (Supplementary Figure 1). The A72 cell–passaged material (P1) was inoculated into A72 cells again, and cytopathic effects were observed within the same time frame (P2). RNA extracted from both P1 and P2 tested CCoV positive; RNA extracted from P1 was used for complete genome sequencing. This virus was visualized using I-TEM (Figure 1) and is referred to as CCoV-HuPn-2018 throughout.

Genomic Organization of CCoV-HuPn-2018

The assembled viral genome was 29 083/29 351 nucleotides long (owing to differences in length between the two 7b forms), excluding the poly(A) tail. The genomic organization and gene order were typical of other *Alphacoronavirus 1* species: ORF1a1b, spike (S), ORF3a, ORF3b, ORF3c, envelope (E), membrane (M), nucleocapsid (N), ORF7a and ORF7b (Supplementary Figure 2 and Table 3). The structural and nonstructural proteins (NSPs) were flanked by 5′ and 3' untranslated regions (UTRs) with a 3′ poly(A) tail.

The 5' UTR consisted of 313 nucleotides, including the leader sequence (nucleotides 1–94) and the conserved core 5-CU(T)AAAC-3 (nucleotides 95–100) of the transcription regulatory sequence (TRS) that controls the messenger RNA synthesis during the subgenomic RNA discontinuous transcription. Similar TRS signals preceded 5 genes: S (nucleotide 20 335), 3a (24 787), E (25 866); M (26 156), N (26 951), and 7a/b (28 072) (Table 3). There were no TRS signals in front of 3b/3c and 7b, suggesting that they may be expressed from polycistronic messenger RNAs. The 3' end of the viral genome consists of a 275-nucleotide 3' UTR, followed by the poly(A) tail. The 20 061 nucleotides following the 5' UTR were occupied by the replicase gene encoding for 2 large polyproteins, polyproteins 1a and 1b, with polyprotein 1ab synthesized through ribosomal slippage at position 12 33, as reported for the highly related CCoV TN-449.

The SMS analysis demonstrated that the genome was mostly similar to CCoV strains TN-449, HLJ-073, and A76 and the TGEV Purdue strain, sharing 93.31%, 91.744%, 90.63% and 91.47% nucleotide identity, respectively, followed by FCoV/feline infectious peritonitis virus (FIPV) strains (83.96%–84.58% nucleotide identity) (Table 2). This suggests

Table 2. Identity Between Canine Coronavirus–Human Pneumonia–2018 and *Alphacoronavirus 1* Reference Strains for Complete Genomic Sequence and Genes for Structural Proteins

			Nucleotide Identity to CCoV-HuPn-2018, %						
Alphacoronavirus 1	Strain	Accession No.	Complete Genome	S	S1	S2	E	M	N
CCoV-IIa	TN-449	JQ404410.1	93.31[a]	93.42[a]	73.22	95.20	93.57	95.08	93.42
CCoV-IIa	HLJ-073	KY063618.2	91.74	93.33	73.32	95.20	93.17	95.08	93.33
CCoV-IIc	A76	JN856008.2	90.63	93.77	53.80	85.42	95.18[a]	97.08[a]	93.77[a]
CCoV	UCD-1	AF116248.1	NA	NA	99.19[a]	NA	NA	NA	NA
TGEV	Purdue (virulent)	DQ811789.2	91.47	92.12	90.93	94.59	93.98	92.65	92.12
FCoV-II	WSU 79-1683	JN634064.1	84.58	74.91	72.80	97.13[a]	93.68	86.25	74.91
FCoV-II/FIPV	79-1146	DQ010921.1	84.04	75.5	73.04	95.04	79.92	81.77	75.5

Abbreviations: CCoV, canine coronavirus; CCoV-HuPn-2018, CCoV–human pneumonia 2018; E, envelope; FCoV, feline coronavirus; FIPV, feline infectious peritonitis virus; HuPN, human pneumonia; M, membrane; N, nucleocapsid; NA, not available; S, spike; TGEV, transmissible gastroenteritis virus.
[a]Highest nucleotide identity between CCoV-HuPn-2018 and given strain.

that CCoV-HuPn-2018 represents a novel strain within the *Alphacoronavirus 1* species.

Similar to the complete genome, CCoV-HuPn-2018 ORF1ab region shared the highest nucleotide identity with those of TN-449 (95.84%), HLJ-073 (95.70%), and A76 (95.40%), followed by other CCoV (89%–94.28%), various TGEV (92.6%–94.49%), and FCoV (82.08%–85.84%) strains. Furthermore, while the full-length S gene of CCoV-HuPn-2018 shared the highest nucleotide identity with CCoV TN-449 (93.42%), its S1 domain was nearly identical to that of CCoV UCD-1 (for which only the S1 sequence is available), sharing 99.19% nucleotide identity, higher than for any other genomic region (Table 2). The S2 domain of CCoV-HuPn-2018 shared the highest identity (97.13%) with FCoV WSU 79-1683, providing additional evidence of the recombinant (feline-canine, canine-TGEV) nature of most CCoV S genes [24]. The remaining 3 genes, encoding for structural proteins E, M, and N, shared the highest nucleotide identities (95.18%, 97.08%, and 93.77%), respectively, with CCoV A76 (Table 2).

Phylogenetic Analysis

Phylogenetic analysis of complete genome sequences demonstrated that the novel CCoV-HuPn-2018 formed a monophyletic branch with CCoV, TGEV, FCoV strains, and swine enteric CoV (TGEV with porcine epidemic diarrhea virus recombinant S gene) (Figure 2A). Furthermore, the full-length S gene of the CCoV-HuPn-2018 was closely related to CCoV strains and TGEV Purdue (Figure 2B), while its S1 and S2 domains were most closely related to CCoV UCD-1 and FCoV WSU 79-1683, respectively (Figure 2C and 2D). Phylogenetic analysis of the E gene confirmed the close relation between CCoV-HuPn-2018 and CCoV A76; however, owing due to the high level of conservation of this gene, all of the analyzed *Alphacoronavirus 1* strains, except FIPV 79-1146, formed a tight cluster (Figure 2E). The M and N gene phylogenetic analysis confirmed that N and M genes were highly similar between CCoV-HuPn-2018 and CCoV A76, followed by other CCoVs and TGEV, while FCoVs formed separate clusters supporting a higher degree of divergence in this genomic region, evident from SMS analysis (Figure 2F and 2G and Table 2).

Recombination Analysis

Potential recombination break points between the background CCoV and TGEV strains were present throughout the ORF1ab, resulting in the short regions sharing more similarity with HLJ-073, A76, and the TGEV Purdue strain (Figure 3A). In addition, while the first two-thirds of the ORF1ab was relatively dissimilar between the CCoV-HuPn-2018 and FCoV WSU 79-1683/FIPV 79-1146, the similarity was greater (and comparable to that in CCoV/TGEV strains) in the last third, with multiple recombination break points (Figure 3A). The 3' end of the genome downstream from the S gene was most similar between CCoV-HuPn-2018 and CCoV strain A76. While the S2 domain shared the highest similarity with that of FCoV WSU 79-1683, the sequence similarity between the CCoV-HuPn-2018 and all the background sequences in the hypervariable S1 region was low. Thus, this finding is consistent with the SMS and phylogenetic analysis results and indicates the recombinant nature of this strain (Figure 3A).

The S gene RIP analysis revealed the presence of the recombination point at approximately 2 kb, with the S2 domain being highly similar to FCoV WSU 79-1683, as noted above (Figure 3B and 3C). The S1 domain RIP analysis allowed us to include the CCoV UCD-1 S1 domain in the analysis and confirm that it indeed shared the highest similarity with the CCoV-HuPn-2018 S1. These observations confirmed that the novel strain carries a recombinant CCoV/FCoV S protein.

Structural/Nonstructural Protein Analysis

The S protein comprised 1448 amino acids, similar to other CCoV II strains and shorter than S proteins of CCoV I characterized elsewhere [25]. Twenty-nine potential glycosylation sites were predicted in the S protein of the newly identified CCoV-HuPn-2018 (Supplementary Figure 3*A*), similar to

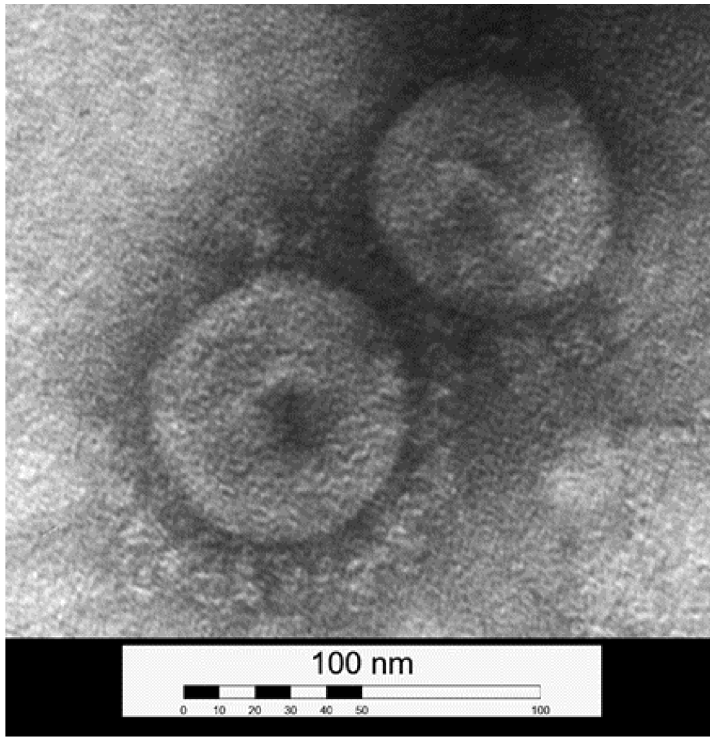

Figure 1. Immune transmission electron microscopic image of canine coronavirus (CCoV)–human pneumonia (HuPn)–2018 from an A72 cell culture. The sample was incubated with anti–CCoV guinea pig serum, leading to the specific viral antibody aggregates. Scale bar represents 100 nm.

findings in other CCoVs [25]. Unlike CCoV I, some FCoV and all betacoronaviruses and gammacoronaviruses, the characteristic multibasic motif (RRXRR)–furin recognition site was absent in the S protein of CCoV-HuPn-2018, suggesting that the virus carries an uncleaved S protein, similarly to most other alphacoronaviruses [15]. Thus, this novel strain shares more similarities with CCoV-II strains.

Surprisingly, there were no unique deletions or insertions in the S protein of CCoV-HuPn-2018. There were also a total of 5 amino acid differences between CCoV-HuPn-2018 and CCoV UCD-1 in the S1 domain; however, these amino acids were identical to those found in the TGEV Purdue S1 and were not unique.

The E protein was 81 amino acids long and did not contain any N-glycosylation sites, whereas 3 N-glycosylated residues have been predicted in each the 261–amino acid M and the 370–amino acid N proteins (Supplementary Figure 3*B* and 3*C*), similar to findings in several other FCoV/CCoV strains. While no evidence of recombination was observed for E, M or N proteins, the N protein contained a unique 12–amino acid deletion within the SR-rich region (located between amino acids 164 and 177 for other CCoV strains). The presence of this deletion was confirmed in the original NSP samples 1116 and 1153.

The 3 ORFs, 3a, 3b, and 3c, between the S and E genes encoded for proteins with sizes of 71, 71, and 244 amino acids, respectively. ORF3, previously found in CCoV I genomes only [14, 25], was not present in the new strain. The 3' end accessory protein gene 7a encoded for 101 amino acids, while there were at least 2 forms of 7b: full-length (213 amino acids) and the one with a 227-nucleotide deletion (leading to a frame shift and premature truncation of the putative protein).

DISCUSSION

A previous study identified 8 patients with pneumonia who had molecular evidence of CCoV in their NPS specimens [17]. Partial sequencing and BLASTn analysis suggested that these were closely related but distinct CCoV variants (Supplementary Table 1). The 8 patients with pneumonia were mainly children living in longhouses or villages in rural or suburban areas, where domestic animal and jungle wildlife exposure with the family is common.

In the current study, we confirmed the presence of CCoV with different, less sensitive 1-step RT-PCR assays in 2 specimens, grew a virus in A72 cells from 1 specimen, and conducted a complete genome sequence analysis of the CCoV. Our results demonstrated that CCoV-HuPn-2018 is a novel canine-feline–like recombinant strain with a unique N. To our knowledge, this is the first report suggesting that a CCoV without major genomic rearrangements or adaptive modifications in the S protein might replicate in association with pneumonia in a human host.

Table 3. Complete Genome, Individual Gene Length, and Other Characteristics of Canine Coronavirus–Human Pneumonia–2018

Genomic Region or ORF No.	Coding Sequence	Length, Nucleotides	Putative TRS Start		Protein Name	Protein Size, Amino Acids	Note
			Nucleotide Position	Sequence			
5′ UTR	No	313	No	No	No	No	Similar to other CCoVs
3′ UTR	No	275	No	No	No	No	
ORF1b	314–20 374	20 061	90	TCGAA*CTAAACGAAAT*	Pp1ab	6686	Putative ribosomal slippage is at position 12 339
ORF2	20 371–24 717	4347	20 335	GTTA*CTAAACTTTG*	S	1448	Recombinant structure with the S1 domain most closely related to CCoV UCD-1 and the S2 domain most closely related to FCoV WSU 79-1683
ORF3a	24 820–25 035	216	24 787	AGAA*CTAAACTTATG*	3a	71	Only 1 TRS before 3a was found; 3a, 3b, and 3c are likely to be expressed from polycistronic mRNAs
ORF3b	24 980–25 195	216	No	No	3b	71	
ORF3c	25 192–25 926	735	No	No	3c	244	
ORF4	25 913–26 158	246	25 866	GGTT*CTAAACGAAAT*	E	81	No unique features
ORF5	26 169–26 954	786	26 156	TGAA*CTAAACAAAAT*	M	261	
ORF6	26 967–28 079	1113	26 951	ATAA*CTAAACTTCTA*	N	370	Contains 36-nucleotide deletion in middle region
ORF7a	28 084–28 389	306	28 072	CGAA*CTAAACGAATG*	7a	101	Only 1 TRS before 7a is located; 7b is expressed from polycistronic mRNAs
ORF7b	28 394–28 808/29 035	415/642	No	No	7b	34/213	Truncated, likely nonfunctional; contains an out-of-frame 227-nucleotide deletion close to its 5′ end, followed by premature stop codons and full-length forms

Abbreviations: CCoV, canine coronavirus; FCoV, feline coronavirus; mRNAs, messenger RNAs; ORF, open reading frame; TRS, transcription regulatory sequence; UTR, untranslated region.

The conducted analyses demonstrated that the newly identified CCoV-HuPn-2018 was most closely related to CCoV TN-449, while its S1 and S2 domains shared the highest nucleotide identity with CCoV UCD-1 and FCoV WSU 79-1683, respectively. These findings are suggestive of the recombinant nature of this strain, similar to many previously characterized CCoVs [24]. Phylogenetic and recombinational analyses confirmed that CCoV-HuPn-2018 was only distantly related to other *Alphacoronavirus* species, including HCoVs (229E and NL63) and bat CoVs, and likely originated via multiple recombination events between different *Alphacoronavirus 1* strains, but not other alphacoronaviruses. The ability of the novel strain to replicate in A72 canine cells, the absence of ORF3, the higher overall similarity with CCoV-II strains (TN-449 and HLJ-073), and the lack of the furin cleavage site between S1 and S2 domains suggest that the strain belongs to CCoV genotype II [25].

The unique feature not found in any other known CCoVs and *Alphacoronavirus 1* species—namely, the 12–amino acid deletion in the middle portion of the N protein—was confirmed in both original NSP samples, 1153 and 1116. While insertions or deletions in the N protein are not found among the known *Alphacoronavirus 1* strains, the deletion of the SR-rich domain within the middle region of SARS-CoV N protein reportedly resulted in dramatic changes in its cellular localization soon after its zoonotic transmission [26]. Thus, similar to SARS-CoV, CCoV-HuPn-2018 possesses some unique genetic features suggestive of recent zoonotic transmission. Notably, such N protein rearrangements are characteristic of SARS-CoV/SARS-CoV-2 with higher case fatality rates [27].

While SARS-CoV and FCoV NSP7b was not essential for viral replication in vitro and in vivo experiments, its deletion or truncation may be associated with attenuated phenotype [28]. Disruption in the expression of the NSPs after zoonotic transmission of SARS-CoV was reported previously, suggesting that it may represent an adaptive mechanism [29]. Finally, deletions unique to FIPVs were found in ORFs 3c and/or 7b and were hypothesized to be responsible for the shift from enteric (FCoV) to FIPV phenotype and increased pathogenicity [30]. The ability of CCoV to evolve quickly through frequent recombination events and induce disease of variable severity is even more concerning, given that these data indicating that circulating CCoV may already be transmittable to humans.

The current study had a number of limitations. First, we have not met recognized standards of causality, such as Koch postulates or Bradford Hill criteria. Second, we recognize that the detected CCoVs could only be "carried" in some of the 8 patients' airways, not causing disease. However, identification of (1) FCoV-like CoVs in influenza-negative patients with acute respiratory symptoms in Arkansas and (2) porcine deltacoronavirus in children in Haiti further emphasizes that *Alphacoronavirus 1* species may be infectious or pathogenic to humans [18, 31].

In conclusion, we recovered and characterized a novel recombinant CoV, CCoV-HuPn-2018, from a hospitalized patient

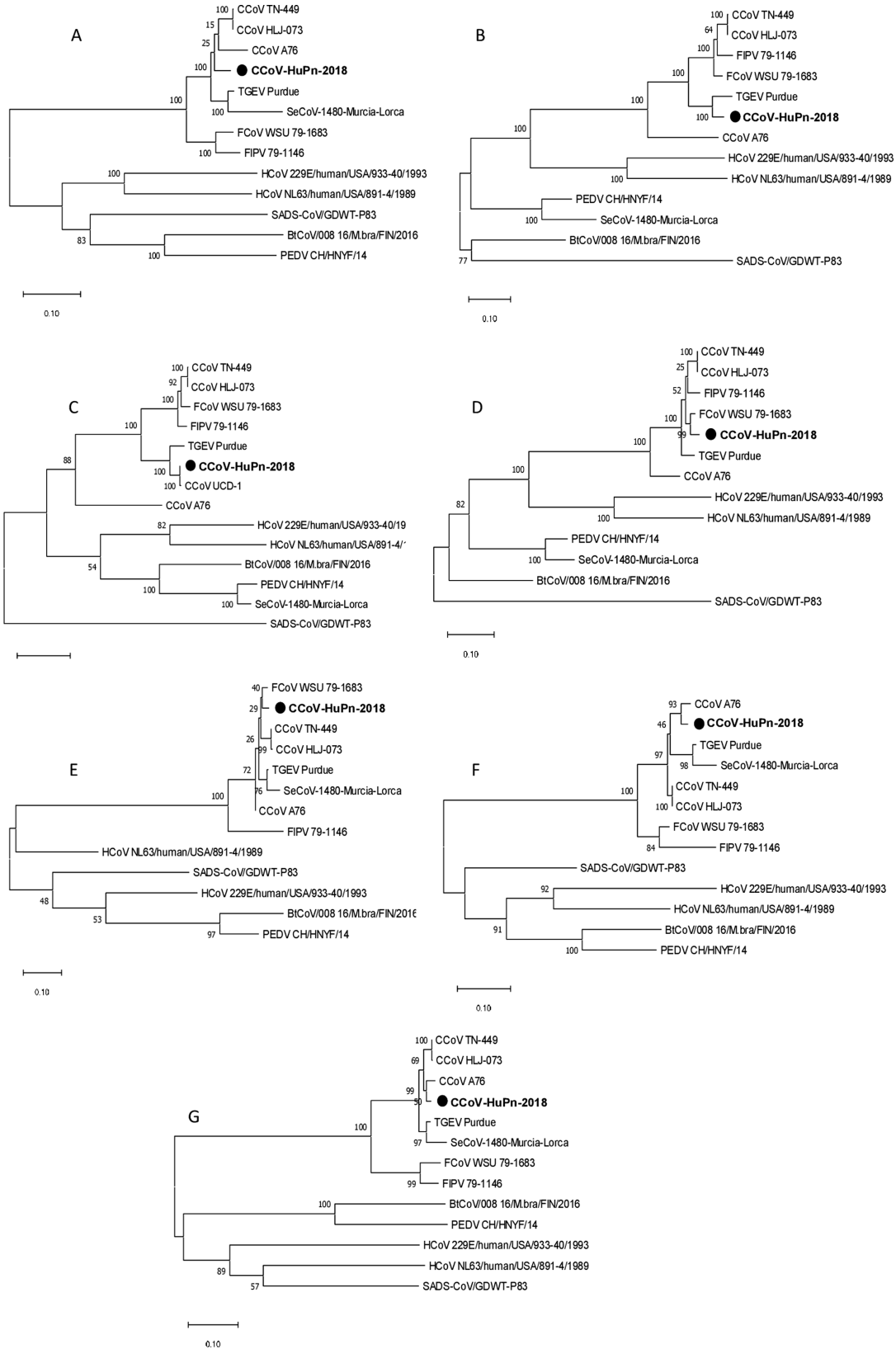

Figure 2. Phylogenetic tree based on complete genome (*A*), S gene (*B*), S1 (*C*), S2 domain (*D*), E gene (*E*), M gene (*F*) and N gene (*G*) sequences of the canine coronavirus (CCoV)–human pneumonia (HuPn)–2018 viral isolate and other *Alphacoronavirus* species. Bootstrap values are represented at key nodes. Scale bar indicates nucleotide substitutions per site. The evolutionary history was inferred using the maximum likelihood method and the general time-reversible model. This analysis involved 13 nucleotide sequences. Evolutionary analyses were conducted using MEGA X software. Black circles represent the newly identified viral isolate, CCoV-HuPn-2018. Abbreviations: BtCoV, bat coronavirus (CoV); FCoV, feline CoV; FIPV, feline infectious peritonitis virus; HCoV, human CoV; PEDV, porcine epidemic diarrhea virus; SADS-CoV, swine acute diarrhea syndrome CoV; SeCoV, Swine enteric CoV; TGEV, transmissible gastroenteritis virus.

