The Proximal Origin of HCoV-19

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Since the first reports of novel pneumonia (COVID-19) in Wuhan, Hubei province, China[1,2] there has been considerable discussion on the origin of the causative virus, human coronavirus 2019 (HCoV-19[3]; also referred to as SARS-CoV-2[4]). Infections with HCoV-19 are now widespread, and as of 29 February 2020, 86,012 cases have been confirmed in 57 countries, with 2,941 deaths[5], although these are likely an underestimate with limited reporting of mild and asymptomatic cases.

HCoV-19 is the seventh coronavirus known to infect humans. Three of these viruses, SARS-CoV, MERS-CoV, and HCoV-19 can cause severe disease; four, HKU1, NL63, OC43 and 229E, are associated with mild respiratory symptoms[7]. Herein, we review what can be deduced about the origin of HCoV-19 from the comparative analysis of genome sequence data. In particular, we offer a perspective on the notable features in the HCoV-19 genome and discuss scenarios by which they could have arisen. Our analysis provides strong evidence that HCoV-19 is not a laboratory construct nor a purposefully manipulated virus.

Notable features of the HCoV-19 genome

Our genomic comparison of alpha- and betacoronaviruses (family *Coronaviridae*) identifies two notable features of the HCoV-19 genome: (*i*) based on structural studies[8–10] and biochemical experiments[1,9,11,12], HCoV-19 appears optimized for binding to the human ACE2 receptor; (*ii*) the highly variable spike (S) protein of HCoV-19 has a functional polybasic (furin) cleavage site at the S1/S2 boundary through the insertion of twelve nucleotides[10,13,14]. Additionally, this led to the predicted acquisition of three O-linked glycans around the polybasic cleavage site.

Mutations in the receptor binding domain of HCoV-19

The receptor binding domain (RBD) in the spike protein is the most variable part of the coronavirus genome[1,2]. Six RBD amino acids have been shown to be critical for binding to ACE2 receptors and determining the host range of SARS-like viruses[8]. Using coordinates based SARS-CoV, they are Y442, L472, N479, D480, T487, and Y4911 corresponding to L455, F486, Q493, S494, N501, and Y505 in HCoV-19[8]. Five of these six residues differ between HCoV-19 and SARS-CoV (**Fig. 1a**). Based on structural studies[8–10] and biochemical experiments[1,9,11,12], HCoV-19 seems to have an RBD that binds with high affinity to ACE2 from human, non-human primate, ferret, pig, and cat, and some other species with high receptor homology[8].

While these analyses suggest that HCoV-19 may be capable of binding human ACE2 with high affinity, computational analyses predict that the interaction is not ideal[8] and the RBD sequence is different from those shown in SARS-CoV to be optimal for receptor binding[16]. Thus, the optimized binding of HCoV-19 spike protein to human ACE2 is most likely the result of natural selection on a human or human-like ACE2 permitting another optimal binding solution to arise. This is strong evidence that HCoV-19 is *not* the product of purposeful manipulation.

Polybasic furin cleavage site and O-linked glycans

The second notable feature of HCoV-19 is a polybasic furin cleavage site (RRAR) at the junction of S1 and S2, the two subunits of the spike (**Fig. 1b**)[13,14]. This allows effective cleavage by furin and other proteases and plays an important role in determining virus infectivity and host range[18]. In addition, a leading proline is also inserted at this site in HCoV-19; thus, the inserted sequence is PRRA (**Fig. 1b**). The strong turn created by the proline is predicted to result in the addition of O-linked glycans to S673, T678, and S686 flanking the cleavage site that are unique to HCoV-19 (**Fig. 1b**). Polybasic cleavage sites have not been observed in related "lineage B" betacoronaviruses, although other human betacoronaviruses, including HKU1 (lineage A), have them and predicted O-linked glycans near the S1/S2 cleavage site[19]. Given the level of genetic variation in the S protein it is likely that HCoV-19-like viruses with partial or full polybasic sites will be discovered in other species.