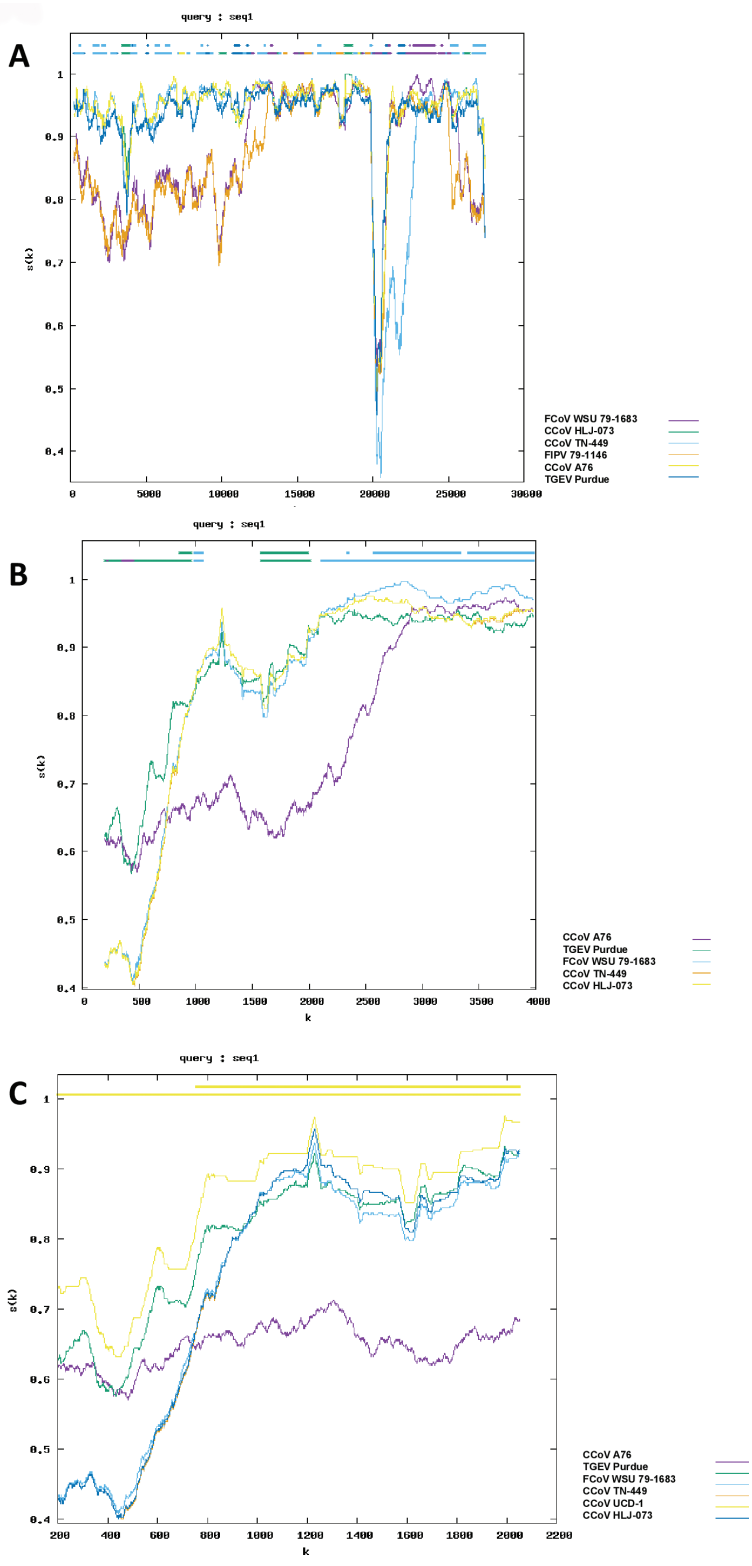

Figure 3. Recombinational analysis of the canine coronavirus (CCoV)–human pneumonia (HuPn)–2018 complete genome (*A*), S1 (*B*) and S2 (*C*) domains. At each position of the window, the query sequence CCoV-HuPn-2018 was compared with background sequences for 6 strains shown in the legend on the right. The x-axes represent the length of the sequence, and the y-axes, the similarity value (Similarity = Match Fraction = 1 - distance). The two bars on the top of the graph represent the "best match" (lower bar), and the significance of this match (upper bar). The "best match" sequence is the background sequence with the highest similarity to the query. The upper bar is also colored at a position when the best match is significantly better than the second match. Arrows represent potential recombination break points. Abbreviations: FCoV, feline coronavirus; FIPV, feline infectious peritonitis virus; TGEV, transmissible gastroenteritis virus.

with pneumonia. While possessing some unique characteristics likely suggestive of a recent zoonotic transmission, this novel strain with recombinant CCoV UCD-1/FCoV WSU 79-1683 S protein shares multiple genomic features of widespread CCoV-II. Further studies are needed to investigate CCoV prevalence, seroprevalence, and pathogenic potential in humans. Additional studies should be conducted to evaluate the biological relevance of the observed deletion in the N protein.

Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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The study—source of nasopharyngeal swab samples—has received a scientific review, and all procedures followed were in accordance with the ethical standards of the Malaysian Ministry of Health's Medical Research and Ethics Committee (protocol NMRR-17-316-34395), the Duke University Health System Institutional Review Board, the Duke-NUS Medical School Ethical Review Board, and the Naval Medical Research Center–Asia Human Research Protection Program (HRPO no. W911QY-16-D-0058).

Author Contributions. A. N. V. designed, oversaw, and provided financial support for the experiments on canine coronavirus (CCoV)–human pneumonia (HuPn)–2018 characterization and sequences, sequenced parts of the genome, analyzed the data, and wrote the manuscript. A. D. conducted most of the experiments on CCoV-HuPn-2018 cell culture isolation and Sanger sequencing. D. D. conducted some of the experiments on Sanger sequencing. L. X. screened 301 samples and identified the 8 samples positive for coronavirus/CCoV. T. H. T. and J. S. Y. L. coordinated sample collection, obtained ethical clearance, and collected and processed the patient demographic data. L. J. S. critically revised the manuscript draft. G. C. G. led the original studies, oversaw the new study, provided financial support, and revised the manuscript.

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Potential conflicts of interest. All authors: No reported conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest.

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From: (b) (6)
To: (b) (6) ; eric.laing (b) (6)
Cc: (b) (6)
Subject: Research discussions regarding CCoV-HuPn-2018
Date: Friday, May 12, 2023 6:21:39 AM
Attachments: Susceptibility of different cell lines to the novel canine (IORV 2021 Abdelqadir).pdf
Isolation of a Novel Recombinant Canine Coronavirus from a Visitor to Haiti (Clin Infect Dis 2021 Lednicky).pdf
Novel Canine Coronavirus Isolated from a Hospitalized Patient With Pneumonia in East Malaysia (Clin Infect Dis 2022 Vlasova).pdf
image001.png

Dear (b) (6)

To avoid duplicated efforts a number of us thought we might want to hold a Zoom meeting to discuss research plans regarding CCoV-HuPn-2018 and HuCCoV_Z19Haiti (see attached). I know some cloning and serologic assay development work is underway.

If you are available and want to join us, this link will show you proposed times. All you need to do is click on when is good for you... http://whenisgood.net/85dtpnx

Thanks much!

(b) (6)

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Rev. 11/17/22				PPT?	Presentation #	Code	Session / Presentation Title	Speaker
THURSDAY - NOV 17, 2022								
Day of Congress	Begin	End	Room			Code	**Workshops (Pre-Congress)**	
					W1		**Developing the Role of the ID pharmacist**	
11/17/22	8:00	12:00	Room (b) (6)		W1.1	W1	**Developing the Role of the ID pharmacist**	(b) (6)
11/17/22	8:00	12:00	Room		W1.2	W1	What does antimicrobial stewardship look like where you are? Narratives on pharmacist and nurse roles from around the world	
11/17/22	8:00	12:00	Room		W1.3	W1	The role of community pharmacists in antimicrobial stewardship – current prac ices and opportunities for improvement	
11/17/22	8:00	12:00	Room		W1.4	W1	Current prac ice in Malaysia and how to develop a strategy for greater pharmacist involvement in AMS	
11/17/22	8:00	12:00	Room		W1.6	W1	Pharmacist led quality improvement initiatives in AM	
11/17/22	8:00	12:00	Room		W1.7	W1	Role of AMS pharmacist in Malaysia	
11/17/22	13:00	17:00	Room		W2	W2	**Innovations in Rapid Diagnostics**	
11/17/22	13:00	17:00	Room		W2.1	W2	Taking an Idea from the Lab to Commercialization	
11/17/22	13:00	17:00	Room		W2.2	W2	R&D Challenges and Emerging Diagnostic Technologies	
11/17/22	13:00	17:00	Room		W2.3	W2	Integration/Deployment of Diagnostic Tools into the Clinic with Particular Focus in Asian Countries	
11/17/22	13:00	17:00	Room		W3	W3	**Misinformation: Responding to Social Media with Science**	
11/17/22	13:00	17:00	Room		W3.1	W3		
11/17/22	13:00	17:00	Room		W3.2	W3		
11/17/22	13:00	17:00	Room		W3.3	W3		
FRIDAY - NOV 18, 2022								
							MEET-THE-EXPERT sessions	
11/18/22	8:00	9:00	Room		M1	M1	**Urinary tract infections - Updates in prevention and management**	
11/18/22	8:00	9:00	Room		M1.1	M1	cUTI and resistance of gram negative bacteria	
11/18/22	8:00	9:00	Room		M1.2	M1	Preventing Urinary tract infections in adults	
11/18/22	8:00	9:00	Room		M2	M2	**Meet-the-Editors**	
11/18/22	8:00	9:00	Room		M2.1	M2	IJID	
11/18/22	8:00	9:00	Room		M2.2	M2	IJID One Health	
11/18/22	8:00	9:00	Room		M2.3	M2	The Lancet Digital Health	
11/18/22	8:00	9:00	Ro		M3	M3	**Bacterial Infections - Meet the expert**	
11/18/22	8:00	9:00	Ro		M3.1	M3	The utility of randomised controlled trials in optimal treatment of bacteraemia	
11/18/22	8:00	9:00	Ro		M3.2	M3	Clinical evidence for therapy of gram-negative bacterial infections	

			Room 306		M3.3	M3	Precision An ibiotic Dosing for Treatment of Severe GN Infections	(b) (6)
11/18/22	9:15	10:00	(b) (6)		P1	P1	**PLENARY I - National COVID-19 Responses**	
11/18/22	9:15	10:00			P1.1	P1	Moderator: (b) (6)	
11/18/22	9:15	10:00			P1.2	P1	Moderator: (b) (6)	
11/18/22	9:15	10:00			P1.3	P1	Malaysia's COVID-19 Response	
11/18/22	9:15	10:00			P1.4	P1	Ireland's COVID-19 Response	
11/18/22	9:15	10:00			P1.5	P1	Barbado's COVID-19 Response	
							PARALLEL SYMPOSIA- Morning	
11/18/22	10:30	12:00			S1	S1	**One Health session: Serosurveillance of High Consequence Zoonotic Viruses at the Human-Animal Interface**	
11/18/22	10:30	12:00			S1.1	S1	**Co-Chair:** (b) (6)	
11/18/22	10:30	12:00			S1.2	S1	Zoonoses at a One Health interface In South Africa- it's the little five not the big five!	
11/18/22	10:30	12:00			S1.3	S1	Insight of zoonotic viruses at human-animal interfaces in Cambodia	
11/18/22	10:30	12:00			S1.4	S1	COVID-19: a multi-host pandemic	
11/18/22	10:30	12:00			S1.5	S1	Bat antibody dynamics in time: what can we learn about pathogen dynamics from multiplexed Luminex serological assays	
11/18/22	10:30	12:00			S2	S2	**Clinical management of hard to treat infections (case-based discussions) – (in collaboration with MSIDC)**	
11/18/22	10:30	12:00			S2.1	S2	**Co-Chair** (b) (6)	
11/18/22	10:30	12:00			S2.2	S2	**Co-Chair:** (b) (6)	
11/18/22	10:30	12:00			S2.3	S2	Management of (recurrent or) persistent MRSA infection	
11/18/22	10:30	12:00			S2.4	S2	Optimising treatment for carbapenemase producing Enterobacterales	
11/18/22	10:30	12:00			S2.5	S2	Drug resistant enteric pathogens	
	10:30	12:00			S2.6	S2	Issues to consider for MDR-Acinetobacter baumannii treatment	
11/18/22	10:30	12:00			S3	S3	**Infectious disease innovations for a digital world**	
11/18/22	10:30	12:00			S3.1	S3	**Co-chairs** (b) (6)	
11/18/22	10:30	12:00			S3.2	S3	**Co-chairs:** (b) (6)	
11/18/22	10:30	12:00			S3.3	S3	**Getting to better pandemic and epidemic preparedness**	
11/18/22	10:30	12:00			S3.4	S3	Spatial epidemiology and malaria elimination strategies	
11/18/22	10:30	12:00			S3.5	S3	Digital Publishing- Preprints and Open Access Publishing: What Happens Next?	
11/18/22	10:30	12:00			Oral1	Oral1	**Oral Abstracts Session 1- Genomics and Infectious Diseases**	
11/18/22	10:30	12:00			Oral1	Oral1	**Co-Chair:** (b) (6)	

11/18/22	10:30	12:00	Room		Oral1.1	Oral1	IDENTIFYING THE NEXT MUTATION OF CONCERN - EXPLOITING GENOMICS FOR TRACKING SARS-COV-2 MUTATIONS AND THEIR BIOLOGICAL IMPACT IN CANADA
11/18/22	10:30	12:00	Room		Oral1.2	Oral1	RESISTOME PROFILES AND GENOME DYNAMICS OF MULTI-DRUG RESISTANT SHIGELLA SPP. ISOLATED IN BANGLADESH
11/18/22	10:30	12:00	Room		Oral1.3	Oral1	GENETIC VARIATION IN PENICILLIN-BINDING GENES 1A, 2B, AND 2X OF STREPTOCOCCUS PNEUMONIAE CAUSING INVASIVE PNEUMOCOCCAL DISEASE IN INDIAN CHILDREN
11/18/22	10:30	12:00	Room		Oral1.4	Oral1	A DECADE OF STUDY ON K.PNEUMONIAE CAPSULAR TYPE DISTRIBUTION IN INDIA – REVEALS HIGH DIVERSITY AND ITS IMPLICATION IN VACCINE DEVELOPMENT
11/18/22	10:30	12:00	Room		Oral1.5	Oral1	DIVERSE GENETIC BACKGROUND OF MULTIDRUG RESISTANT PSEUDOMONAS AERUGINOSA CIRCULATING IN INDIA
11/18/22	10:30	12:00	Room		Oral1.6	Oral1	THE GENOMIC POPULATION STUDY OF BLOODSTREAM ASSOCIATED ESCHERICHIA COLI IN 2020 IN SOUTHWEST, UK
11/18/22	10:30	12:00	Room		Oral1.7	Oral1	GENOMIC ANALYSIS TO UNDERSTAND NON-TYPHOIDAL SALMONELLA CARRIAGE: SALMONELLA AGONA – THE BUG THAT WON'T GO AWAY
11/18/22	10:30	12:00	Room		Oral1.8	Oral1	GENOMIC DIVERSITY AND RESISTOME PROFILING OF MULTI-DRUG RESISTANT SALMONELLA ENTERICA SUBSP. ENTERICA ISOLATED IN BANGLADESH
11/18/22	10:30	12:00	Room		Oral1.9	Oral1	A PROSPECTIVE CLINICAL STUDY ON THE USE OF A NON-INVASIVE WEARABLE DEVICE AND NEURAL NETWORK MODELS FOR PATIENTS WITH DENGUE
11/18/22	10:30	12:00	Room		**S4**	**S4**	**HIV - Hot Topics**
11/18/22	10:30	12:00	Room		**S4.1**	**S4**	**Co-chairs:** (b) (6)
11/18/22	10:30	12:00	Room		**S4.2**	**S4**	**Co-chairs** (b) (6)
11/18/22	10:30	12:00	Room		**S4.3**	**S4**	HIV Pre-Exposure Prophylaxis (PrEP) Updates for the Infectious Disease (ID) Physician
11/18/22	10:30	12:00	Room		**S4.4**	**S4**	Updates on ART
11/18/22	10:30	12:00	Room		**S4.5**	**S4**	Self Testing in HIV
	10:30	12:00	Room		**S4.6**	**S4**	A people centred health systems approach to living long with HIV
11/18/22	10:30	12:00	T		**PD1**	**PD1**	**Policy Discussion - Health systems resilience**
11/18/22	10:30	12:00	T		**PD1.1**	**PD1**	Moderator: (b) (6)
11/18/22	10:30	12:00	T		**PD1.2**	**PD1**	TBD
11/18/22	10:30	12:00	T		**PD1.3**	**PD1**	ID training and capacity building
							WORKING GROUP
11/18/22	12:30	13:45	Room		**W4**	**W4**	**Guide to Infection Control Working Group**
11/18/22	12:30	13:45	Room		**W4.1**	**W4**	The Guide- Last 2 years in review
11/18/22	12:30	13:45	Room		**W4.2**	**W4**	Fungal Outbreaks: Implications for IPC

							PARALLEL SYMPOSIA - Afternoon	
11/18/22	14:00	15:30	(b) (6)		**S5**	**S5**	**Perspectives from Emerging Leaders Session**	
11/18/22	14:00	15:30			**S5.1**	**S5**	**Chair:** (b) (6)	
11/18/22	14:00	15:30			**S5.2**	**S5**	Challenges And Solutions Towards Antimicrobial Stewardship Implementation	(b) (6)
11/18/22	14:00	15:30			**S5.3**	**S5**	Infectious Diseases in the COVID-19 era	
11/18/22	14:00	15:30			**S5.4**	**S5**	One Health perspectives for responding to pandemics	
11/18/22	14:00	15:30			**S6**	**S6**	**Debate: Antibiotics for diarrheal disease**	
11/18/22	14:00	15:30			**S6.1**	**S6**	**Moderator:** (b) (6)	
11/18/22	14:00	15:30			**S6.2**	**S6**		
11/18/22	14:00	15:30			**S6.3**	**S6**		
11/18/22	14:00	15:30			**S7**	**S7**	**Respiratory infections**	
11/18/22	14:00	15:30			**S7.1**	**S7**	**Co-chair:** (b) (6)	
11/18/22	14:00	15:30			**S7.2**	**S7**	**Co-chair:** (b) (6)	
11/18/22	14:00	15:30			**S7.3**	**S7**	Impact of influenza in Sou h East Asia	
11/18/22	14:00	15:30			**S7.4**	**S7**	USA H5N1 HPAI response	
11/18/22	14:00	15:30			**S7.5**	**S7**	Pan-respiratory disease surveillance	
11/18/22	14:00	15:30			**Oral2**	**Oral2**	**Oral Abstracts Session 2 - Tuberculosis & Other Mycobacterial Infections**	
11/18/22	14:00	15:30			**Oral2**	**Oral2**	Co-Chairs: Sean Wasserman, Cynthia Chee	
11/18/22	14:00	15:30			**Oral2.1**	**Oral2**	CARE SEEKING AND DELAYS IN CASE OF DRUG-RESISTANT TUBERCULOSIS PATIENTS IN BANGLADESH	
11/18/22	14:00	15:30			**Oral2.2**	**Oral2**	HEALTH SYSTEM RELATED BARRIERS TO MULTIDRUG-RESISTANT TUBERCULOSIS (MDR-TB) CARE IN AN INDIAN SETTING: FROM PATIENTS' PERSPECTIVE	
11/18/22	14:00	15:30			**Oral2.3**	**Oral2**	COMBINATION OF LIGAND-BASED PHARMACOPHORE MODELLING, MOLECULAR DYNAMICS, AND DEEP LEARNING APPROACH TO IDENTIFY SELECTIVE PANK INHIBITORS AS ANTITUBERCULAR AGENTS.	
11/18/22	14:00	15:30			**Oral2.4**	**Oral2**	CENTRAL NERVOUS SYSTEM TUBERCULOSIS IMMUNOPATHOLOGY IS DRIVEN BY MATRIX DESTRUCTION WITH MATRIX METALLOPROTEINASES INHIBITION REDUCING INFLAMMATION AND MPROVING SURVIVAL	
11/18/22	14:00	15:30			**Oral2.5**	**Oral2**	TUBERCULOSIS IN PATIENTS CO-INFECTED WITH VISCERAL LEISHMANIASIS AND HIV – A NEW DIAGNOSTIC AND MANAGEMENT CHALLENGE	
11/18/22	14:00	15:30			**Oral2.6**	**Oral2**	EXPLORING INTER-REGULATORY GENE NETWORK DERIVED CANDIDATE GENES MPARTING RESISTANCE IN MYCOBACTERIUM TUBERCULOSIS	
11/18/22	14:00	15:30			**Oral2.7**	**Oral2**	IMMUNOPATHOLOGY OF DIABETES MELLITUS IN PULMONARY TUBERCULOSIS IS DRIVEN BY NEUTROPHIL HYPER-INFLAMMATORY DYSFUNCTION: DATA FROM A CELLULAR MODEL AND HUMAN COHORT.	