The functional consequence of the furin cleavage site in HCoV-19 is unknown and it will be important to determine what impact the feature has on transmissibility and pathogenesis in animal models[21]. Experiments with SARS-CoV have shown that insertion of a furin cleavage site at the S1/S2 junction enhances cell–cell fusion without affecting virus entry[22]. In addition, efficient cleavage of the MERS-CoV spike enables MERS-like coronaviruses from bats to infect human cells[23]. In avian influenza viruses, rapid virus replication and transmission in highly dense chicken populations selects for the acquisition of polybasic cleavage sites in the haemagglutinin (HA) protein , [24–26]. HA serves a similar function in cell-cell fusion and viral entry as the coronavirus spike protein. Acquisition polybasic cleavage sites in HA, by insertion or recombination, converts low pathogenicity avian influenza viruses into highly pathogenic forms[24–26]. The acquisition of polybasic cleavage sites by HA has also been observed after repeated passage in cell culture or through animals[27,28].

The function of the predicted O-linked glycans is less clear, but they could create a "mucin-like domain" shielding potential epitopes or key residues on the HCoV-19 spike protein[30,31]. Several viruses employ mucin-like domains as part of a glycan shield involved in immune evasion[30]. Although prediction of O-linked glycosylation is robust[32], biochemical analyses or structural studies are required to determine whether or not these sites in HCoV-19 are utilized.

Theories of HCoV-19 origins

It is improbable that HCoV-19 emerged through laboratory manipulation or engineering of a related SARS-like coronavirus. As noted above, the RBD of HCoV-19 is optimized for human ACE2 binding with an efficient solution different from those previously predicted[8,16]. Further, had genetic manipulation had been performed, one of the several reverse genetic systems available for betacoronaviruses would likely have been used[20]. However, the genetic data irrefutably show that HCoV-19 is not derived from any previously used virus backbone[33]. Instead, we propose two scenarios that can plausibly explain the origin of HCoV-19: (*i*) natural selection in a non-human animal host prior to zoonotic transfer, and (*ii*) natural selection in humans following zoonotic transfer. We also discuss whether selection during passage in culture could have given rise to the same observed features.

Natural selection in an animal host prior to zoonotic transfer

As many of the early cases of COVID-19 were linked to the Huanan seafood and wildlife market in Wuhan[1,2], it is possible that an animal source was present at this location. Given the similarity of HCoV-19 to bat SARS-like coronaviruses[2], it is likely that bats serve as reservoir hosts for its progenitor. Although RaTG13, sampled from a *Rhinolophus affinis* bat, is ~96% identical overall to HCoV-19[1], its spike diverges in the RBD suggesting that it may not bind efficiently to the human ACE2 receptor (**Fig. 1a**)[8].

Malayan pangolins (*Manis javanica*) illegally imported into Guangdong province contain coronaviruses similar to HCoV-19[15,37–39]. Although the RaTG13 bat virus remains the closest relative to HCoV-19 across the whole genome[1], some pangolin coronaviruses exhibit strong similarity to HCoV-19 in the RBD, including all six key RBD residues (**Fig. 1**)[15,39]. This clearly shows the HCoV-19 spike protein optimized for binding to human-like ACE2 occurs in nature and is the result of natural selection. Similarly, neither the bat nor pangolin betacoronaviruses sampled to date carry polybasic cleavage sites. Although a non-human animal coronavirus, sufficiently similar to HCoV-19 across its entire genome that it could have served as the direct progenitor of the virus, has yet to be identified, the diversity of coronaviruses in bats and other species is massively undersampled. Mutations, including point mutations, insertions and deletions, can occur near the S1/S2 junction of coronaviruses[34,40–43] suggesting that the polybasic site could arise by a natural evolutionary process. For a precursor virus to acquire both the polybasic cleavage site and mutations in the spike protein suitable for human ACE2 receptor binding, an animal host would likely have to have a high population density – to allow natural selection to proceed efficiently – and an ACE2 gene that is similar to the human orthologue.

Natural selection in humans following zoonotic transfer

It is possible that a progenitor to HCoV-19 jumped into humans, acquiring the genomic features described above through adaptation during (undetected) human-to-human transmission. Once acquired, these adaptations would enable the epidemic to take off, producing a sufficiently large and unusual cluster of pneumonia cases to trigger the surveillance system that ultimately detected it[1,2].

All HCoV-19 genomes sequenced so far have the genomic features derived above and are thus derived from a common ancestor that had them too. The presence in pangolins of an RBD very similar to that in HCoV-19 means we can infer this was also likely in the virus that jumped to humans. This leaves the polybasic cleavage site insertion to occur during human-to-human transmission.

Estimates of the timing of the most recent common ancestor of HCoV-19 using currently available genome sequence data point to virus emergence in late November to early December 2019[44-46], compatible with the earliest retrospectively confirmed cases[47]. Hence, this scenario presumes a period of unrecognised transmission in humans between the initial zoonotic transfer event and the acquisition of the polybasic cleavage site. Sufficient opportunity could occur if there had been many prior zoonotic events producing short chains of human-to-human transmission over an extended period. This is essentially the situation for MERS-CoV in the Arabian Peninsula where all the human cases are the result of repeated jumps of the virus from dromedary camels, producing single infections or short transmission chainsthat eventually resolve, with no adaptation to sustained human transmission[48].

Metagenomic studies of banked human samples could provide important information on whether this cryptic spread has occurred, although given the relatively short period of viremia it may be impossible to detect low level HCoV-19 circulation in historical samples. Retrospective serological studies could also be informative and a few such studies have been conducted. One found that animal importation traders had a 13% seropositivity to coronaviruses[49], while another noted that 3% residents of a village in Southern China were seropositive to SARS-like coronaviruses[50]. Critically, however, these studies could not have distinguished whether positive serological responses were due to prior infections with SARS-CoV, HCoV-19,

or other SARS-like coronaviruses. Further serological studies should be conducted to determine the extent of prior human exposure to HCoV-19.

Selection during passage

Basic research involving passage of bat SARS-like coronaviruses in cell culture and/or animal models have been ongoing in BSL-2 for many years in laboratories across the world[51–54]. There are also several documented instances of laboratory escapes of SARS-CoV[55–57]. We must therefore examine the possibility of a inadvertent laboratory release of HCoV-19.

In theory, it is possible that HCoV-19 acquired RBD mutations (**Fig. 1a**) during adaptation to passage in cell culture, as has been observed in studies with SARS-CoV[16] and MERS-CoV[58]. The finding of SARS-like coronaviruses from pangolins with near-identical RBDs, however, provides a much stronger and parsimonious explanation for how HCoV-19 acquired these via recombination or mutation[20].

The acquisition of both the polybasic cleavage site and predicted O-linked glycans also argues against any type of culture-based scenario. New polybasic cleavage sites have only been observed after prolonged passage of low pathogenicity avian influenza virus *in vitro* or *in vivo*[24,26–28]. Furthermore, a hypothetical generation of HCoV-19 by cell culture or animal passage would have required prior isolation of a progenitor virus with a very high genetic similarity, which has not been described. Subsequent generation of a polybasic cleavage site would have then required repeated passage in cell culture or animals with ACE2 receptors similar to humans (e.g. ferrets), but such work has also not previously been described. Finally, the generation of the predicted O-linked glycans is also unlikely to have occured due to cell culture passage, as such features suggest the involvement of an immune system[30].

Conclusions

In the midst of the global COVID-19 public health emergency it is reasonable to wonder why the origins of the epidemic matter. A detailed understanding of how an animal virus jumped species boundaries to infect humans so productively will help in the prevention of future zoonotic events. For example, if HCoV-19 pre-adapted in another animal species then we are at risk of future re-emergence events. In contrast, if the adaptive process we describe occurred in humans, then even if we have repeated zoonotic transfers they are unlikely to take-off without the same series of mutations. In addition, identifying the closest animal relatives of HCoV-19 will greatly assist studies of virus function. Indeed, the availability of the RaTG13 bat sequence helped reveal the key mutations in the RBD as well as the polybasic cleavage site insertion.

The genomic features described here may in part explain the infectiousness and transmissibility of HCoV-19 in humans. Although the evidence shows that HCoV-19 is not a purposefully manipulated virus, it is currently impossible to prove or disprove the other theories of its origin described here. However, since we observe all notable HCoV-19 features - including the optimized RBD and furin cleavage site - in related coronaviruses in nature, we do not believe that selection during passage, or any other type of laboratory-based scenario, is necessary.

More scientific data could swing the balance of evidence to favor one hypothesis over another. Obtaining virus sequences from any immediate non-human animal source would be the most definitive way of revealing virus origins. For example, a future observation of an intermediate or fully formed polybasic cleavage site in an HCoV-19 related virus from animals would lend very strong support to the natural selection hypotheses. It would also be helpful to obtain more genetic and functional data about HCoV-19, including experimental studies of receptor binding and the role of the polybasic cleavage site and predicted O-linked glycans. The identification of a potential intermediate host of HCoV-19, as well as the sequencing of very early cases would similarly be highly informative. Irrespective of the exact mechanisms of how

HCoV-19 originated via natural selection, the ongoing surveillance of pneumonia in humans and other animals is clearly of utmost importance.