			(b) (6)				
11/18/22	14:00	15:30			S8	S8	**Enterovirus Infections in Asia Pacific – Critical Updates (Member-proposed Symposium)**
11/18/22	14:00	15:30			S8.1	S8	**Co-chair:** (b) (6)
11/18/22	14:00	15:30			S8.2	S8	**Co-chair:** (b) (6)
11/18/22	14:00	15:30			S8.3	S8	Asia-Pacific Network for Enterovirus Surveillance overview and development of enterovirus A71 vaccines
11/18/22	14:00	15:30			S8.4	S8	Laboratory Diagnosis and Management of Enterovirus Infections
11/18/22	14:00	15:30			S8.5	S8	Antiviral strategies for human enteroviruses.
11/18/22	14:00	15:30			S9	S9	**Dengue Updates**
11/18/22	14:00	15:30			S9.1	S9	**Co-Chair:** (b) (6)
11/18/22	14:00	15:30			S9.2	S9	**Co-Chair:** (b) (6)
11/18/22	14:00	15:30			S9.3	S9	Innovations in Monitoring Patients with Dengue
11/18/22	14:00	15:30			S9.4	S9	Immune Responses & Severe Dengue
11/18/22	14:00	15:30			S9.5	S9	New proactive paradigm for dengue surveillance using GOS traps and NS1 kits
11/18/22	14:00	15:30			S9.6	S9	Dengue Vaccine Implementation Updates
11/18/22	16:00	17:00			P2	P2	**PLENARY II**
11/18/22	16:00	17:00			P2	P2	**Stopping the next pandemic before it strikes**
11/18/22	16:00	17:00			P2.1	P2	**Chair:** (b) (6)
11/18/22	16:00	17:00			P2.2	P2	
11/18/22	16:00	17:00			P2.3	P2	
SATURDAY - NOV 19, 2022							
11/19/22	8:00	9:00					**MEET-THE-EXPERT sessions**
11/19/22	8:00	9:00			M4	M4	**Surgical infections and antibiotic use**
11/19/22	8:00	9:00			M4.1	M4	
11/19/22	8:00	9:00			M4.2	M4	
11/19/22	8:00	9:00			M5	M5	**Career in Public Health/Global Health**
11/19/22	8:00	9:00			M5.1	M5	
11/19/22	8:00	9:00			M5.2	M5	
11/19/22	8:00	9:00			M6	M6	**Meet the ProMED Moderators**
11/19/22	8:00	9:00			M6.1	M6	ProMED moderators
11/19/22	8:00	9:00			M6.2	M6	
11/19/22	8:00	9:00			M6.3	M6	
11/19/22	8:00	9:00			Oral3	Oral3	**Oral Abstracts Session 3: Vaccines Developments**
11/19/22	8:00	9:00			Oral3	Oral3	**Co-Chairs:** (b) (6)
11/19/22	8:00	9:00			Oral3	Oral3	**Co-Chair:** (b) (6)

(b) (6)

11/19/22	8:00	9:00	(b) (6)		~~Oral3.1~~	~~Oral3~~	~~CUBAN VACCINES ABDALA AND MAMBISA AGAINST COVID-19~~
11/19/22	8:00	9:00			~~Oral3.2~~	~~Oral3~~	~~HEPATITIS B NUCLEOCAPSID PARTICLE AS IMMUNOPOTENTIATOR OF INNATE AND ADAPTIVE IMMUNITY FOR NASAL VACCINES CIGB 2020 AND MAMBISA AGAINST COVID-19~~
11/19/22	8:00	9:00			Oral3.3	Oral3	GLOBAL AND REGIONAL BURDEN OF ATTRIBUTABLE AND ASSOCIATED BACTERIAL ANTIMICROBIAL RESISTANCE AVERTABLE BY VACCINATION: MODELLING STUDY
11/19/22	8:00	9:00			Oral3.4	Oral3	NUCLEOSIDE-MODIFIED MRNA VACCINES PROTECT IFNAR -/- MICE AGAINST CRIMEAN-CONGO HEMORRHAGIC FEVER VIRUS INFECTION
11/19/22	8:00	9:00			Oral3.5	Oral3	BRIDGING THE IMMUNOGENICITY OF A TETRAVALENT DENGUE VACCINE CANDIDATE (TAK-003) FROM CHILDREN AND ADOLESCENTS TO ADULTS
11/19/22	8:00	9:00			Oral3.6	Oral3	IMMUNOGENICITY OF A TETANUS TOXOID CONJUGATED QUADRIVALENT MENINGOCOCCAL VACCINE (MENACYW-TT) IN MENINGOCOCCAL VACCINE-NAÏVE TODDLERS, CHILDREN, ADOLESCENTS AND ADULTS USING AN RSBA ASSAY
11/19/22	9:15	10:00			P3	P3	**PLENARY III**
11/19/22	9:15	10:00			P3	P3	**MONKEYPOX**
11/19/22	9:15	10:00			P3.1	P3	**Chair:** (b) (6)
11/19/22	9:15	10:00			P3.2	P3	
11/19/22	9:15	10:00			P3.3	P3	
							PARALLEL SYMPOSIA - Morning
11/19/22	10:30	12:00			S10	S10	**Hepatitis C Elimination – Global Opportunities**
11/19/22	10:30	12:00			S10.1	S10	**Chair:** (b) (6)
11/19/22	10:30	12:00			S10.2	S10	Malaysia's Hepatitis C Elimination Journey – Global Implications and Opportunities
11/19/22	10:30	12:00			S10.3	S10	Egypt's Experience in the Elimination of Hepatitis C
11/19/22	10:30	12:00			S10.4	S10	Innovation and integration of HCV diagnostics to facilitate linkage to care
11/19/22	10:30	12:00			S11	S11	**AMR Challenges in the Post COVID-19 Era**
11/19/22	10:30	12:00			S11.1	S11	**Co-Chair: Prof** (b) (6)
11/19/22	10:30	12:00			S11.2	S11	Global burden of bacterial antimicrobial resistance
11/19/22	10:30	12:00			S11.3	S11	Patient-centered surveillance of drug-resistant infections
11/19/22	10:30	12:00			S11.4	S11	Learning from COVID-19 to tackle antibiotic resistance
11/19/22	10:30	12:00			S12	S12	**Global Genomic Infectious Disease Surveillance and Response**
11/19/22	10:30	12:00			S12.1	S12	**Co-chair: tbd**
11/19/22	10:30	12:00			S12.2	S12	Spotting unseen outbreaks using WGS
11/19/22	10:30	12:00			S12.3	S12	TB genomics for public health
11/19/22	10:30	12:00			S12.4	S12	Towards building capacity and accelerating genomic surveillance: Lessons from Banglades

(b) (6)

11/19/22	10:30	12:00	(b) (6)		**Oral4**	**Oral4**	**Oral Abstracts Session 4 - Antimicrobial Resistance**	(b) (6)
11/19/22	10:30	12:00			**Oral4**	**Oral4**	**Co-Chair:** (b) (6)	
11/19/22	10:30	12:00			**Oral4**	**Oral4**	**Co-Chair:** (b) (6)	
11/19/22	10:30	12:00			**Oral4.1**	**Oral4**	EXTENT OF ANTIMICROBIAL RESISTANCE (AMR) IN AN ECOSYSTEM WITH ORGANIZED LIVESTOCK FARMING IN SRI LANKA.	
11/19/22	10:30	12:00			**Oral4.2**	**Oral4**	IN-VITRO ACTIVITY OF CEFIDEROCOL AGAINST CARBAPENEM-RESISTANT GRAM-NEGATIVE BACILLI: FIRST STUDY FROM INDIA	
11/19/22	10:30	12:00			**Oral4.3**	**Oral4**	THE CONTEXT OF ANTIBIOTIC USE IN BROILER POULTRY FARMS IN BANGLADESH: A QUALITATIVE EXPLORATION	
11/19/22	10:30	12:00			**Oral4.4**	**Oral4**	PREVALANCE OF LINEZOLID-RESISTANT VANCOMYCIN-RESISTANT ENTEROCOCCUS SPECIES (LRVRE) IN CLINICAL ISOLATES FROM TERTIARY CARE HOSPITAL OF NORTH INDIA – A REAL THREAT	
11/19/22	10:30	12:00			**Oral4.5**	**Oral4**	PRE-CLINICAL DEVELOPMENT OF HUMAN MONOCLONAL ANTIBODIES TARGETING NOVEL, CELL WALL PROTEINS IN DRUG RESISTANT FUNGAL PATHOGENS	
11/19/22	10:30	12:00			**Oral4.6**	**Oral4**	EMERGENCE OF RESISTANCE TO FLUOROQUINOLONES AND THIRD-GENERATION CEPHALOSPORINS IN SALMONELLA TYPHI IN LAHORE, PAKISTAN	
11/19/22	10:30	12:00			**Oral4.7**	**Oral4**	LARGE RETROSPECTIVE WGS STUDY DESCRIBES GENOMIC EPIDEMIOLOGY OF S. AUREUS IN INDIA AND REVEALS TWO NOVEL MULTI-DRUG RESISTANT SUB-LINEAGES OF S. AUREUS CLONAL COMPLEX 22	
11/19/22	10:30	12:00			**Oral4.8**	**Oral4**	INTRAVENOUS DOXYCYCLINE OR AZITHROMYCIN OR A COMBINATION OF THE TWO FOR TREATMENT OF SEVERE SCRUB TYPHUS: A RANDOMISED, DOUBLE-BLIND, PLACEBO-CONTROLLED TRIAL	
11/19/22	10:30	12:00			**S13**	**S13**	**The global rise of sexually transmitted infections**	
11/19/22	10:30	12:00			**S13.1**	**S13**	**chair:** (b) (6)	
11/19/22	10:30	12:00			**S13.2**	**S13**	STI Diagnostics and the Multi-verse	
11/19/22	10:30	12:00			**S13.3**	**S13**	Resurgence of syphilis	
11/19/22	10:30	12:00			**S13.4**	**S13**	Ongoing public efforts to develop new treatments for gonorrhea	
11/19/22	10:30	12:00			**PD2**	**PD2**	**Policy Discussion - Pandemic Centers**	
11/19/22	10:30	12:00			**PD2.1**	**PD2**	Moderator: (b) (6)	
11/19/22	10:30	12:00			**PD2.2**	**PD2**		
11/19/22	10:30	12:00			**PD2.3**	**PD2**		
11/19/22	10:30	12:00						
							WORKSHOP	
11/19/22	12:30	13:45			**W5**	**W5**	**Bring your manuscript - discuss it with the Editors**	
11/19/22	12:30	13:45			**W5.1**	**W5**	IJID Editors	
11/19/22	12:30	13:45			**W5.2**	**W5**	IJID One Health	
11/19/22	12:30	13:45			**W5.4**	**W5**	Lancet Digital Heal h	

							PARALLEL SYMPOSIA - Afternoon	
11/19/22	14:00	15:30	(b) (6)		**S14**	**S14**	**Innovation in Infectious Diseases - Adoption and Implementation**	(b) (6)
11/19/22	14:00	15:30			**S14**	**S14**	Organized by (b) (6) and colleagues	
11/19/22	14:00	15:30			**S14.1**	**S14**	Chair: (b) (6)	
11/19/22	14:00	15:30			**S14.2**	**S14**	Digitisation & Health - Reflections from the COVID Pandemic	
11/19/22	14:00	15:30			**S14.3**	**S14**	Overview on mRNA vaccines beyond COVID-19	
11/19/22	14:00	15:30			**S14.4**	**S14**	Rapid Diagnostics	
11/19/22	14:00	15:30			**S15**	**S15**	**Panel Discussion: Antimicrobials and Vaccines - Equitable Global Access**	
11/19/22	14:00	15:30			**S15.1**	**S15**	**Moderator:** (b) (6)	
11/19/22	14:00	15:30			**S15.2**	**S15**	**Moderator:** (b) (6)	
11/19/22	14:00	15:30			**S15.3**	**S15**	Equitable Access	
11/19/22	14:00	15:30			**S15.4**	**S15**	Global vaccine equity	
11/19/22	14:00	15:30			**S15.5**	**S15**	Global access to antimicrobials and the emergence of AMR in the COVID-19 era	
11/19/22	14:00	15:30			**S16**	**S16**	**Fungal resistance, diagnostics and therapy updates**	
11/19/22	14:00	15:30			**S16.1**	**S16**	**Co-Chairs:** (b) (6)	
11/19/22	14:00	15:30			**S16.2**	**S16**	TBD	
11/19/22	14:00	15:30			**S16.3**	**S16**	Chronic histoplasmosis	
11/19/22	14:00	15:30			**S16.4**	**S16**	Burden of serious human fungal infections in Malaysia	
11/19/22	14:00	15:30			**Oral5**	**Oral5**	**Oral Abstracts Session 5- Emerging & Re-emerging Infectious Diseases**	
11/19/22	14:00	15:30			**Oral5**	**Oral5**	**Co-Chair:** (b) (6)	
11/19/22	14:00	15:30			**Oral5**	**Oral5**	**Co-Chair:** (b) (6)	
11/19/22	14:00	15:30			**Oral5.1**	**Oral5**	EMERGING VIRUSES ARE AN UNDERESTIMATED CAUSE OF UNDIAGNOSED FEBRILE ILLNESS IN UGANDA	
11/19/22	14:00	15:30			**Oral5.2**	**Oral5**	PHYLOGENETIC ANALYS S OF DENGUE VIRUS TYPE 2 IN RECURRING OUTBREAKS IN MALAYSIA	
11/19/22	14:00	15:30			**Oral5.3**	**Oral5**	NEUTROPHIL-LYMPHOCYTE RATIO IN GUILLAIN-BARRÉ SYNDROME: A PROGNOSTIC MARKER TO PREDICT MECHANICAL VENTILATION IN EARLY STAGE OF THE DISEASE	
11/19/22	14:00	15:30			**Oral5.4**	**Oral5**	THE COST OF DENGUE SHOCK AND SEPTIC SHOCK IN VIETNAM	
11/19/22	14:00	15:30			**Oral5.5**	**Oral5**	CROSS-LINEAGE PROTECTION BETWEEN CHIKV PRIMARY INFECTION AND MAYV SECONDARY EXPOSURE IN MICE	
11/19/22	14:00	15:30			**S17**	**S17**	**Rabies - Key Areas of Focus for Successful Elimination**	
11/19/22	14:00	15:30			**S17.1**	**S17**	**Co-chair:** (b) (6) **(ProMED Moderator)**	

11/19/22	14:00	15:30	(b) (6)		**S17.2**	**S17**	Do we Need Oral Bait Vaccination to Eliminate Dog Mediated Human Rabies?	(b) (6)
11/19/22	14:00	15:30			**S17.3**	**S17**	Mass canine vaccination: a proven One Health context for the elimination of human rabies caused by dogs	
11/19/22	14:00	15:30			**S17.4**	**S17**	Feasibility and Effectiveness Studies of Oral Rabies	
11/19/22	16:00	17:00			**P4**	**P4**	**PLENARY IV**	
11/19/22	16:00	17:00			**P4**	**P4**	**Malaria**	
11/19/22	16:00	17:00			**P4.1**	**P4**	**Chair:** (b) (6)	
11/19/22	16:00	17:00			**P4.2**	**P4**	The Threat of Multidrug Resistant Falciparum Malaria	
11/19/22	16:00	17:00			**P4.3**	**P4**	Getting back on track: the tools and strategies needed to achieve malaria elimination and eradication	
11/19/22	16:00	17:00			**P4.4**	**P4**	Plasmodium knowlesi - Malaria at the One Health interface	
11/19/22	17:15	18:45			**Oral6**	**Oral6**	**Oral Abstracts Session 6 - Parasitology and Parasitic Infections**	
11/19/22	17:15	18:45			**Oral6**	**Oral6**	**Co-Chair:** (b) (6)	
11/19/22	17:15	18:45			**Oral6**	**Oral6**	**Co-Chair** (b) (6)	
11/19/22	17:15	18:45			**Oral6.1**	**Oral6**	EFFECT OF BI-ANNUAL COMMUNITY-DIRECTED DISTRIBUTION WITH IVERMECTIN (CDTI) AND A COMMUNITY-BASED AWARENESS PROGRAMME ON THE INCIDENCE OF ONCHOCERCIASIS-ASSOCIATED EPILEPSY IN MAHENGE, TANZANIA.	
11/19/22	17:15	18:45			**Oral6.2**	**Oral6**	ASSOCIATION OF ONCHOCERCIASIS NODULE PREVALENCE IN MOTHERS AND THE DEVELOPMENT OF EPILEPSY IN THEIR CHILDREN IN MAHENGE, AN ONCHOCERCIASIS-ENDEMIC AREA OF TANZANIA: A CASE-CONTROL STUDY	
11/19/22	17:15	18:45			**Oral6.3**	**Oral6**	EVALUATION OF A COMMUNITY-BASED PREVENTION PROGRAMME TO DECREASE ONCHOCERCIASIS-ASSOCIATED EPILEPSY IN ONCHOCERCIASIS ENDEMIC VILLAGES IN SOUTH SUDAN	
11/19/22	17:15	18:45			**Oral6.4**	**Oral6**	A MOLECULAR AND IMMUNOLOGICAL SIGNATURE OF IL-10 PRODUCING CD4+ T CELLS IN VISCERAL LEISHMANIASIS	
11/19/22	17:15	18:45			**Oral6.5**	**Oral6**	PREVALENCE OF ASYMPTOMATIC LEISHMANIA INFECTION IN PEOPLE LIVING WITH HIV AND PROGRESSION TO SYMPTOMATIC VISCERAL LEISHMANIASIS IN BIHAR, INDIA	
11/19/22	17:15	18:45			**Oral6.6**	**Oral6**	LEISHMANIA DONOVANI AND WUCHERERIA BANCROFTI CO-INFECTION IN AN ASYMPTOMATIC POPULATION OF VISCERAL LEISHMANIASIS	
11/19/22	17:15	18:45			**Oral6.7**	**Oral6**	MONITORING OF LEISHMANIA TRANSMISSION IN THE PERI-ELIMINATION PHASE: THE POTENTIAL OF SEROLOGICAL SURVEYS	
11/19/22	17:15	18:45			**Oral6.8**	**Oral6**	VISCERAL LEISHMANIASIS -HIV COINFECTED PATIENTS ARE HIGHLY INFECTIOUS TO SAND FLIES IN ENDEMIC AREA OF BIHAR, INDIA	
11/19/22	17:15	18:45			**Oral6.9**	**Oral6**	NOVEL METHODS FOR RAPID IDENTIFICATION OF BACTERIAL PATHOGENS	
SUNDAY - NOV 20, 2022								

11/20/22			[redacted]				**MEET-THE-EXPERT sessions**
11/20/22	8:00	9:00			M7	M7	**Travel Medicine Updates**
11/20/22	8:00	9:00			M7.1	M7	
11/20/22	8:00	9:00			M7.2	M7	
11/20/22	8:00	9:00			M8	M8	**Management of Infective endocarditis**
11/20/22	8:00	9:00			M8.1	M8	
11/20/22	8:00	9:00			M9	M9	**Early childhood diarrheal disease: Causes, consequences, and control strategies**
11/20/22	8:00	9:00			M9.1	M9	
11/20/22	8:00	9:00			M9.2	M9	
11/20/22	8:00	9:00			Oral7	Oral7	**Oral Abstracts Session 7 - COVID**
11/20/22	8:00	9:00			Oral7	Oral7	**Co-Chair:** [redacted]
11/20/22	8:00	9:00			Oral7	Oral7	**Co-Chair:** [redacted]
11/20/22	8:00	9:00			Oral7.1	Oral7	CLOSTRIDIUM DIFFICILLAE INFECTION IN POST-COVID PATIENTS
11/20/22	8:00	9:00			Oral7.2	Oral7	MODELLING THE IMPACT OF COVID-19 AND ROUTINE MENACWY VACCINATION ON MENINGOCOCCAL CARRIAGE AND DISEASE IN THE UK.
11/20/22	8:00	9:00			Oral7.3	Oral7	BURDEN AND SEVERITY OF COVID-19 IN CHILDREN HOSPITALISED OVER FIVE COVID-19 WAVES IN SOWETO, SOUTH AFRICA
11/20/22	8:00	9:00			Oral7.4	Oral7	EFFECT OF HYBRID IMMUNITY, SCHOOL REOPENING, AND THE OMICRON VARIANT ON TRAJECTORY OF COVID-19 EPIDEMIC IN INDIA: A MODELLING STUDY
~~11/20/22~~	~~8:00~~	~~9:00~~			~~Oral7.5~~	~~Oral7~~	~~HIGH RATE OF MULTIDRUG RESISTANT BACTERIAL INFECTIONS IN CRITICALLY ILL COVID-19 PATIENTS ADMITTED AT THE PEAK OF PANDEMIC IN A NATIONAL REFERRAL HOSPITAL, KENYA~~
11/20/22	8:00	9:00			Oral7.6	Oral7	A RAPID POINT-OF-CARE DIPSTICK ASSAY FOR DIFFERENTIATION OF SARS-COV-2 VARIANTS IN COVID-19 PATIENTS
11/20/22	8:00	9:00			Oral7.7	Oral7	COVID-19 ASSOCIATED HEPATITIS IN CHILDREN (CAH-C) DURING THE RISE OF DELTA VARIANT IN INDIA: A NEW COVID-19 COMPLICATION OR A SUPERINFECTION.
11/20/22	9:15	10:00			P5	P5	**PLENARY V**
11/20/22	9:15	10:00			P5.1	P5	**Chair:** [redacted]
11/20/22	9:15	10:00			P5.2	P5	Pancoronavirus vaccines
							PARALLEL SYMPOSIA - Morning
11/20/22	10:30	12:00			S18	S18	**Personalized/integrated approaches across ID therapy and prevention**
11/20/22	10:30	12:00			S18.1	S18	**Co-chairs:** [redacted]
11/20/22	10:30	12:00			S18.2	S18	Transla ing genomics into practice