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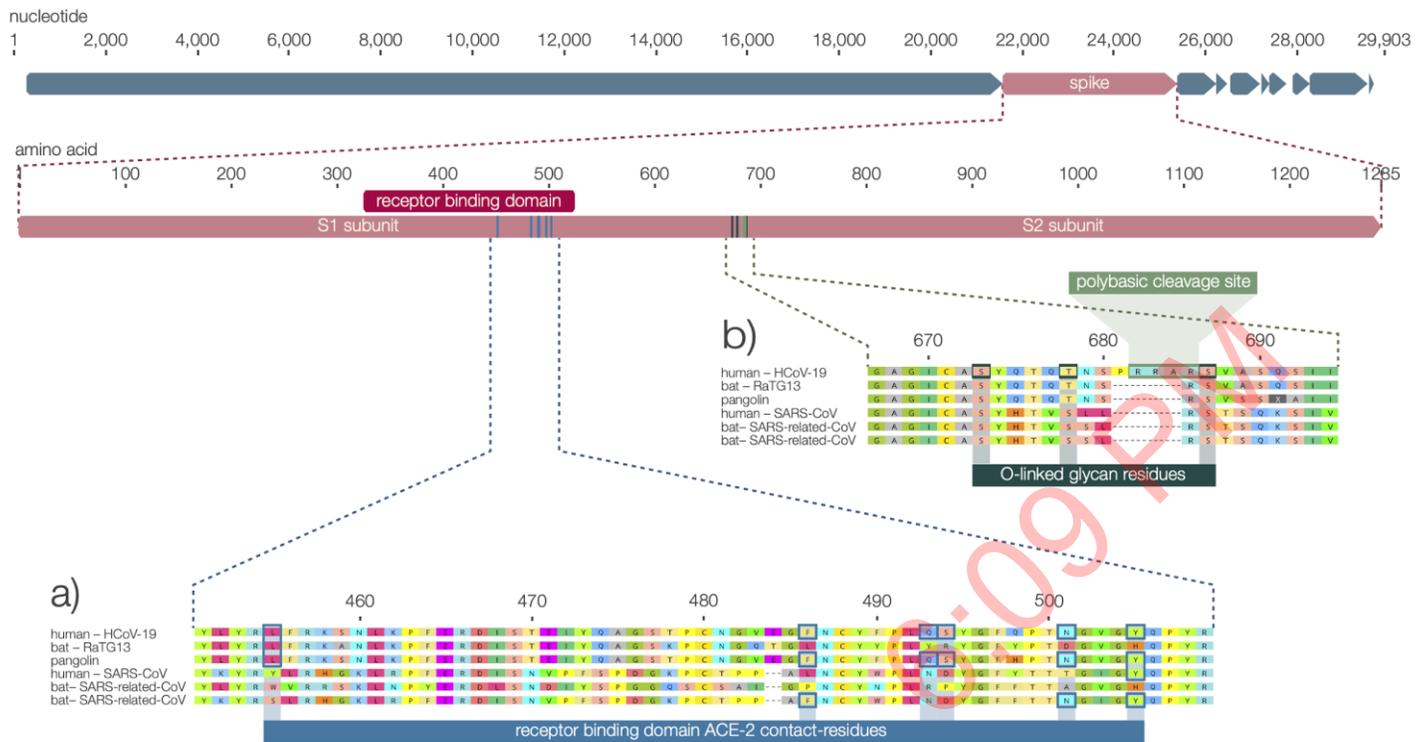
Figure Legends

Figure 1. (a) Mutations in contact residues of the HCoV-19 spike protein. The spike protein of HCoV-19 (top) was aligned against the most closely related SARS-like CoVs and SARS-CoV. Key residues in the spike protein that make contact to the ACE2 receptor are marked with blue boxes in both HCoV-19 and the SARS-CoV Urbani strain. **(b)** Acquisition of polybasic cleavage site and O-linked glycans. Both the polybasic cleavage site and the three adjacent predicted O-linked glycans are unique to HCoV-19 and not previously seen in lineage B betacoronaviruses. Sequences shown are from NCBI GenBank, accession numbers MN908947, MN996532, AY278741, KY417146 and MK211376. The pangolin coronavirus sequences are a consensus generated from SRR10168377 and SRR10168378 (NCBI BioProject PRJNA573298)[37,59].

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