11/20/22	10:30	12:00	(b) (6)		**S18.3**	**S18**	Personalized/Integrated Approaches Across Infectious Dise
11/20/22	10:30	12:00			**S18.4**	**S18**	Alternatives to Antibiotics - Microbiome modulation and others
11/20/22	10:30	12:00			**S19**	**S19**	**Developing Vaccine Confidence**
11/20/22	10:30	12:00			**S19.1**	**S19**	**Co-chairs:** (b) (6)
11/20/22	10:30	12:00			**S19.2**	**S19**	How to build confidence in vaccines
11/20/22	10:30	12:00			**S19.3**	**S19**	Childhood vaccines and the pandemic
11/20/22	10:30	12:00			**S19.4**	**S19**	Social Media, Misinformation, and Health Literacy
11/20/22	10:30	12:00			**Oral8**	**Oral8**	**Oral Abstracts Session 8: IPC/Healthcare Associated Infections & Critical Care**
11/20/22	10:30	12:00			**Oral8**	**Oral8**	**Co-Chair:** (b) (6)
11/20/22	10:30	12:00			**Oral8**	**Oral8**	**Co-Chair:** (b) (6)
11/20/22	10:30	12:00			**Oral8.1**	**Oral8**	MIXED-SPECIES BIOFILMS AND RESISTANCE TO HEAVY METALS AND DISINFECTANTS: IMPLICATIONS FOR UPEC AND UTIS
11/20/22	10:30	12:00			**Oral8.2**	**Oral8**	SPATIAL-TEMPORAL DETERMINANTS OF MDRO TRANSMISSION DYNAMICS: IMPLICATIONS FOR INFECTION CONTROL
11/20/22	10:30	12:00			**Oral8.3**	**Oral8**	SCREENING PREGNANT WOMEN FOR ASYMPTOMATIC BACTERIURIA USING MULTIREAGENT URINE DIPSTICKS AT PRIMARY HEALTH CENTRES: IMPLEMENTATION EXPERIENCE FROM INDIA
11/20/22	10:30	12:00			**Oral8.4**	**Oral8**	IN-HOSPITAL MORTALITY OF HEART FAILURE PATIENTS ASSOCIATED WITH COMMUNITY-ACQUIRED SEPSIS AND HOSPITAL-ACQUIRED SEPSIS
11/20/22	10:30	12:00			**Oral8.5**	**Oral8**	CARBAPENEM RESISTANT ENTEROBACTERALES COLONIZATION & RISK OF INFECTION IN ICU PATIENTS IN A TERTIARY CARE CENTRE
11/20/22	10:30	12:00			**Oral8.6**	**Oral8**	MAPPING TEAM DYNAMICS AND TRAFFIC IN THE OPERATING THEATRE: IDENTIFICATION OF ROLES AND STRESSORS IN INFECTION RELATED PRACTICE AND ITS COMMUNICATION WITH SURGICAL TEAMS
11/20/22	10:30	12:00			**S20**	**S20**	**Current updates in tuberculosis**
11/20/22	10:30	12:00			**S20.1**	**S20**	**Co-Chair:** (b) (6)
11/20/22	10:30	12:00			**S20.2**	**S20**	MDRTB treatment update
11/20/22	10:30	12:00			**S20.3**	**S20**	Targeting Tuberculosis and Tissue destruction
11/20/22	10:30	12:00			**S20.4**	**S20**	Advances in shortening treatment for drug-susceptible TB
11/20/22	10:30	12:00			**S20.5**	**S20**	Updates on HIV and Tuberculosis co-infection
11/20/22	10:30	12:00			**PD3**	**PD3**	**Policy Discussion - Advancing Clinical Trials**
11/20/22	10:30	12:00			**PD3.1**	**PD3**	Moderator: (b) (6)
11/20/22	10:30	12:00			**PD3.2**	**PD3**	
11/20/22	10:30	12:00			**PD3.3**	**PD3**	

(b) (6)

			(b) (6)				**PARALLEL SYMPOSIA - Afternoon**	(b) (6)
11/20/22	14:00	15:30			**S21**	**S21**	**Neglected Infectious Diseases**	
11/20/22	14:00	15:30			**S21.1**	**S21**	**Chair:** (b) (6)	
11/20/22	14:00	15:30			**S21.2**	**S21**	The role of animal reservoirs in spreading human leptospirosis in Sou heast Asia	
11/20/22	14:00	15:30			**S21.3**	**S21**	Sarcocystis: From snakes to humans	
11/20/22	14:00	15:30			**S21.4**	**S21**	Helminth infections	
11/20/22	14:00	15:30			**S21.5**	**S21**	Chikungunya Surveillance Updates	
11/20/22	14:00	15:30			**S22**	**S22**	**Hospital-Acquired Infections - How Much Can We Prevent**	
11/20/22	14:00	15:30			**S22.1**	**S22**	**Co-Chair:** (b) (6)	
11/20/22	14:00	15:30			**S22.2**	**S22**	**Co-Chair:** (b) (6)	
11/20/22	14:00	15:30			**S22.3**	**S22**	HAI's - How much can we prevent and how to manage expectations	
11/20/22	14:00	15:30			**S22.4**	**S22**	Screening strategies in different resource settings	
11/20/22	14:00	15:30			**S22.5**	**S22**	The Integration of IPC and Stewardship: Key Strategies	
11/20/22	14:00	15:30			**Oral9**	**Oral9**	**Oral Abstracts Session 9 - Hot Topics**	
11/20/22	14:00	15:30			**Oral9**	**Oral9**	Co-Chairs: (b) (6)	
11/20/22	14:00	15:30			**Oral9.1**	**Oral9**	STRUCTURAL AND BIOCHEMICAL ELUCIDIATION OF MOSQUITO HEAT SHOCK PROTEIN 70	
11/20/22	14:00	15:30			**Oral9.2**	**Oral9**	INVESTIGATION OF 3-BENZOYLBENZOFURANS AND THEIR METHYLATED AND PYRAZOLE DERIVATIVES FOR POTENTIAL INHIBITION OF HIV-1 REPLICATION	
11/20/22	14:00	15:30			**Oral9.3**	**Oral9**	AN ESTERASE-LIKE PROTEIN (ELP) CONFERRED MALATHION AND DELTAMETHRIN RESISTANCE IN THE INDIAN FIELD POPULATION OF ANOPHELES STEPHENSI	
11/20/22	14:00	15:30			**Oral9.4**	**Oral9**	SYPHILIS REINFECTION IN NEW SOUTH WALES, 2014-2021	
11/20/22	14:00	15:30			**Oral9.5**	**Oral9**	DEVELOPMENT OF CONJUGATED SECONDARY ANTIBODIES FOR WILDLIFE DISEASE SURVEILLANCE	
11/20/22	14:00	15:30			**Oral9.6**	**Oral9**	THE USE OF IMAGERY IN GLOBAL HEALTH: AN ANALYSIS OF INFECTIOUS DISEASE DOCUMENTS AND A FRAMEWORK FOR ETHICAL STANDARDS	
11/20/22	14:00	15:30			**Oral9.7**	**Oral9**	ROLE OF ILCS IN THE PATHOPHYSIOLOGY OF ACUTE RESPIRATORY DISTRESS SYNDROME (ARDS)	
11/20/22	14:00	15:30			**Oral9.8**	**Oral9**	TRACKING ROSS RIVER VIRUS HOST DIVERSITY USING MOSQUITOES AS 'FLYING SYRINGES'	
11/20/22	14:00	15:30			**Oral9.9**	**Oral9**	STUDY OF VARIOUS METHODS FOR DETECTING HLA-B* 57:01 ALLELE IN PLHIV IN EASTERN UP, INDIA.	
11/20/22	14:00	15:30			**S23**	**S23**	**Pediatric infectious diseases - Challenges and Opportunities**	
11/20/22	14:00	15:30			**S23.1**	**S23**	**Chair:** (b) (6)	
11/20/22	14:00	15:30			**S23.2**	**S23**	Re-emergence of measles	
11/20/22	14:00	15:30			**S23.3**	**S23**	A Multiyear Journey Towards Reduced Water NICU	
11/20/22	14:00	15:30			**S23.4**	**S23**	Enhancing the prevention of mother-to-child transmission of Hepati is B	

11/20/22	14:00	15:30	(b) (6)	**S23.5**	**S23**	The continuing crisis of neonatal sepsis: lessons from 19 years of surveillance in Bangladesh	(b) (6)
11/20/22	15:45	16:30		**P6**	**P6**	**PLENARY VI**	
11/20/22	15:45	16:30		**P6**	**P6**	**Dengue**	
11/20/22	15:45	16:30		**P6.1**	**P6**	**Chair:** (b) (6)	
11/20/22	15:45	16:30		**P6.2**	**P6**		(b) (6)
11/20/22	15:45	16:30		**P6.3**	**P6**		
11/20/22	16:30	16:45		**Close**	**Close**	Closing remarks: (b) (6)	

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Cc: (b) (6)
Subject: ISID Congress 2022 - Upload your Presentation
Date: Thursday, November 17, 2022 8:25:52 AM
Attachments: Technical Time Table_2022_11_17.xlsx - Scientific Sessions.pdf

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Greeting Speakers. If you are able, please send your presentations to the following email address with your last name and session number(s) (or any acceptable format) to the following email address(b) (6)

Attached is a list of all sessions with the dates and times and locations for your reference.

You can also upload your presentations at the **Speaker Ready Room** on level 3 of the KLCC. You can ask for directions to it at the Registration counter on the same level 3. Thank you. - Christopher

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(b) (6)

Cooperative Biological Research
Final Biannual Scientific Report

PROJECT INFORMATION	
Project Number:	PO: 18-0507 Project Number: 042959
Project Title:	Surveillance for emerging infectious disease pathogens at the animal-human interfaces in Thailand, in coordination with PREDICT USAID project and the bat serology study
Award Effective Date:	01 June 2018
Award End Date:	01 December 2019
Contractor:	**CHULALONGKORN UNIVERSITY** 254 Phayathai Road, Pathumwan, Bangkok 10330 Thailand
Report #:	**FINAL REPORT**
REPORT DATE:	01 December 2021
Prepared by:	Dr. Supaporn Wacharapluesadee Dr. Eric Liang (Serology data analysis)
Organization:	Faculty of Medicine, Chulalongkorn University King Chulalongkorn Memorial Hospital, Thai Red cross Society

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Overall Project Summary

In this final report, 1,173 specimens from bats were collected from 5 sites (Ratchaburi (n=280), Chachoengsao (n=252), Chonburi (n=172), Bangkok (n=64), and Chanthaburi (n=405) provinces) from June 2019 through to September 2020. Samples from rodents (n=90) and macaques (n=100) were collected from Ratchaburi province from August to September 2020. All samples were tested for three viral families, namely Paramyxoviruses, Coronaviruses, and Filoviruses using the molecular technique. Seven Coronavirus species were identified, including Sarbecovirus, the SARS-CoV-2 related virus, from horseshoe bats. Nipah virus RNA was tested from pooled bat urine of Lyle flying foxes collected in May 2018. Additionally, a serology study was conducted using multiplex microsphere immunoassay (MMIA) to test antibodies against 16 viruses in 1,002 *P. lylei* bat plasma from Chonburi in 2012, 2016, 2017, and 2018; 128 archived human serum from Chonburi in 2018; and new bat plasma specimens (n=1,0360) collected in 2019 and 2020.

Background and Justification

Bats play a critical role in the transmission of zoonotic diseases, primarily viral zoonoses associated with high case-fatality rates, including Nipah virus (NiV), severe acute respiratory syndrome (SARS)-like coronavirus (CoV) including SAR-CoV-2, and Middle East Respiratory Syndrome (MERS)-like CoV infections. Ratchaburi, Chonburi, and Loei provinces in Thailand are hotspots for emerging zoonotic viruses. MERS-like CoV has previously been found in bat guano fertilizer in Ratchaburi; NiV has been previously identified in Lyle's flying fox roosting in the village in Chonburi since 2002; however, no outbreak has been reported. This research aims to understand better the role of bats and interfaces from these areas in harbouring and transmitting emerging infectious diseases (EIDs), including known and novel EID viral pathogens. This is also a disease surveillance research of wildlife-domestic animal-human interfaces, in coordination with PREDICT USAID project. Bats and animals (rodents and macaques) were sampled around the bat cave area to test NiV, MEES-like-, SARS-like CoVs, and filovirus. In addition to animal surveillance, this study also includes human subjects. Thus, the overall scope is to detect and characterize NiV and MERS like-CoVs in potentially high-risk communities and respiratory pathogens.

Project Objectives and Hypotheses

Objective/Task 0: Detect and characterize new and known epidemic and pandemic viruses in wildlife (bats, rodents, and macaques) and high-risk communities; identify animal reservoirs and amplification hosts for zoonotic viruses.

Objective/Task 1: Study bat serology for its immune response against Nipah virus, MERS-CoV, and other bat-borne viruses.

Objective/Task 2: Enhance biosecurity and serological diagnostic capabilities in Thailand

SCIENTIFIC REPORT

Overview of Scientific Achievements

1. Viral zoonotic molecular study

1.1 Sampling (oral, feces or rectal swab, blood and/or urine)

1,173 bat samples were collected from 6 sites in 5 provinces (Table 1):

100 samples from Ratchaburi in June 2019
54 samples from Chachoengsao in July 2019
60 samples from Chonburi in July 2019
80 samples from Ratchaburi in August 2019
64 samples from Bangkok in September 2019
112 samples from Chonburi in October 2019
206 samples from Chanthaburi in November 2019
199 samples from Chanthaburi in June 2020
100 samples from Chachoengsao in July 2020
98 samples from Chachoengsao in July 2020
100 samples from Ratchaburi in September 2020

90 Rodent samples were collected from 1 site

90 samples from Ratchaburi in August 2020

100 Macaque samples were collected from 1 site

100 samples from Ratchaburi in September 2020

1.2 Results: Molecular testing for viral detection

1.2.1 Nipah Virus & Paramyxovirus Family

- **197 pooled bat urine samples from Chonburi in November 2017, February 2018 and May 2018** were tested for Nipah virus using Nipah specific primers PCR.

  5/197 (2.54%) samples tested positive for Nipah virus. The nucleotide sequences of nucleocapsid protein gene showed 99.21 to 99.47 % identity to Nipah virus isolated from Bangladesh patients.

- **975 bat rectal swabs from 5 sites collected in 2019 and 2020** were tested for paramyxoviruses using PCR.

  21/975 (2.15%) samples tested positive for paramyxoviruses (Table 1). Three positive specimens were from *Hiposideros larvatus* bat from Chantaburi (n=2, and Chachoengsao (n=1), from phylogenetic analysis (Figure 2) they belong to bat Paramyxovirus found from Hipposideros in Myanmar and Thailand.

While the other 18 samples were from *Cherephon plicatus* (n=2), *Eonycteris spelaea* (n=12), *Rousettus leschenaulti* (n=2) and *Rousettus* sp (n=2), they belong to different lineage to Hipposideros virus but shared similarity to bat Paramyxovirus from Rwanda, Congo, China and Indonesia. However, there is no report of a threat to humans or other animals from the bat paramyxovirus found in this study.

- **90 rodent rectal swabs from the year 2020** were tested for paramyxoviruses using PCR.
  - No sample tested positive for paramyxoviruses.
- **100 macaque rectal swabs from the year 2020** were tested for paramyxoviruses using PCR.
  - No sample tested positive for paramyxoviruses.

1.2.2 Coronaviruses

A. Coronaviruses Quan Protocol[1]

- **1,173 bat rectal swabs from 2019 and 2020** were tested for coronaviruses using PCR.
  - 192/1,173 (16.36%) samples tested positive for coronaviruses (Table 1).
- **90 rodent rectal swabs from 2020** were tested for coronaviruses using PCR.
  - 3/90 (3.33%) samples tested positive for coronaviruses (Table 1).
- **100 macaque rectal swabs from 2020** were tested for coronaviruses using PCR.
  - No sample tested positive for coronaviruses.

B. Coronaviruses Watanabe Protocol[2]

- **1,075 bat rectal swabs from 2019 and 2020** were tested for coronaviruses using PCR.
  - 226/1,075 (21.02%) samples tested positive for coronaviruses (Table 1).
- **90 rodent rectal swabs from 2020** were tested for coronaviruses using PCR.
  - 3/90 (3.33%) samples tested positive for coronaviruses (Table 1).
- **100 macaque rectal swabs from 2020** were tested for coronaviruses using PCR.
  - No sample tested positive for coronavirus

1.2.3 Filoviruses

975 bat rectal swabs from 2019 and 2020 were tested for filoviruses using PCR.

- No sample tested positive for filovirus

90 rodent rectal swabs from 2020 were tested for filoviruses using PCR.

- No sample tested positive for filovirus

[1] PREDICT protocol modified from Quan PL, et al. Identification of a severe acute respiratory syndrome coronavirus-like virus in a leaf-nosed bat in Nigeria. MBio. 2010 Oct 29;1(4).
[2] PREDICT protocol modified from Watanabe T, et al. Development of a dose-response model for SARS coronavirus. Risk Analysis: An International Journal. 2010 Jul;30(7):1129-38.

100 macaque rectal swabs from 2020 were tested for filoviruses using PCR.

- No sample tested positive for filovirus

1.3 Whole-genome sequencing (WGS)

Five Sarbecovirus PCR positive specimens were further characterized for the whole-genome sequence. WGS was performed using enrichment library preparation (Respiratory Viral Oligos Panel, RVOP) and an Illumina MiSeq 3000 sequencer, according to the manufacturer instructions.using the RVOP enrichment library preparation protocol (Illuminar, USA). The complete genome sequence was a success in one specimen; No. RacCS203. All five genome sequences were submitted and can be accessed via NCBI GenBank; accession number MW251308 (complete genome; RacCS203), MW251310-12 (partial genome; RacCS224, RacCS253, RacCS264, and RacCS271).

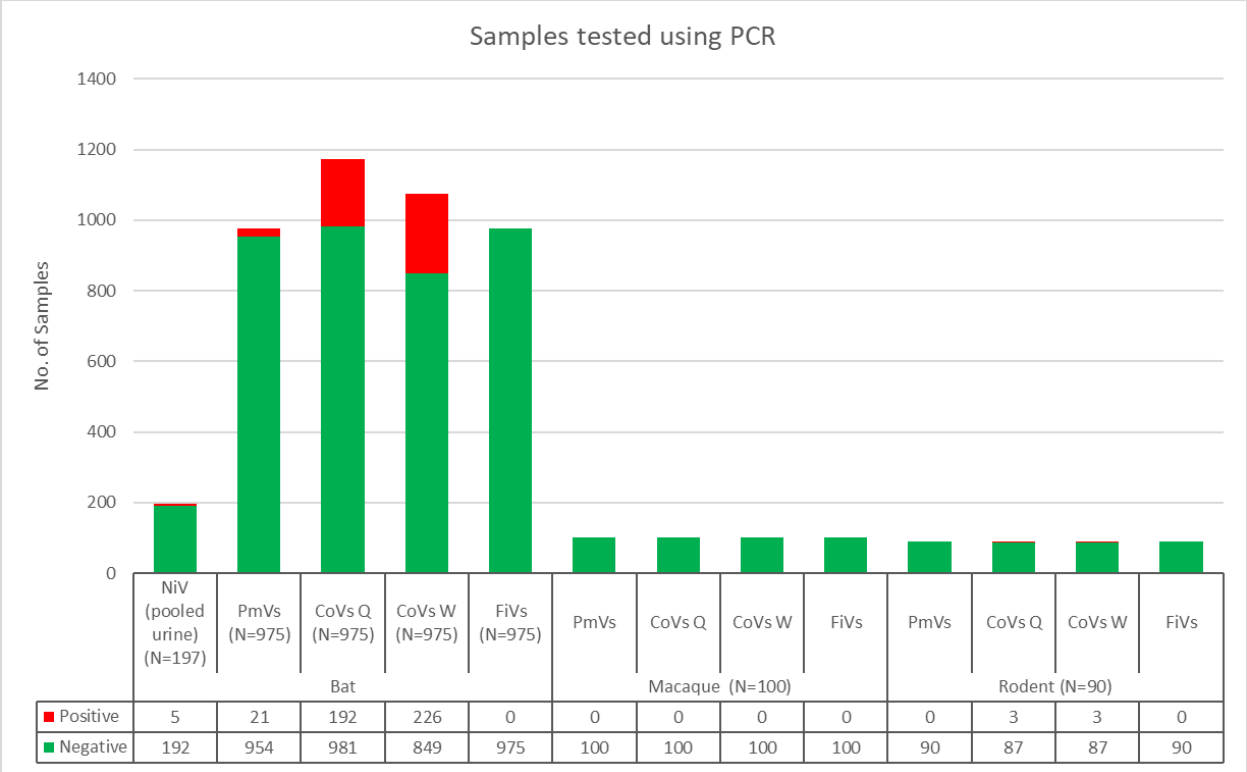

Figure 1: Summary of samples tested using PCR

Table 1. PCR results of Coronavirus (CoV) (2 protocols) and Paramyxovirus (PmV) from rectal swab specimens of bats, rodents, and macaques.

Event Name	Collected Date	Animal ID	No. tested	No. of Positive sample-viral group		
				CoV (Q protocol)	CoV (W protocol)	PmV
Bat						
Ratchaburi-Wat Khao Chong Pran	2019Jun14	B19043-142	100	All Neg	All Neg	1
Chachoengsao-1 (Wat Khao Tham Raet)	2019Jul23	B19143-196	54	5-Hibecovirus 9-unclassified AlphaCoV	3- unclassified AlphaCoV	1
Chonburi-Wat Khao Cha-ang	2019Jul24	B19197-256	60	1-Nobecovirus (HKU9) 1-Nobecovirus (GCCDC1) 2-Minunacovirus 1-unclassified Decavirus	5-Nobecovirus (HKU9) 4-Nobecovirus (GCCDC1)	2
Ratchaburi-Wat Khao Chong Pran	2019Aug16	B19257-336	80	27-unclassified AlphaCoV	20-unclassified AlphaCoV	All Neg
Bangkok-Kasetsart university	2019Sep14	B19337-389 B19391-401	64	2-Nobecovirus (HKU9) 1-Pedacovirus [99.3% identity to Porcine epidemic diarrhea virus (PEDV), GenBank accession no. MN314264]	3-Nobecovirus (HKU9)	All Neg
Chonburi-Wat Khao Cha-ang	2019Oct17	B19402-513	112	10-Nobecovirus (GCCDC1) 1-Minunacovirus	30-Nobecovirus (GCCDC1)	1
Chantaburi-Khao Soi Dao	2019Nov08	B19514-719	206	3-Nobecovirus (HKU9) 10-Nobecovirus (GCCDC1) 5-unclassified Decacovirus	53 32-Nobecovirus (HKU9) 21-Nobecovirus (GCCDC1)	6
Chantaburi-Khao Soi Dao	2020Jun11	B20001-004 B20006-200	199	82 30-Nobecovirus (HKU9) 18-Nobecovirus (GCCDC1) 1-Hibecovirus 26-unclassified Decacovirus 6-unclassified AlphaCoV	102 68-Nobecovirus (HKU9) 29-Nobecovirus (GCCDC1) 5- unclassified AlphaCoV	9

Event Name	Collected Date	Animal ID	No. tested	No. of Positive sample-viral group		
				CoV (Q protocol)	**CoV (W protocol)**	**PmV**
Chachoengsao-2 (Ang Rue Nai)	2020Jun19	B201-300	100	**13-Sarbecovirus**	Negative	notdone
Chachoengsao-2 (Ang Rue Nai)	2020Jul	B543-640	98	**14-Sarbecovirus**	Not done	notdone
Ratchaburi-Wat Khao Chong Pran	2020Sep12	B20838-937	100	4-unclassified AlphaCoV 2-Hibecovirus	1-Nobecovirus (HKU9) 2-Nobecovirus (GCCDC1) 3-unclassified AlphaCoV	1
TOTAL			1,173	192	226	21
Macaque						
Ratchaburi-Wat Tham Nam	2020Sep01	P20001-100	100	All Negative	All Negative	All Negative
Rodent						
Ratchaburi-Wat Khao Chong Pran	2020Aug29	R20001-090	90	3-Embecovirus	3-Embecovirus	All Negative

1.4 Discussion

Specimens from the bat (13 species), rodent, and macaque were collected during 2019 and 2020 and tested for three viral families to detect EID bat-borne viruses. Three viral families included coronavirus, paramyxovirus and filovirus.

No tested virus was found from macaque rectal swab specimens. Three positive murine coronaviruses were from rodent rectal specimens. However, to our knowledge, there is no report of any threat from this virus to humans.

Coronavirus was found in bat from all studied sites. At Ratchaburi-Wat Khao Chong Pran site where specimens from *Cherephon plicatus* bats were collected three times, CoV was not found in June 2019, but unidentified AlphaCoV was detected in August 2019, and unclassified AlphaCoV, Hibecovirus, and Nobevirus were detected in September 2020 (Table 1).

Sabecovirus (SARS-CoV-2 related virus) was detected from horseshoe bat (*Rhinolophus accuminatus*) from Chachoengsao province (**Figure S4a)**. The small polymerase gene fragment (290 base pairs) showed 96% similarity to human SAR-CoV-2, but its whole genome (RacCS203) showed 91% identity (**Figure S4b)**. The ability of the virus to enter the human cell using ACE-2 receptor was performed at Professor Linfa's laboratory, Duke-NUS, Singapore. The RacCS203 virus could not bind to the human ACE-2 receptor (Wacharapluesadee, et al., 2021). However, Sarbecovirus did not identify in the other 12 bat species from this study.

Nobecovirus was primarily found in fruit bat species; *Cynopterus sphinx*, *Eonycteris spelaea*, *Rousettus leschenaultia*, *Rousettus amplexicaudatus* and *Rousettus* sp (Table 2, supplement figures). In contrast, unclassified decavirus was detected in insect-eating and fruit bats species; *Eonycteris spelaea*, *Hipposideros armiger*, *Hipposideros larvatus*, *Rousettus leschenaultia*, *Rousettus amplexicaudatus* and *Rousettus* sp. In addition, unclassified AlphaCoV was identified in *Cherephon plicatus*, and *Hipposideros larvatus*. Minunacovirus was detected only from *Miniopterus magnate*. Finally, Pedacovirus was found in *Myotis horsfieldii* and showed 99% identity to the virus from porcine (Porcine epidemic diarrhea virus).

Nobecovirus is the most abundant found from this study. It is the subgenus of viruses in the genus Betacoronavirus, previously known as group 2d coronaviruses (HKU9 strain). It originates in (fruit) bat but not other animal species as far as our knowledge. There is no evidence that it can cause disease to humans or other animals.

Two PCR protocols for the detection of CoV were performed in this study. The Watanabe protocol could detect Nobevirus better than Quan PCR protocol. Whereas Sarbecovirus and Hebecovirus (Betacoronavirus) could be identified by Quan but not Watanabe protocol. Combining both protocols for CoV detection in bat gives better sensitivity and avoids the false-negative result.

Wacharapluesadee S, Tan CW, Maneeorn P, Duengkae P, Zhu F, Joyjinda Y, Kaewpom T, Chia WN, Ampoot W, Lim BL, Worachotsueptrakun K, Chen VC, Sirichan N, Ruchisrisarod C, Rodpan A, Noradechanon K, Phaichana T, Jantarat N, Thongnumchaima B, Tu C, Crameri G, Stokes MM, Hemachudha T, Wang LF. Evidence for SARS-CoV-2 related coronaviruses circulating in bats and pangolins in Southeast Asia. Nat Commun. 2021 Feb 9;12(1):972. doi: 10.1038/s41467-021-21240-1. Erratum in: Nat Commun. 2021 Feb 25;12(1):1430. PMID: 33563978; PMCID: PMC7873279.

Table 2 Coronaviruses (sub-genus) found from 13 bat species in the study

Bat species	Location	No. tested	unclassified AlphaCoV	unclassified Decacovirus	Minuna covirus	Peda covirus	Hibe covirus	Nobe covirus	Sarbe covirus	Total	%
Cherephon plicatus	Ratchaburi	280	32	nf	nf	nf	2	3	nf	37	13.21
Cynopterus sphinx	Choburi	3	nf	nf	nf	nf	nf	2	nf	2	66.67
Cynopterus sphinx	BKK	63	nf	nf	nf	nf	nf	3	nf	3	4.76
Eonycteris spelaea	Chantaburi	170	nf	1	nf	nf	nf	67	nf	68	40.00
Eonycteris spelaea	Chonburi	143	nf	nf	nf	nf	nf	37	nf	37	25.87
Hipposideros armiger	Chantaburi	17	nf	1	nf	nf	nf	nf	nf	1	5.88
Hipposideros armiger	Chachoengsao-1	9	nf	nf	nf	nf	1	nf	nf	1	11.11
Hipposideros armiger	Chonburi	7	nf	nf	nf	nf	nf	nf	nf	0	0.00
Hipposideros larvatus	Chantaburi	20	4	1	nf	nf	1	1	nf	7	35.00
Hipposideros larvatus	Chachoengsao-1	44	9	nf	nf	nf	4	nf	nf	13	29.55
Hipposideros lekaguli	Chonburi	7	nf	nf	nf	nf	nf	nf	nf	0	0.00
Miniopterus magnate	Chonburi	5	nf	nf	3	nf	nf	nf	nf	3	60.00
Myotis horsfieldii	BKK	1	nf	nf	nf	1	nf	nf	nf	1	100.00
Rhinolophus accuminatus	Chachoengsao -2	198	nf	nf	nf	nf	nf	nf	**27**	27	13.64
Rhinolophus shameli	Chonburi	2	nf	nf	nf	nf	nf	nf	nf	0	0.00
Rousettus amplexicaudatus	Chantaburi	70	nf	4	nf	nf	nf	20	nf	24	34.29
Rousettus leschenaultia	Chantaburi	37	nf	1	nf	nf	nf	8	nf	9	24.32
Rousettus leschenaultia	Chonburi	4	nf	1	nf	nf	nf	1	nf	2	50.00
Rousettus sp	Chantaburi	91	nf	25	nf	nf	nf	37	nf	62	67.39
Taphozous melanopogon	Chachoengsao	1	nf	nf	nf	nf	nf	nf	nf	0	0.00
Taphozous melanopogon	Chonburi	1	nf	nf	nf	nf	nf	nf	nf	0	0.00
TOTAL	297	1173	45	34	3	1	8	179	27	297	25.30

2. Viral zoonotic serological study

2.1 Specimens: plasma separated from collected blood samples

2.1.1 Archived specimens (n=1,130)

- 358 bats' plasma samples were collected from Chonburi in January 2012 through to January 2013
- 104 bats' plasma samples were collected from Chonburi in November 2016
- 302 bats'plasma samples were collected from Chonburi in February 2017 through to November 2017
- 238 bats'plasma samples were collected from Chonburi in February 2018 through to May 2018
- 128 humans' plasma samples were collected from Chonburi in May 2018

2.1.2 New specimens (n=1,036)

- 540 **bats'** plasma samples were collected from Ratchaburi, Chachoengsao, Chanthaburi and Bangkok in June 2019 through to November 2019
- 298 **bats'** plasma samples were collected from Chanthaburi and Ratchaburi in June 2019 through to November 2019
- 88 **rodents'** plasma samples were collected from Ratchaburi in August 2020
- 100 **macaques'** plasma samples were collected from Ratchaburi in September 2020

2.2 Results: Viral detection using MMIA Serology testing

Human and bat sera samples were screened in a multiplex microsphere-based immunoassay (MMIA). 16 viruses and two additional human ACE-2-using bat SARS-related CoVs (Table 3) were prepared and provided by the Department of Microbiology and Immunology, Uniformed Services University, Bethesda, MD, USA.

We detected 23.4% (234/1002) of flying foxes had NiV-specific IgG, and that henipavirus RBP (NiV/GhV)-reactive IgG were detected in 43.9% of flying foxes (Figure 2 and Table 4). Suprsingly, we detected GhV RBP-reactive IgG in several flying fox serum samples. Comparaively, no other bat species had detectable henipavirus RBP-binding IgG.

Flying foxes also had 15.1% (151/1002) seroprevalence for any fiovirids (Table 4). The highest preferential reactivity was observed against Bundibugyo virus GP (Figure 3). Cross-reaction among the ebolaviruses GPs were observed, in addition several flying fox serum samples possessed IgG that reacted with Lloviu virus and marburgviruses. Rousette bats were positive for Mengla virus IgG, but no other filovirids. Additionally, *Chaerephon spp.* sera had some IgG reactivity across ebolavirus GPs but reacitivty was low.

Futhermore, flying foxes had evidence of IgG anitbodies that bound to bat SARS-related CoVs, Rs4784 and Rs4231, spike proteins.

One human and one non-human primate serum sample possessed IgG that were most reactive with Ebola virus GP, however, reactivity was low relative to the upper limits of detection.

2.3 Discussion

The serology data supports the well-characterized host-virus relationship between Lyle's flyig foxes and NiV. NiV seroprevalance of 23 – 44% is consistent with HeV in Australian flying foxes and NiV in Bangladesh sampled Indian flying foxes. Using this confirmed natural reservoir of NiV and a robust sample size of sera, we were able to apply LCA to define threshold cutoffs that were broadly applicable to the multiplex serology assay. We found minimal evidence of henipavirus, filovirid, and betacoronavirus infection outside of Lyle's flying foxes. The negative serology data is further supportive that there may be virus host-restrictions and that ecological or behavorial barriers exist that limit enzootic transmission among bat species.

The specific detection of Mengla virus reactive IgG in rousette bats is consistent with rousette bats being a natural host of Megnla virus and dianloviruses across South and Southeast Asia. However, the IgG levels to Mengla virus were low, suggesting that other dianloviruses may be circulating in rousette bats located with Thailand and distinct from Mengla virus, though retaining conserved antigenic similarities. Serology data indicates that *Pteropus, Chaerephon,* and *Rousettus spp* should be the focus of continued serological and nucleic acid detection for novel Asiatic filovirids.

We observed no evidence of subclinical human exposure Nipah virus or other henipavirses.

Lastly, Lyle's flying foxes were reactive with *Sarbecovirus* spike proteins (14%), whereas, no other bats had evidence of prior infection. Human SARS-CoV-2 research has indicaed that cross-reactions exist between SARS-CoV-2 (*Sarbecovirus*) and other distantly related betacoronaviruses such as HCoV-OC43 *(Embecovirus*). Thus, in the absence of other coronavirus spike proteins we are limited in our interpretation of coronavirus serology in flying foxes. Horseshoe bats are the known source and host of SARS-CoVs and bat SARS-related CoVs (*Sarbecoviruses*). It is more likely that flying foxes are host of antigenically-related betacoronaviruses as opposed to CoVs in the *Sarbecovirus* lineage.

Table 3. List of virus antigen used in this study.

Virus species	Abbreviation	Soluble Glycoprotein	Bead No.
Ebolaviruses			
Ebola virus	EBOV	$sGP_{(1,2)}$	34
Bundibugyo virus	BDBV	$sGP_{(1,2)}$	64
Bombali virus	BOMV	$sGP_{(1,2)}$	55
Tai forest virus	TAFV	$sGP_{(1,2)}$	57
Sudan virus	SUDV	$sGP_{(1,2)}$	77
Reston virus (monkey isolate)	RESTVm	$sGP_{(1,2)}$	85
Reston virus (pig isolate)	RESTVp	$sGP_{(1,2)}$	72
Marburgviruses			
Marburg virus	MARV	$sGP_{(1,2)}$	37
Ravn virus	RAVV	$sGP_{(1,2)}$	62
Cuevavirus			
Lloviu virus	LLOV	$sGP_{(1,2)}$	66
Dianloviruses			
Měnglà virus	MLAV	$sGP_{(1,2)}$	22
Henipaviruses			
Hendra virus	HeV	sG	43
Nipah virus (Malaysia strain)	NiV	sG	46
Cedar virus	CedV	sG	53
Mojiang virus	MojV	sG	29
Ghana virus	GhV	sG	35

Table 4. Multiplex microsphere-based immunoassay (MMIA) results against three viral families.

		Filovirus			Henipavirus			Coronavirus
Genus	n	Positive	Single Positive	Multiple Positive	Positive	Single Positive	Multiple Positive	Positive
Pteropus	1002	15.07% (151/1002)	7.39% (74/1002)	7.68% (77/1002)	43.91% (440/1002)	23.35% (234/1002)	20.56% (206/1002)	13.17% (132/1002)
Hipposideros	103	0% (0/103)	-	-	0% (0/103)	-	-	0% (0/103)
Rhinolophus	7	0% (0/7)	-	-	0% (0/7)	-	-	0% (0/7)
Rousettus	190	2.63% (5/190)	2.63% (5/190)	-	0% (0/190)	-	-	0% (0/190)
Charephon	175	6.29% (11/175)	5.14% (9/175)	1.29% (2/175)	0.57% (1/175)	0% (0/175)	0.57% (1/175)	0% (0/175)
Cynopterus	64	0% (0/64)	-	-	0% (0/64)	-	-	0% (0/64)
Eonycteris	195	0% (0/195)	-	-	0% (0/195)	-	-	0% (0/195)
Miniopterus	4	0% (0/4)	-	-	0% (0/4)	-	-	0% (0/4)
Myotis	5	0% (0/5)	-	-	0% (0/5)	-	-	0% (0/5)
Tazophus	2	0% (0/2)	-	-	0% (0/2)	-	-	0% (0/2)
Unspeciated	100	4% (4/100)	3% (3/100)	1% (1/100)	0% (0/100)	-	-	0% (0/100)
Human	128	2.34% (3/128)	1.56% (2/128)	0.78% (1/128)	0.78% (1/128)	-	0.78% (1/128)	0% (0/128)
Macaque	100	1% (1/100)	1% (1/100)	-	0% (0/100)	-	-	0% (0/100)
Rodent	88	0% (0/88)	-	-	0% (0/88)	-	-	0% (0/88)

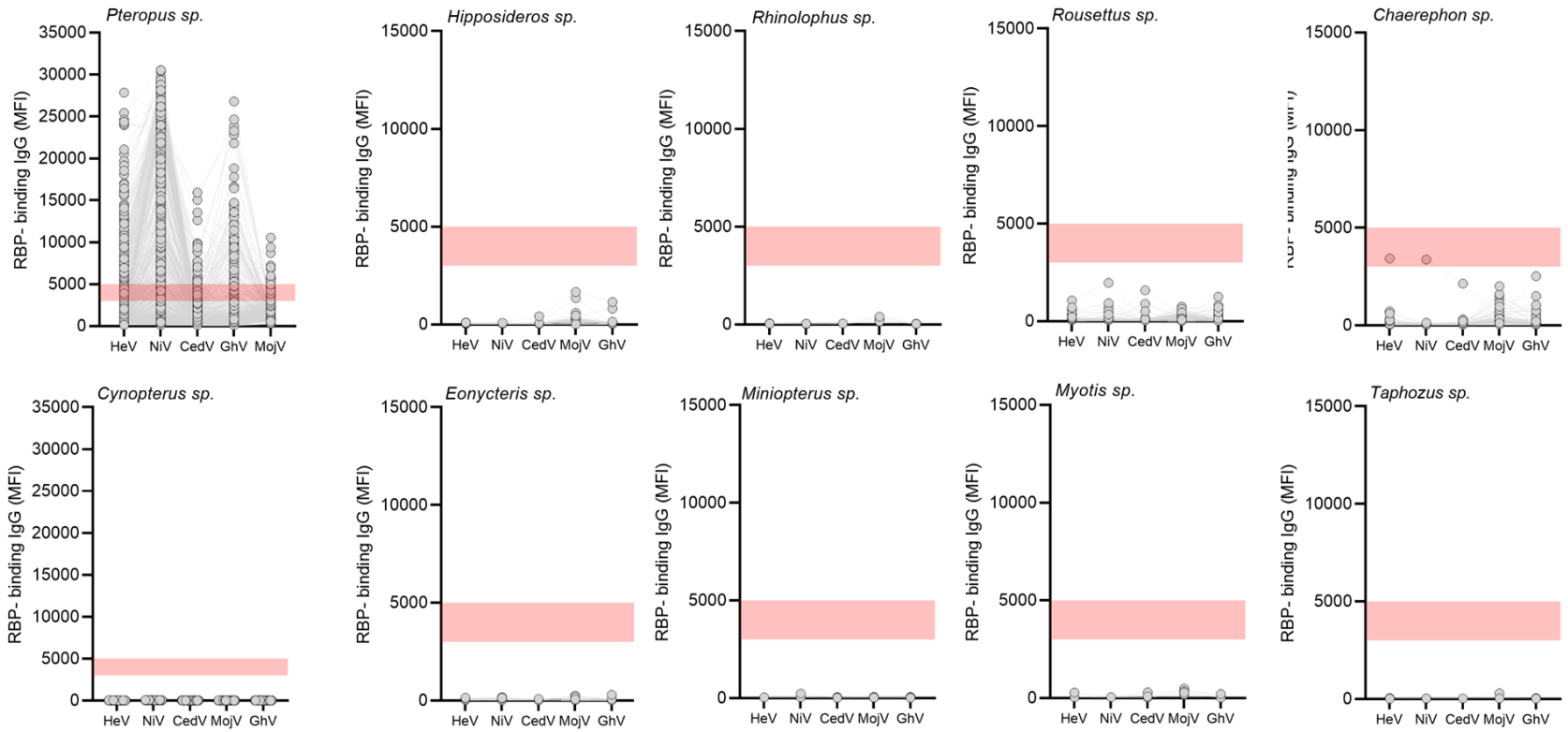

Figure 2 Multiplex microsphere-based immunoassay (MMIA) results of 10 bat genus against five antigen beads; HeV, Hendra virus; NiV, Nipah virus; CedV, Cedar virus; MojV, Mojiang virus; GhV, Ghana virus

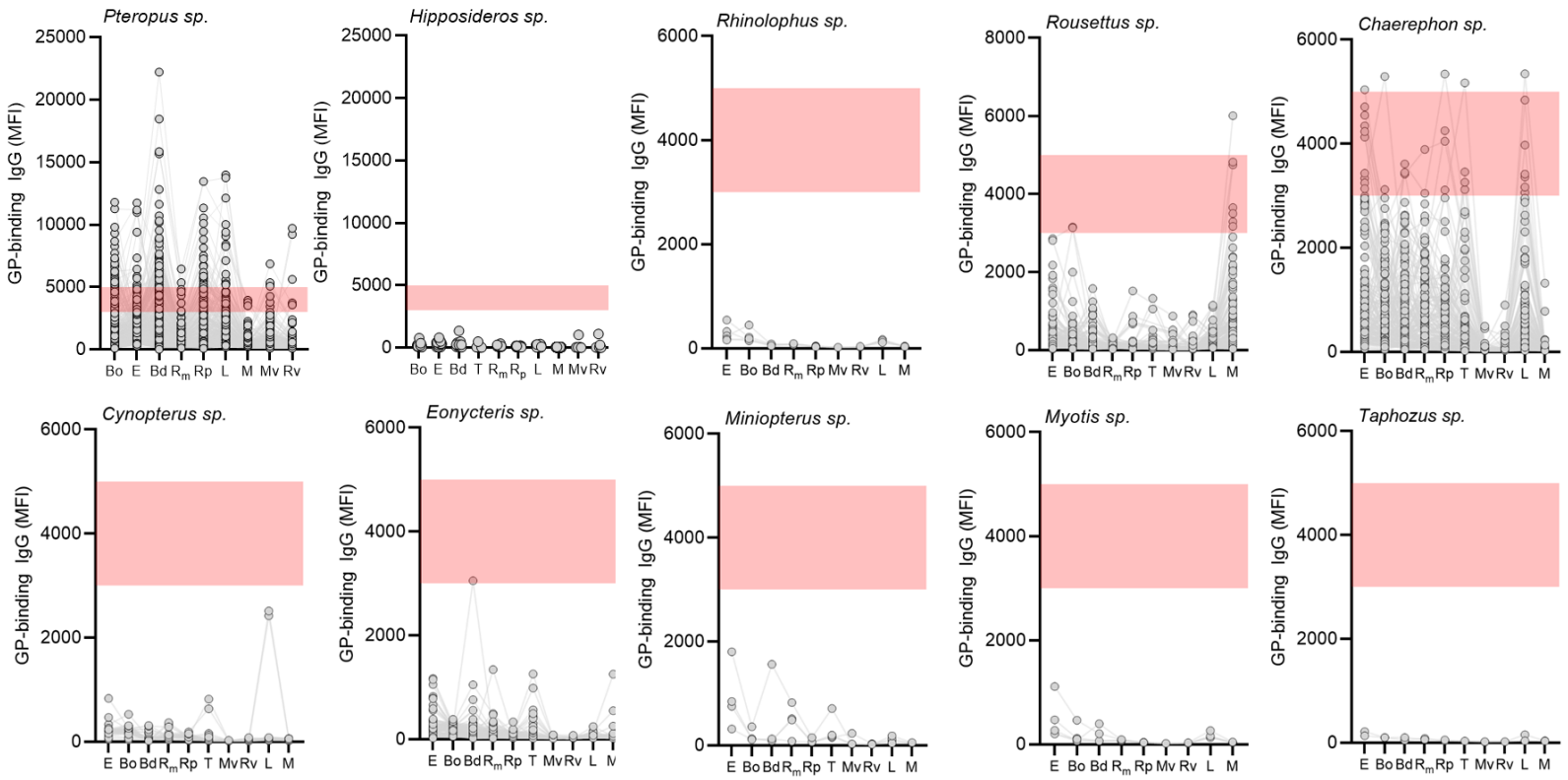

Figure 3 Multiplex microsphere-based immunoassay (MMIA) results of 10 bat genus against ten antigen beads; Bo, Bombali virus; E, Ebola virus; Bd, Bundibugyo virus; T, Tai Forest virus; Rm, Reston virus; Rp, Reston virus; L, Lloviu virus; M, Mengla virus; Mv, Marburg virus; Rv, Ravn virus

Methods

Bat capture and sample collection

Bats were captured each time using mist net for flying foxes or butterfly net for small bats. Captured animals were removed immediately and put into cotton bag individually. Bats were not euthanized, and they were released after measurements were taken and samples were collected. Bats were identified morphometrically, and species, sex, reproductive status, FA length and body mass were determined. Rectal swab was collected from each individual bat and immediately put into Lysis buffer. The samples were transported to laboratory on ice within 48 hours and stored at -80^{o}C until further analysis.

Bat Pooled urine sample collection

Bat urine samples were collected using a plastic sheet. Plastic sheets were laid at 26 spots under the trees where the urine and faeces of fruit bats were expected to be deposited as indicated by the presence of previous droppings. Each sheet was 1.5 x 1.5 meters. Sterile cotton swabs were used to soak up the urine on the plastic sheet. These were immersed immediately into 9 mL of Lysis buffer. Two cotton swabs were pooled in each Lysis buffer tube. The tubes were kept cold by placing them in a cooled box and transported back to the laboratory within 24 hours.

Bats' packed red blood cells and serum collection

Non-heparinized capillary tubes were used in blood collection from brachial vein. Bats were bled with caution to maintain a ratio no greater than 10 µL of collected blood to 1 g of bat body weight (equivalent to 1% of bodyweight). The capillary tubes were kept vertically for 30 minutes. Then the capillary tubes were centrifuged at 1,000xg for 5 minutes. Sterile pipette tips were used to separate packed red blood cells from serum in each sample. The packed red blood cells were placed in 500 µL VTM, and the serum was stored in 0.5 mL sterile tubes.

Both the packed red blood cells in VTM and the serum sample were kept in -80˚C freezer until further analyses.

Rodent capture and sample collection

Free ranging rodents were captured through pit traps and box traps. Captured rodents were removed immediately and put into cotton bags individually. Rodents were not euthanized, and were released after measurements were taken and samples were collected. Sampling included venipuncture; fecal, urine & external parasite collection; skin scrape of skin lesions; oropharyngeal, urogenital & rectal swabs; hair clipping; physical measurements (weight, height), photos, and dentition examination. Blood was drawn from the orbital vein and collected into a vial. This was only performed on anesthetized rodents. Femoral, ventral tail vein or jugular venipuncture were used for larger rodents (e.g. grass cutters). In all rodents, blood volumes of no more than 1% of body weight were drawn (example 0.5 ml blood from a 50 g rodent).

Macaques capture and sample collection

Free ranging and captive macaques will be chemically restrained by darting with anesthetic or through manual chemical injection, and handled only for the duration of sampling, thorough physical examination (PE), PIT tagging or other marking, and morphometrics. Macaque will be captured using net cages (made of rope) or metal traps placed on flat ground in a secure area or on a pallet constructed on a tree. Trapped animals will be transferred to a transfer cage with a sliding door and covered. Sampling procedures for non-human primates will include venipuncture; fecal, urine, milk (if a lactating female), and external parasite collection; oral, nasal, urogenital and anal swabs, plucked hair and milk if/when available. Blood samples from macaques will primarily be collected from the forearm veins cephalic, radial, median, and ulnar veins sampling will include non-invasive specimen collection of oral swabs from specially designed dental ropes and opportunistic collection of fresh feces and urine.

Human serum collection

Blood samples from healthy humans in hotspots were collected. After blood collection in red top vacutainers, the tubes were incubated at room temperature to let blood clot for 30 mins. To remove clots, blood was centrifuged at 1,000xg for 5 min. Following centrifugation, the separated serum was immediately transferred into 1.5 mL sterile tubes. Serum samples were immediately frozen in aliquots of 100 L at 80°C.

PCR assays

1. MERS-like CoV PCR

Hemi-nested Reverse Transcription PCR (RT-PCR) was performed using broadly reactive consensus PCR primers for CoV, targeting the RNA-dependent RNA polymerase (RdRp) gene. A total of 5µl of extracted nucleic acid was added to 50µl of reaction mixture of OneStep RT-PCR kit (QIAGEN, Hilden, Germany), per manufacturer's instructions, and reacted with each forward primer and reverse primer[3]. Hemi-nested PCR amplifications were performed using 2µl of first amplification product and 48µl of reaction mixture containing 1.0 unit of Platinum Taq DNA polymerase in 2.5mM MgCl2, 400µM dNTPs, 0.6µM of second forward primer and 0.6µM of the same reverse primer as the first round of RT-PCR. Amplification product of 282 bp was visualized using 2% agarose gel electrophoresis. All positive PCR products were further sequenced for confirmation and strain characterization.

[3] Corman VM, Müller MA, Costabel U, Timm J, Binger T, Meyer B, Kreher P, Lattwein E, Eschbach-Bludau M, Nitsche A, Bleicker T. Assays for laboratory confirmation of novel human coronavirus (hCoV-EMC) infections. Eurosurveillance. 2012 Dec 6;17(49):20334.

2. NiV nested RT-PCR

NiV nucleoprotein (N)-specific primers were used for first-round and nested PCR[4]. The PCR product was sized by gel electrophoresis in 2% agarose (NiV-PCR product is 227-bp). All PCR positive samples were re-amplified with heminested PCR[5]. The heminested primer pairs were NP1F/NP2R and NP1R/NP2F, which resulted in 342 bp and 283 bp PCR products, respectively. PCR products were purified and sequenced using direct sequencing.

3. Paramyxovirus Nested RT-PCR

Polymerase (*pol*)-specific primers were used for first-round and nested PCR[6]. The PCR product was sized by gel electrophoresis in 1.5% agarose (*pol* -PCR product is 561-bp). PCR products were purified and sequenced using direct sequencing.

4. Filoviruses Nested RT-PCR

Protocol for Filovirus detection was modified[7]. RNA polymerase L (L)-specific primers were used for first-round and nested PCR. The PCR product was sized by gel electrophoresis in 1.5% agarose (L-PCR product is <630-bp). PCR products were purified and sequenced using direct sequencing.

5. Coronavirus Nested RT-PCR

Alphacoronaviruses

RNA-dependent RNA polymerase (RdRp) gene specific primers were used for first-round and nested PCR[8]. The PCR product was sized by gel electrophoresis in 1.5% agarose (RdRp-PCR product is 434-bp). PCR products were purified and sequenced using direct sequencing.

Betacoronaviruses; severe acute respiratory syndrome coronaviruses (SARS-CoVs)

RNA-dependent RNA polymerase (RdRp) gene specific primers were used for first-round and nested PCR (Quan P, *et al.*, 2010). The PCR product was sized by gel electrophoresis in 2% agarose (RdRp-PCR product is 328-bp). PCR products were purified and sequenced using direct sequencing.

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[4] Wacharapluesadee S, Lumlertdacha B, Boongird K, Wanghongsa S, Chanhome L, Rollin P, Stockton P, Rupprecht CE, Ksiazek TG, Hemachudha T. Bat Nipah virus, Thailand. Emerging infectious diseases. 2005 Dec;11(12):1949.
[5] Wacharapluesadee S, Hemachudha T. Duplex nested RT-PCR for detection of Nipah virus RNA from urine specimens of bats. Journal of virological methods. 2007 Apr 1;141(1):97-101.
[6] Tong S, Chern SW, Li Y, Pallansch MA, Anderson LJ. Sensitive and broadly reactive reverse transcription-PCR assays to detect novel paramyxoviruses. Journal of clinical microbiology. 2008 Aug 1;46(8):2652-8.
[7] Zhai J, Palacios G, Towner JS, Jabado O, Kapoor V, Venter M, Grolla A, Briese T, Paweska J, Swanepoel R, Feldmann H. Rapid molecular strategy for filovirus detection and characterization. Journal of clinical microbiology. 2007 Jan 1;45(1):224-6.
[8] Watanabe S, Masangkay JS, Nagata N, Morikawa S, Mizutani T, Fukushi S, Alviola P, Omatsu T, Ueda N, Iha K, Taniguchi S. Bat coronaviruses and experimental infection of bats, the Philippines. Emerging infectious diseases. 2010 Aug;16(8):1217.

6. Sequencing

The positive PCR products were gel purified using the NucleoSpin® Gel and PCR Clean-up kit and sequenced directly using an automated ABI PRISM 377 DNA sequencer. Sequences were cleaned using the Bio-edit program and aligned with reference sequences collected from GenBank.

Genome characterization by next generation sequencing (NGS)

Whole genome sequencing (WGS) using NGS technology was performed on five nucleic acid specimens with relatively strong PCR positive signals. WGS was performed using enrichment library preparation (Respiratory Viral Oligos Panel, RVOP) and an Illumina MiSeq 3000 sequencer, according to the manufacturer instructions.

Genome data analysis (collaborated with Prof. Linfa's team)

Raw reads were first imported into Geneious Prime (version 2020.2.3) for downstream analysis and trimmed of adaptors with BBDuk (version 38.84). De novo assembly was conducted with clean reads by SPAdes (version 3.13.0, http://cab.spbu.ru/software/spades/) in Metagenome mode. The longest contig for each sample was then blasted against SARS-CoV-2 reference genome (MN908947) to evaluate the completeness of genome. The name RacCS203 was assigned to the best contig (29,853 nt). Each sample was then individually mapped to the reference RacCS203 genome using Geneious assembler. Coverage map, low coverage and Variant/SNP was further analyzed in Geneious. Annotation of RacCS203 was done by comparing and transferring the annotation of human SARS-CoV-2 and other related CoVs (RaTG13, BJ01, GX-P4L, SL-ZXC21, SL-ZC45 and RmYN02) after nucleotide sequence alignment done by MAFFT in Geneious Prime software. Individual gene alignment was generated by Geneious alignment and used to plot the phylogeny tree by the maximum-likelihood method with the general-time-reversible (GTR) model and 1,000 bootstrap replicates in PHYML 3.0 software. Similarity plot was generated by SimPlot (version 3.5.1). The accession number of the genome sequences used in the phylogeny analysis are tabulated in Supplementary Table 1.

Serology assay

Virus Glycoprotein Antigen-Base Multiplex Serology Assay

Human and bat sera samples were screened in a multiplex microsphere-based immunoassay (MMIA). Envelope attachment glycoporteins from henipaviruses (RBP, receptor-binidng protein), filovirids (GP), and bat SARS-related CoVs (spike) were expressed in native-like quaternary conformations then coupled to magnetic microspheres. The antigen-coupled microspheres representing 16 viruses and two additional human ACE-2-using bat SARS-related CoVs (Table 3) were prepared and provided by the Department of Microbiology and Immunology, Uniformed Services University, Bethesda, MD, USA. Bat and human samples were diluted at 1:500 in PBS and incubated with antigen-coupled microspheres. After sera incubation with antigen-coupled microspheres, samples were washed, incubated with biotinylated-Protein A and biotinylated Protein G (1:1 ratio) (Thermo Fisher Scientific, Waltham, MA, USA), washed and then finally incubated with streptavidin-phycoerythrin (PE) (Bio-Rad, Hercules, CA, USA). Antigen-antibody complexes were screend on a Bio-Rad Bio-Plex 200 HTF multiplexing system (Bio-Rad), and IgG levels were measured as median fluorescence intensities (MFI).

Analysis method

In the absence of a true control group the assay cutoff for antigen-positive IgG was generated using the assay results for NiV serology in Lyle's flying foxes (*Pteropus lylei*), a confirmed NiV reservoir and a well-studied host-virus relationship. A latent cluster analysis (LCA) was preformed on 1,002 serum and plasma samples using R-Studio. This LCA generated four distinct clusters of IgG data with three threshold cutoffs; clusters between 0-300 MFI, 301-3,357 MFI, 3,358-30,549 MFI, and above 30,550 MFI, represent naïve, cross-reactive exposures/IgG decay, recent antigen-specific exposure, and saturating positives, respectively. IgG values above 3,357 MFI were regarded as likely positive. To standardize this cutoff across antigens, and to account for the a range of cerntainity/uncerntanity between clusters and variance, we established an indeterminate range of 3,000 – 5,000 MFI. Thus, samples with IgG levels < 3,000 MFI were negative, those > 5,000 MFI were positive and those falling in between are indeterminate. Indetermine may relate to cross-reactive IgG responses with the antigens included in the serology panels, or decay of antigen-specific IgG responses to levels that are below what we would consider positive of circulating antibodies.

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Supplement data

Phylogenetic tree analysis of Paramyxovirus, Coronavirus (Quan and Watanabe protocols).

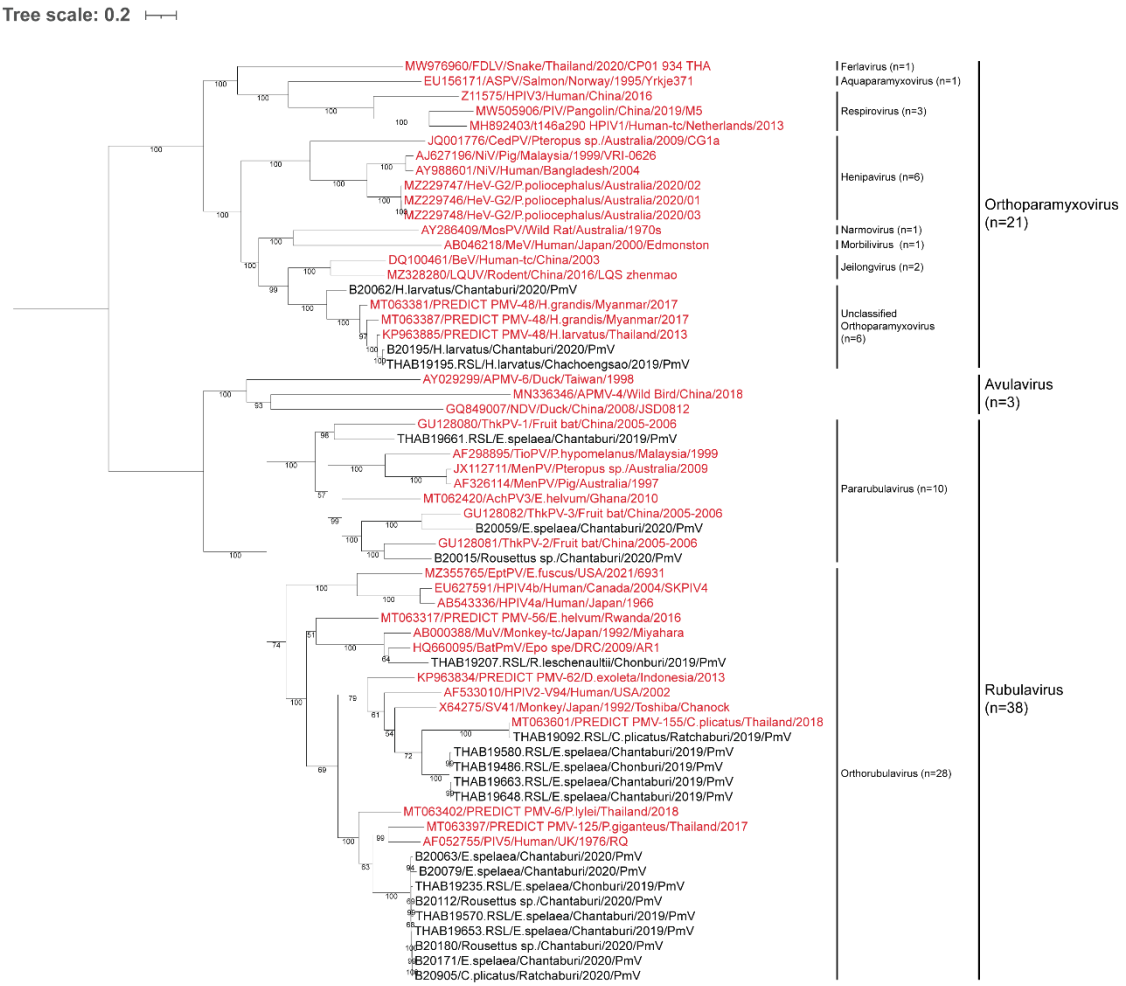

Figure S1. Phylogenetic tree generated using 530 nucleotides of the polymerase gene sequences of **Paramyxovirus (PmV)** *Hiposideros larvatus (n=3), Cherephon plicatus* (n=2), *Eonycteris spelaea* (n=12), *Rousettus leschenaulti* (n=1), and *Rousettus* sp (n=3) from this study (21 specimens, black) and reference strains of PmV (red). The location of the sampled bat in this study was indicated in the sequence name. The maximum-likelihood method, with 1000 replicates bootstrap test, and ModelFinder automatically selected GTR+F+I+G4 substitution model during running IQ-TREE version 2.1.4 was used for analysis. The tree was visualized with the iTOL web application.

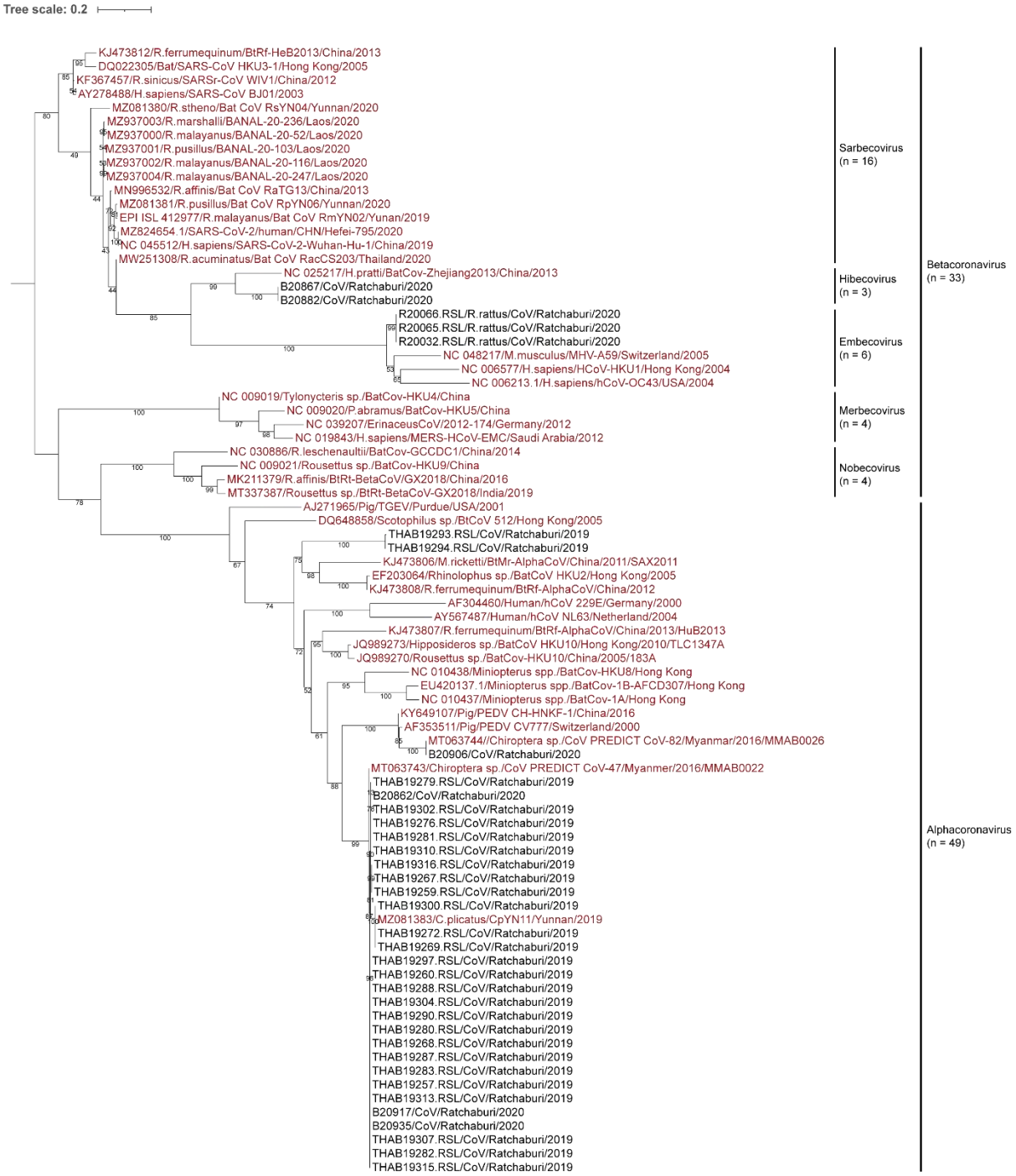

Figure S2. Phylogenetic tree generated using 286 nucleotides of the polymerase gene sequences of Coronavirus (CoV) from the bat (*Cherephon plicatus,* n=33) and rodent(Rodentia Rattus, n=3) from **Ratchaburi** province in this study (black) and reference strains of CoV (red). The maximum-likelihood method, with 1000 replicates bootstrap test, and ModelFinder automatically selected TIM2+F+I+G4 substitution model during running IQ-TREE version 2.1.4 was used for analysis. The tree was visualized with the iTOL web application. PCR protocol was modified from Quan et al.

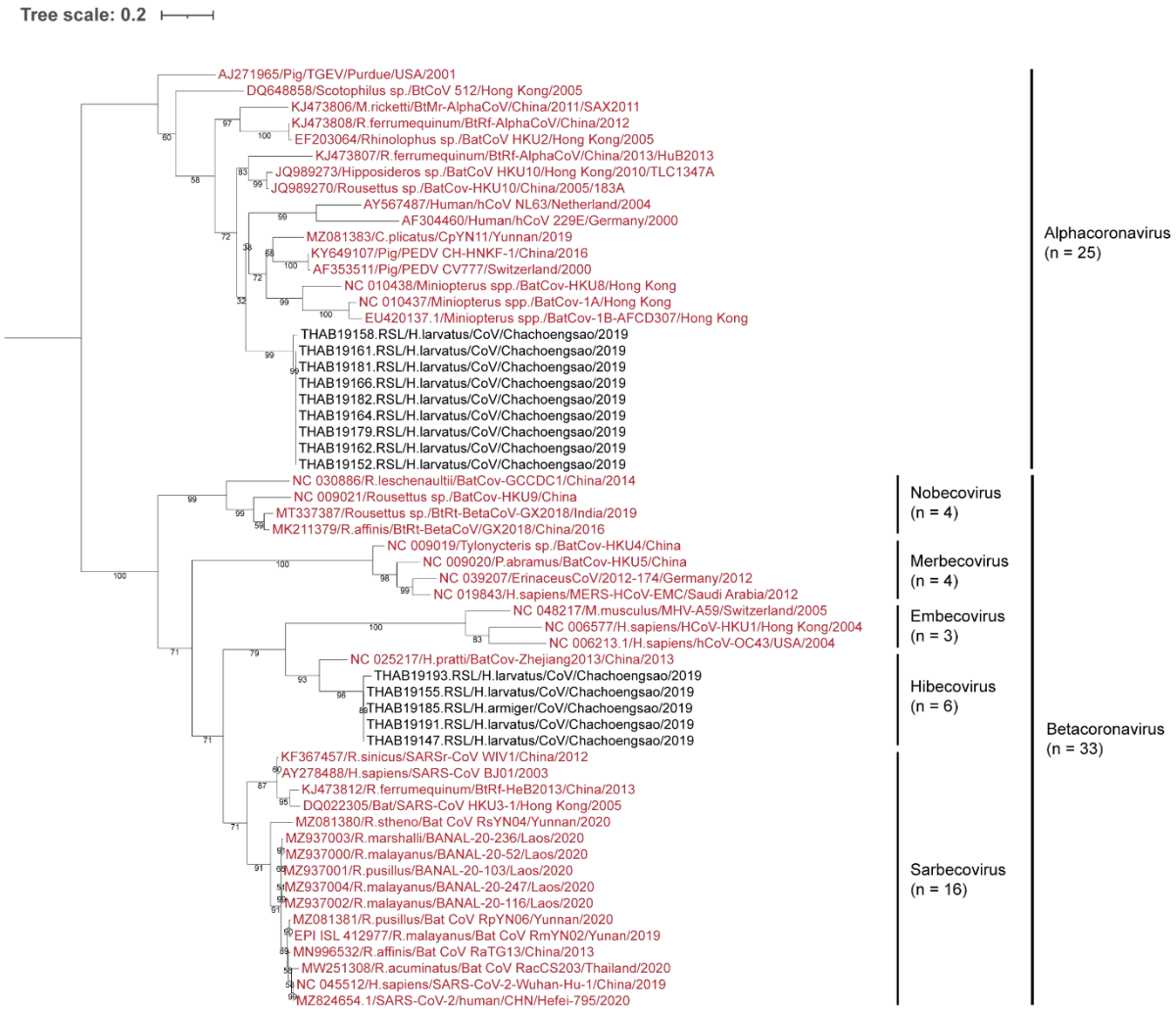

Figure S3. Phylogenetic tree generated using 252 nucleotides of the polymerase gene sequences of Coronavirus (CoV) from *Hiposideros larvatus* bat (n=13) from Chachoengsao province (Wat Khao Tham Raet) in this study (black) and reference strains of CoV (red). The maximum-likelihood method, with 1000 replicates bootstrap test, and ModelFinder automatically selected TIM2+F+I+G4 substitution model during running IQ-TREE version 2.1.4 was used for analysis. The tree was visualized with the iTOL web application. PCR protocol was modified from Quan et al.

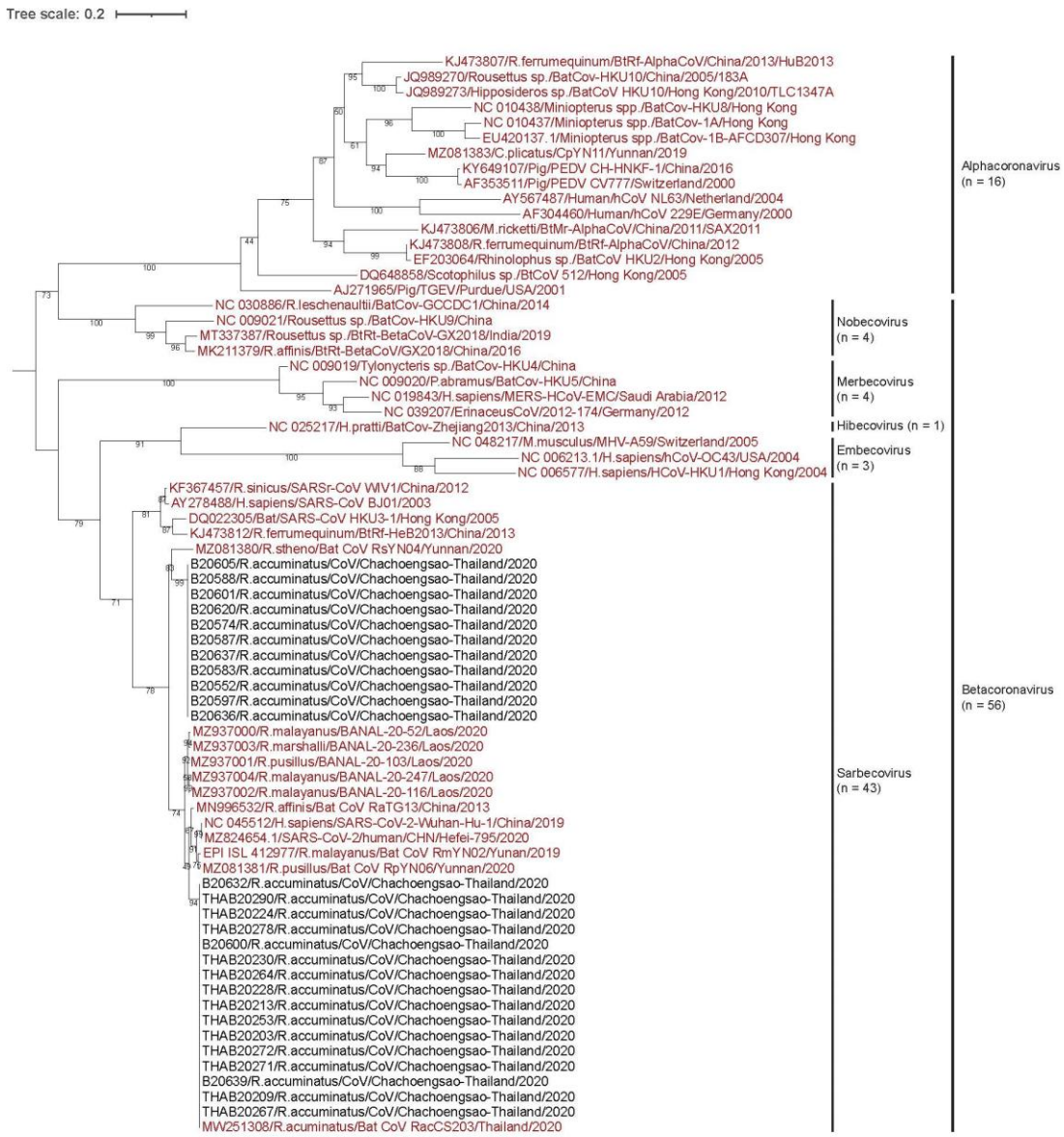

Figure S4a. Phylogenetic tree generated using 290 nucleotides of the polymerase gene sequences of Coronavirus (CoV) from *Rhinolophus accuminatus* bat (n=17) from Chachoengsao province (Khao Ang Rue Nai) in this study (black) and reference strains of CoV (red). The maximum-likelihood method, with 1000 replicates bootstrap test, and ModelFinder automatically selected TIM2+F+I+G4 substitution model during running IQ-TREE version 2.1.4 was used for analysis. The tree was visualized with the iTOL web application. PCR protocol was modified from Quan et al.

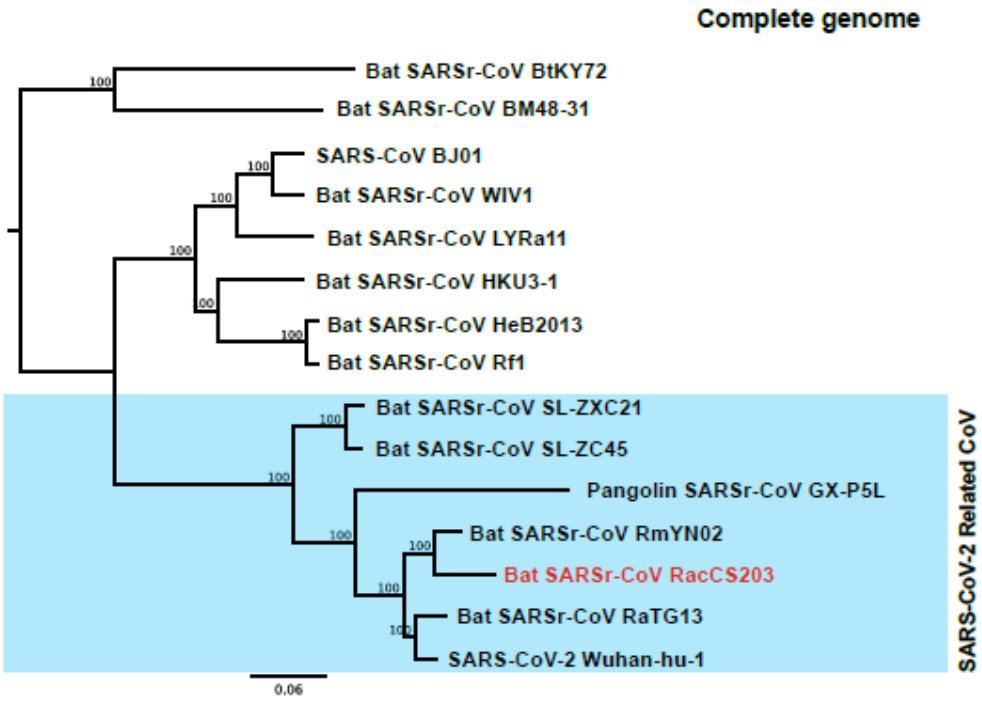

Figure S4b. Phylogenetic tree based on whole-genome sequences of SARS-related CoV from *Rhinolophus accuminatus* (RacCS203) from Khao Ang Rue Nai National park, Chachoengsao province.

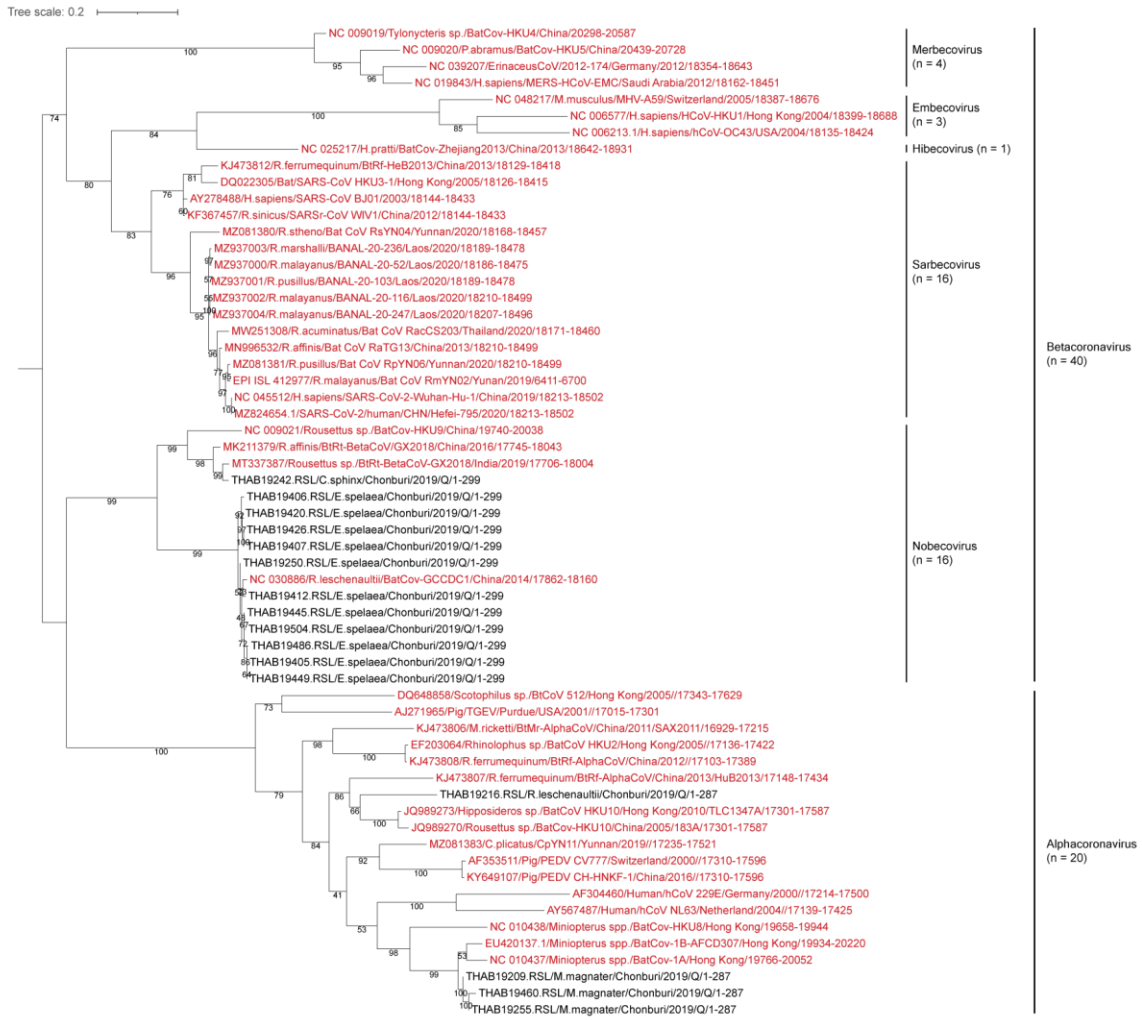

Figure S5. Phylogenetic tree generated using 287 nucleotides of the polymerase gene sequences of Coronavirus (CoV) from *Miniopterus magnater* (n=3), *Rousettus leschenaultii* (n=1), *Cynopterus sphinx* (n=1), *Eonycteris spelaea* (n=11) from Chonburi province (Wat Khao Cha-Ang) in this study (black) and reference strains of CoV (red). The maximum-likelihood method, with 1000 replicates bootstrap test, and ModelFinder automatically selected TIM2+F+I+G4 substitution model during running IQ-TREE version 2.1.4 was used for analysis. The tree was visualized with the iTOL web application. PCR protocol was modified from Quan et al.

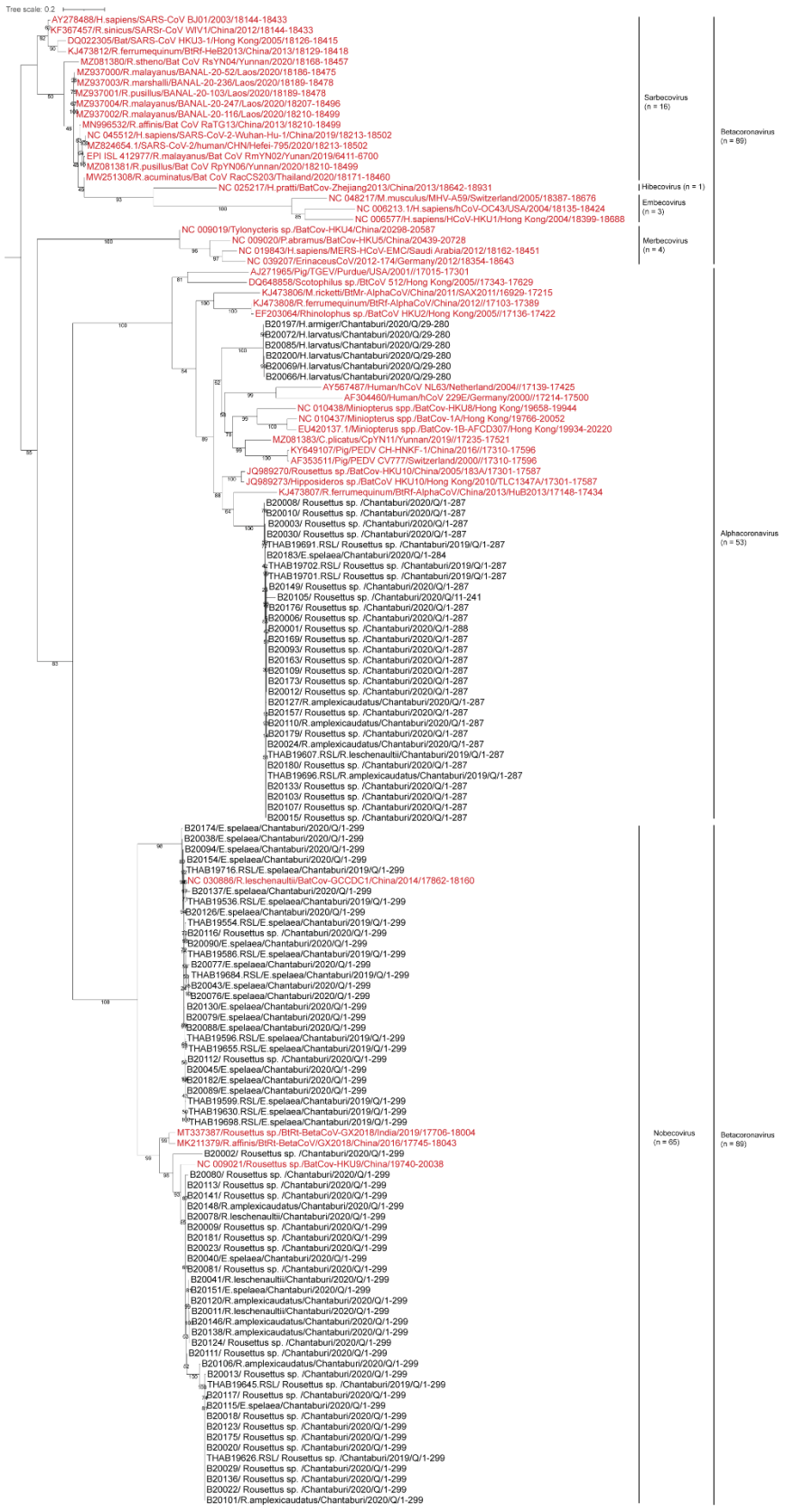

Figure S6. Phylogenetic tree generated using 142 nucleotides of the polymerase gene sequences of Coronavirus (CoV) *Hipposideros larvatus* (n=5), *Hipposideros armiger* (n=1), *Rousettus* sp. (n=48), *Eonycteris spelaea* (n=30), *Rousettus amplexicaudatus* (n=10), and *Rousettus leschenaultii* (n=4) from Chantaburi province (Khao Soi Dao) in this study (black) and reference strains of CoV (red). The maximum-likelihood method, with 1000 replicates bootstrap test, and ModelFinder automatically selected TIM2+F+I+G4 substitution model during running IQ-TREE version 2.1.4 was used for analysis. The tree was visualized with the iTOL web application. PCR protocol was modified from Quan et al.

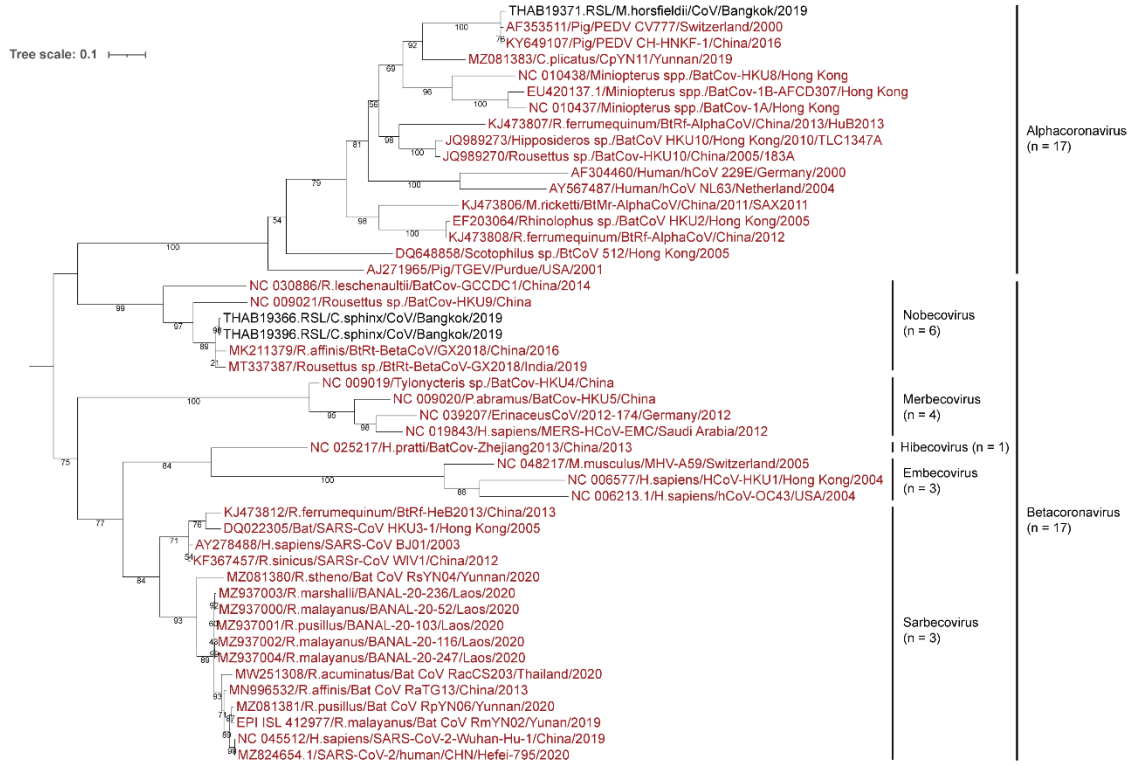

Figure S7. Phylogenetic tree generated using 287 nucleotides of the polymerase gene sequences of Coronavirus (CoV) from *Myotis horsfieldii* (n=1) and *Cynopterus sphinx* (n=2) from Bangkok (Kasetsart University) in this study (black) and reference strains of CoV (red). The maximum-likelihood method, with 1000 replicates bootstrap test, and ModelFinder automatically selected TIM2+F+I+G4 substitution model during running IQ-TREE version 2.1.4 was used for analysis. The tree was visualized with the iTOL web application. PCR protocol was modified from Quan et al.

Figure S8. Phylogenetic tree generated using 387 nucleotides of the polymerase gene sequences of Coronavirus (CoV) from the bat (*Cherephon plicatus,*n=26) and rodent (*Rodentia Rattus*, n=3) from Ratchaburi in this study (black) and reference strains of CoV (red). The maximum-likelihood method, with 1000 replicates bootstrap test, and ModelFinder automatically selected GTR+F+I+G4 substitution model during running IQ-TREE version 2.1.4 was used for analysis. The tree was visualized with the iTOL web application. PCR protocol was modified from Watanabe et al.

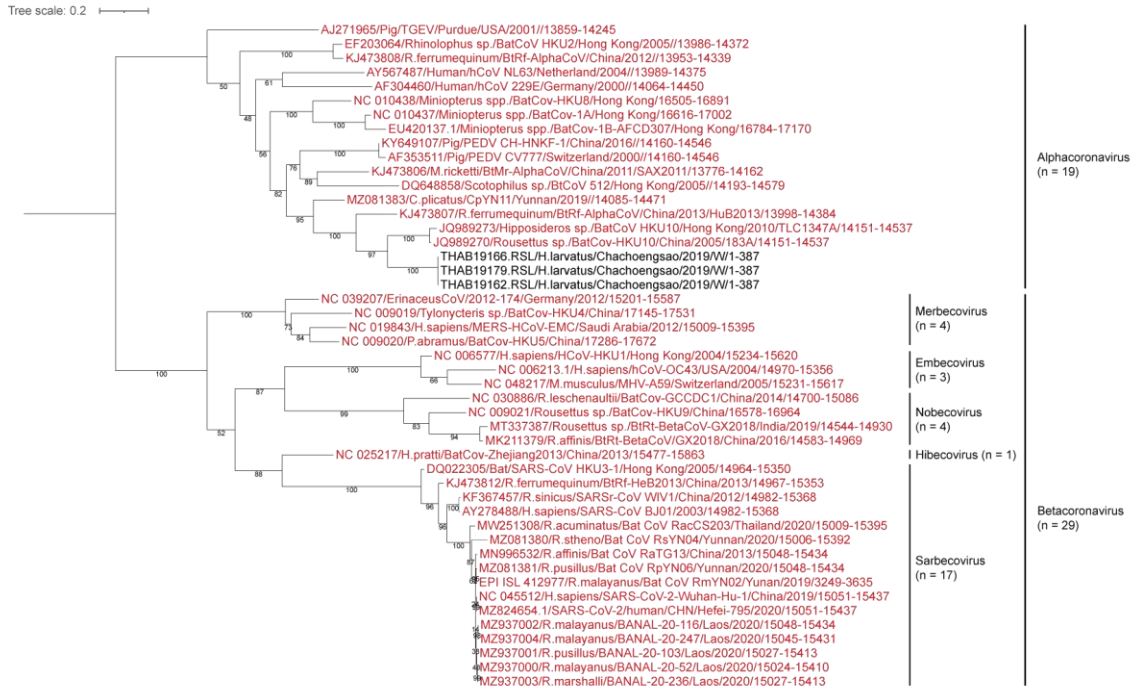

Figure S9. Phylogenetic tree generated using 387 nucleotides of the polymerase gene sequences of Coronavirus (CoV) from *Hiposideros larvatus* bat (n=3) from Chachongsao (Wat Khao Tham Raet) in this study (black) and reference strains of CoV (red). The maximum-likelihood method, with 1000 replicates bootstrap test, and ModelFinder automatically selected GTR+F+I+G4 substitution model during running IQ-TREE version 2.1.4 was used for analysis. The tree was visualized with the iTOL web application. PCR protocol was modified from Watanabe et al.

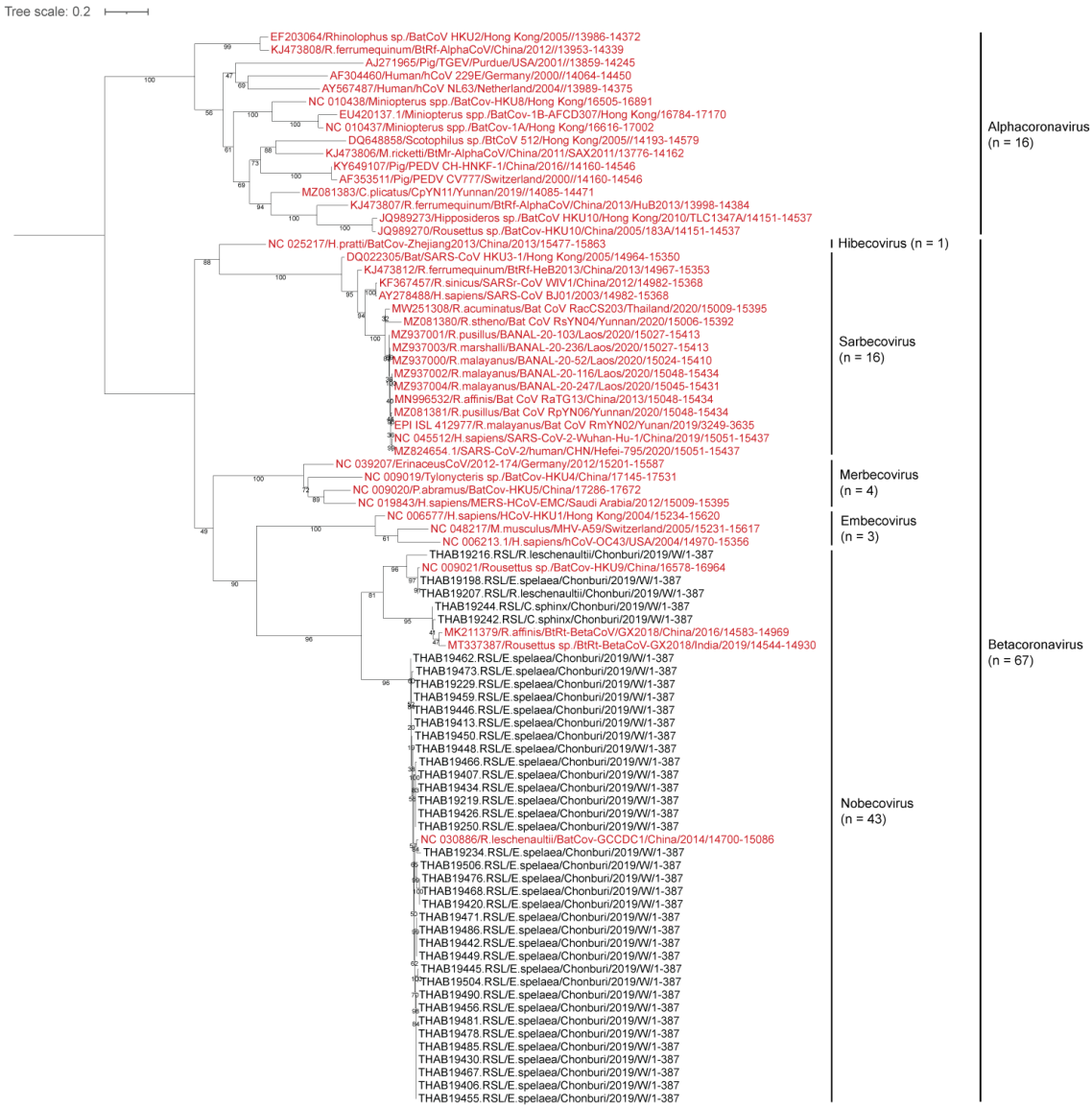

Figure S10. Phylogenetic tree generated using 387 nucleotides of the polymerase gene sequences of Coronavirus (CoV) from *Rousettus leschenaultii* (n=2), *Eonycteris spelaea* (n=35), and *Cynopterus sphinx* (n=2) from Chonburi (Wat Khao Cha-ang) in this study (black) and reference strains of CoV (red). The maximum-likelihood method, with 1000 replicates bootstrap test, and ModelFinder automatically selected GTR+F+I+G4 substitution model during running IQ-TREE version 2.1.4 was used for analysis. The tree was visualized with the iTOL web application. PCR protocol was modified from Watanabe et al.

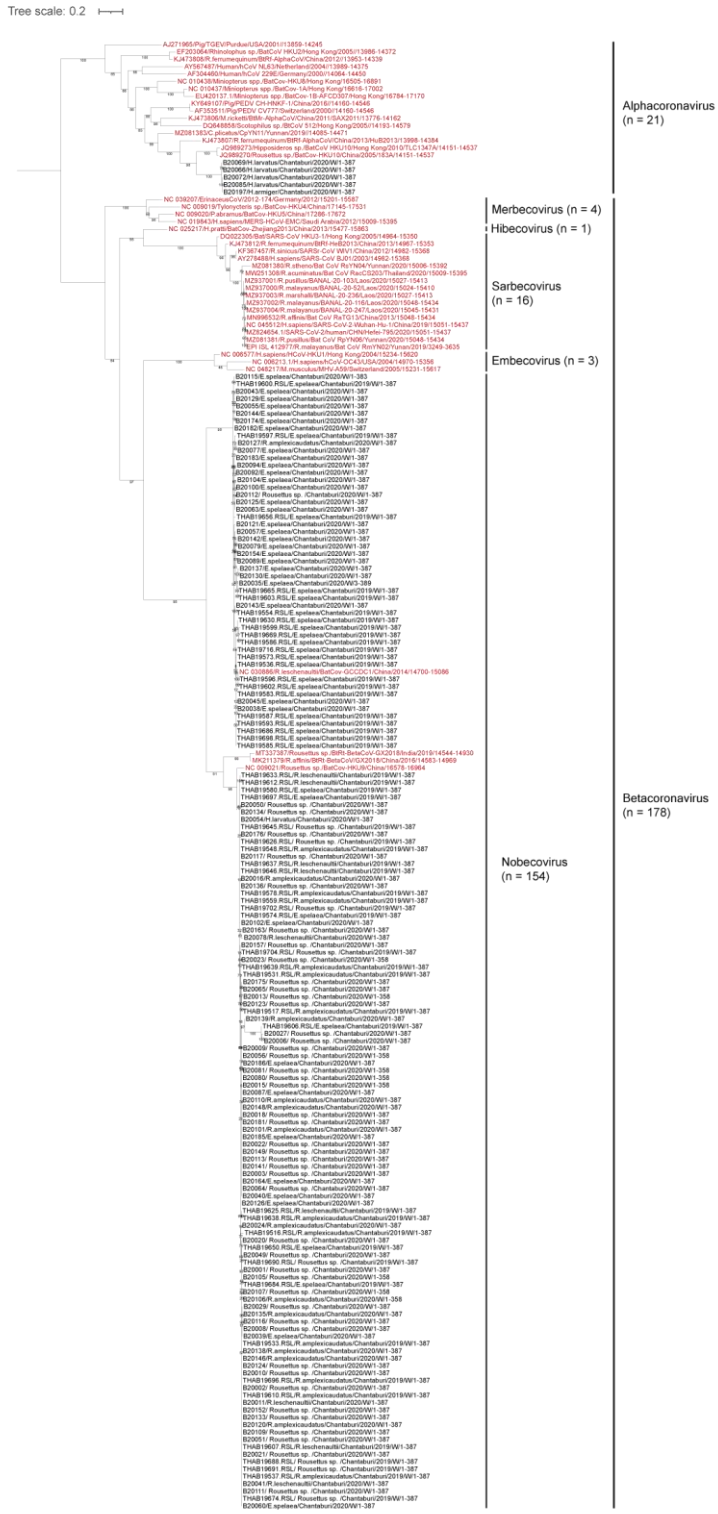

Figure S11. Phylogenetic tree generated using 387 nucleotides of the polymerase gene sequences of Coronavirus (CoV) from *Hipposideros larvatus* (n=5), *Hipposideros armige*r (n=1), *Eonycteris spelaea* (n=63), *Rousettus* sp. (n=53), *Rousettus amplexicaudatus* (n=24), and *Rousettus leschenaultii* (n=9) from Chantaburi (Khao Soi Dao) in this study (black) and reference strains of CoV (red). The maximum-likelihood method, with 1000 replicates bootstrap test, and ModelFinder automatically selected GTR+F+I+G4 substitution model during running IQ-TREE version 2.1.4 was used for analysis. The tree was visualized with the iTOL web application. PCR protocol was modified from Watanabe et al.

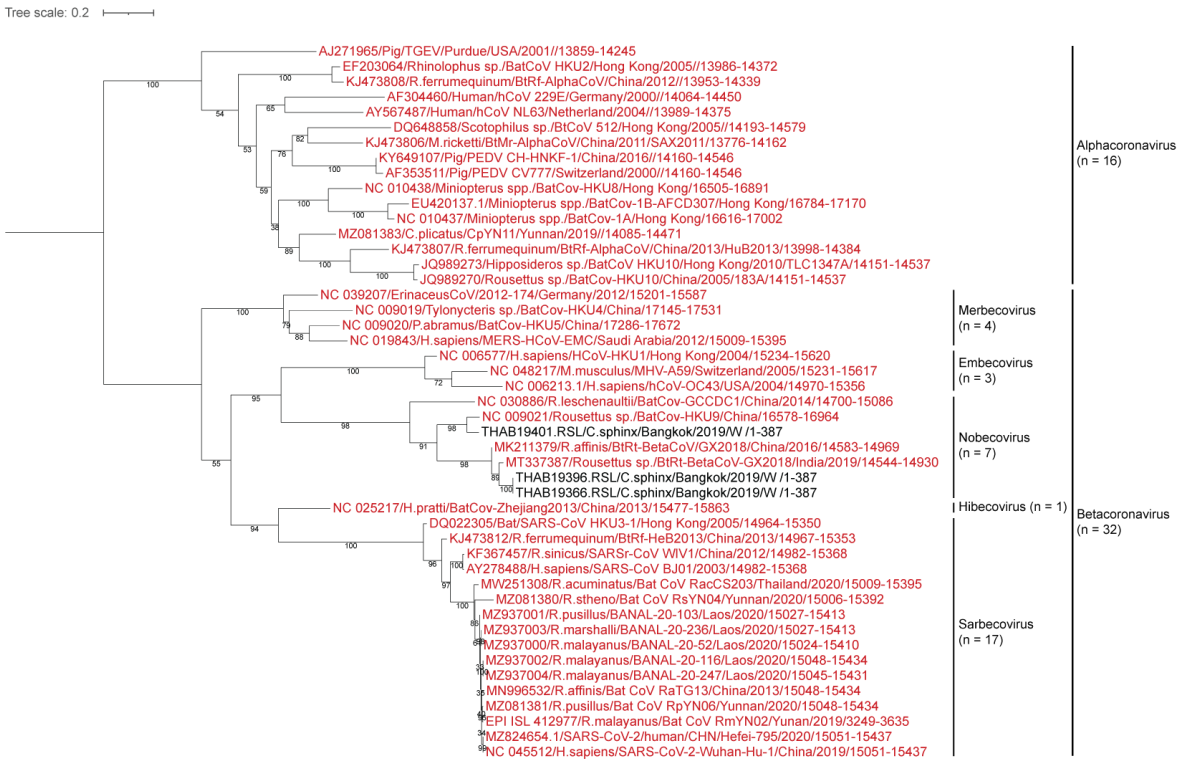

Figure S12. Phylogenetic tree generated using 387 nucleotides of the polymerase gene sequences of Coronavirus (CoV) from *Cynopterus sphinx* (n=3) from Bangkok (Kasetsart University) in this study (black) and reference strains of CoV (red). The maximum-likelihood method, with 1000 replicates bootstrap test, and ModelFinder automatically selected GTR+F+I+G4 substitution model during running IQ-TREE version 2.1.4 was used for analysis. The tree was visualized with the iTOL web application. PCR protocol was modified from Watanabe et al.

From: (b) (6)
To: (b) (6)
Cc: (b) (6) ; Eric D Laing; (b) (6)
Subject: Final Report Project no. 042959: Deliverables for Bat Serology Study (CHULA)
Date: Wednesday, December 1, 2021 5:56:32 AM
Attachments: Final Scientific Report(b) (6) Proj 042959.pdf

Dear Dr. (b) (6)

I've attached the Final Biannual Scientific Report of project number 042959 (PO: 18-0507, BLACK & VEATCH), project title "Surveillance for emerging infectious disease pathogens at the animal-human interfaces in Thailand, in coordination with PREDICT USAID project and the bat serology study" .

For your kind consideration and approval for the final (8th) payment.

I apologize for the delay in sending the final report.

Best,
(b) (6)

From: (b) (6)
Sent: Sunday, September 15, 2019 4:33 PM
To: (b) (6)
Cc: (b) (6)
Subject: Re: Deliverables for Bat Serology Study (CHULA)

Dear (b) (6) ,

Please see the attached 6th deliverable, project status update report.

Best Regards,
(b) (6)

(b) (6)

On Wed, 10 Jul 2019 at 14:30, (b) (6) wrote:

> Great news (b) (6) thank you for the update.
>
> (b) (6)
>
> **Please consider the environment before printing my email**
> Please note that the information and attachments in this email are intended for the exclusive use of the addressee and may contain confidential or privileged information. If you are not the intended recipient, please do not forward, copy or print the message or its attachments. Notify me at the above address, and delete this message and any attachments. Thank you.
>
> **From:** (b) (6)
> **Sent:** Wednesday, July 10, 2019 2:10 PM
> **To:** (b) (6)
> **Cc:** (b) (6)
> **Subject:** Re: Deliverables for Bat Serology Study (CHULA)
>
> Dear (b) (6),
>
> We confirm receipt of payment. Please see the attached.
>
> Best Regards,
> (b) (6)

(b) (6) [redacted]

On Sun, 23 Jun 2019 at 05:45, (b) (6) [redacted] wrote:

> Thanks (b) (6) I will get payment processed as soon as possible.
>
> (b) (6) [redacted]
>
> [redacted]
>
> [redacted]
>
> **Please consider the environment before printing my email**
> Please note that the information and attachments in this email are intended for the exclusive use of the addressee and may contain confidential or privileged information. If you are not the intended recipient, please do not forward, copy or print the message or its attachments. Notify me at the above address, and delete this message and any attachments. Thank you.
>
> **From:** (b) (6) [redacted]
> **Sent:** Friday, June 21, 2019 3:21 PM
> **To:** (b) (6) [redacted]
> **Cc:** (b) (6) [redacted]
> **Subject:** Re: Deliverables for Bat Serology Study (CHULA)
>
> Dear (b) (6) [redacted],
>
> Please see the signed invoice attached.
>
> Best Regards,
> (b) (6) [redacted]
>
> [redacted]
>
> [redacted]

On Thu, 20 Jun 2019 at 12:22, (b) (6) wrote:

> Hello (b) (6)
>
> I am very sorry to say that I just found this submittal along with the earlier submittal of CHULA's Project Status Update Report in my quarantined messages.
>
> Please find the attached invoice for CHULA's review and signature for these two submittals. As soon as you can return the signed invoice I will get the payment processed.
>
> Thank You
>
> (b) (6)
>
> [redacted]
>
> [redacted]
>
> **Please consider the environment before printing my email**
> Please note that the information and attachments in this email are intended for the exclusive use of the addressee and may contain confidential or privileged information. If you are not the intended recipient, please do not forward, copy or print the message or its attachments. Notify me at the above address, and delete this message and any attachments. Thank you.
>
> **From:** (b) (6)
> **Sent:** Wednesday, June 12, 2019 9:12 PM
> **To:** (b) (6)
> **Cc:** (b) (6)
> [redacted]
> **Subject:** Re: Deliverables for Bat Serology Study (CHULA)
>
> Dear (b) (6),
>
> Please see the attached 5th deliverable, 2nd Biannual Scientific report.
>
> Best Regards,
> (b) (6)
>
> [redacted]
>
> [redacted]

(b) (6)

On Wed, 20 Mar 2019 at 11:22, (b) (6) wrote:

> Thanks (b) (6)!
>
> Sent from my iPhone
>
> On 20 Mar 2019, at 11:37 AM, (b) (6) > wrote:
>
> > Dear (b) (6) ,
> >
> > Please see the attached 4th deliverable, project status update report.
> >
> > Please let us know if you have any questions/comments. Thanks!
> >
> > Best Regards,
> > (b) (6)
> >
> > On Tue, 1 Jan 2019 at 00:48, (b) (6) wrote:
> >
> > > Dear (b) (6) ,
> > >
> > > Please see the attached deliverables 2 and 3, status update and biannual scientific reports.
> > >
> > > Please let us know if you have any questions/comments. Thanks!
> > >
> > > Best Regards,
> > > (b) (6)

(b) (6)

On Tue, 2 Oct 2018 at 19:08, (b) (6) wrote:

> Thank you (b) (6),
>
> Receipt acknowledges.
>
> v/r
>
> (b) (6)
>
> **Please consider the environment before printing my email**
> Please note that the information and attachments in this email are intended for the exclusive use of the addressee and may contain confidential or privileged information. If you are not the intended recipient, please do not forward, copy or print the message or its attachments. Notify me at the above address, and delete this message and any attachments. Thank you.
>
> **From:** (b) (6)
> **Sent:** Monday, October 01, 2018 7:49 AM
> **To:** (b) (6)
> **Cc:** (b) (6)
> **Subject:** Deliverables for Bat Serology Study (CHULA)
>
> Dear (b) (6),
>
> Please see the attached project plan and schedule for the serology study (1st deliverable).
>
> Please let us know if you have any questions or comments. Thank you!

Best Regards,
(b) (6)

[redacted]

<PROJECT STATUS UPDATE REPORT_9months 19Mar2019.docx>

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From: (b) (6)
To: (b) (6) Laing, Eric; (b) (6)
Cc: (b) (6)
Subject: RO1 submitted
Date: Tuesday, June 8, 2021 12:37:04 PM
Attachments: specific_aims.pdf
research_strategy.pdf
references.pdf

Colleagues,

I'm writing to share that our RO1 application has been submitted. The specific aims and research strategy we submitted is attached here, for your records.

Special thanks to (b) (6) for his tireless efforts to get things together, and the really lovely figures.

All the best,
(b) (6)