BIBLIOGRAPHICAL ANNEX .. Link between the start of pandemic SARS-CoV/2 (COVID19) and the Huanan Seafood Wholesale Market in Wuhan (Hubei: China): the furin cleavage site of spike protein.

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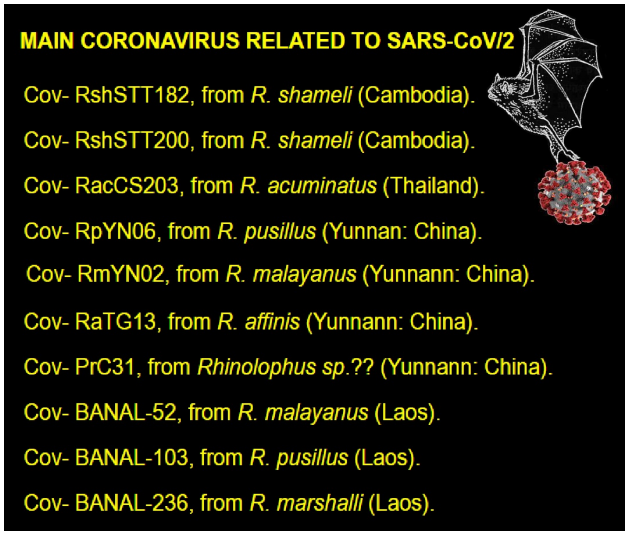

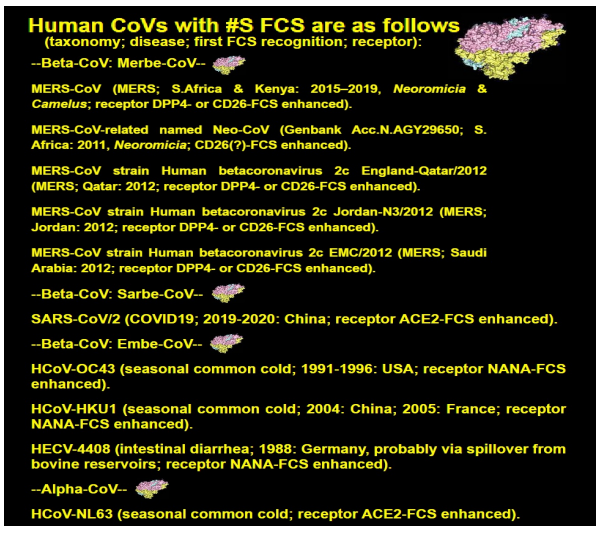

►► Structural Analysis of Neutralizing Epitopes of the SARS-CoV-2 Spike to Guide Therapy and Vaccine Design Strategies. Viruses 2021, DOI:10.3390/v13010134

Abstract

Coronavirus research has gained tremendous attention because of the COVID-19 pandemic, caused by the novel severe acute respiratory syndrome coronavirus (nCoV or SARS-CoV-2). In this review, we highlight recent studies that provide atomic-resolution structural details important for the development of monoclonal antibodies (mAbs) that can be used therapeutically and prophylactically and for vaccines against SARS-CoV-2. Structural studies with SARS-CoV-2 neutralizing mAbs have revealed a diverse set of binding modes on the spike's receptor-binding domain and N-terminal domain and highlight alternative targets on the spike. We consider this structural work together with mAb effects in vivo to suggest correlations between structure and clinical applications. We also place mAbs against severe acute respiratory syndrome (SARS) and Middle East respiratory syndrome (MERS) coronaviruses in the context of the SARS-CoV-2 spike to suggest features that may be desirable to design mAbs or vaccines capable of conferring broad protection.

Conclusions and Future Perspectives

The isolation of antibodies from convalescent individuals, together with their structural characterization through cryo-EM and X-ray crystallography, has accelerated the process of identifying features of SARS-CoV-2 S necessary for neutralization by mAbs and for eliciting such mAbs. Additional technologies, including the use of synthetic libraries, have helped identify neutralizing nanobodies that can be more rapidly generated against a desired antigen than those from llama or alpaca immunizations. It still remains to be determined how effective such nanobodies are in vivo. Moreover, humanized mouse platforms have shown promise in that S mAbs can be produced that bind in a similar way to those produced in humans. MAb REGN10933 represents such an example [68]. Further engineering of both nanobodies and mAbs to increase multivalency and/or improve effector functions may also improve therapies until the COVID-19 vaccine becomes widely accessible. In any case, a thorough understanding of critical residues for neutralization on various regions of SARS-CoV-2 S is crucial for vaccine design as well as for the selection of antibodies for passive administration.

Many of the mAbs produced against SARS-CoV-2 S have low rates of somatic hypermutations and use a diverse set of variable domain genes, demonstrating promise that similar mAbs can be produced rapidly via vaccination. Consistent with this, the BioNTech/Pfizer and Moderna vaccines, which produce different spike-related constructs, have shown to offer protection, even after the first of two doses

[120,121]. Nevertheless, longitudinal analyses of antibody responses are still necessary to determine the long-term efficacy of these vaccine-induced antibodies against any potential viral variants that may arise. The notion of conferring protection against SARS-CoV-2 mutants raises the question of the possibility of a universal coronavirus vaccine that protects against other betacoronaviruses. Additional analyses would need to be done to address this, and the development of a coronavirus vaccine is a critical first step. Due to sequence and structural differences between various coronavirus spikes, other vaccine design strategies would need to be employed to design a universal vaccine. It may be the case that seasonal vaccines, such as those for influenza, may become necessary until such a universal vaccine is available, should other coronaviruses spread as much as the current one.

In the absence of a safe, effective, and widely available vaccine, passive antibody administration is an appealing alternative, despite likely having short-term beneficial effects. Escape mutations are a concern with passive administration of antibodies [106], but mAb cocktails and multi-specific antibodies targeting multiple distinct regions on SARS-CoV-2 S may eliminate this concern and offer synergistic effects. Additionally, careful selection for strongly neutralizing mAbs, along with engineered mutations in the Fc region, may reduce the risk of ADE, if it were to occur in humans. Nevertheless, with any potential antibody treatment, rigorous testing in clinical trials must be pursued.

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- Spikes (#S) of SARS-CoV-2 (as MERS-CoV, SARS-CoV), is a trimeric class I fusion protein and can be divided into the receptor-binding #S1 and the membrane-anchored #S2 subunits; each S1 contains an N-terminal domain (NTD) and a receptor-binding domain (RBD or C-terminal domain CTD). RBD can be subdivided into a fairly conserved core region and a more variable receptor-binding motif (RBM). The RBM of SARS-CoV-2 interacts with the host cell receptor, angiotensin-converting enzyme 2 or ACE2. S2 subunit contains the fusion machinery of #S.

https://www.mdpi.com/viruses/viruses-13-00134/article_deploy/html/images/viruses-13-00134-g001.png

- #S can only bind ACE2 (or DPP4 in the case of MERS-CoV) when the RBD is in the up state.
- Both SARS-CoV-2 and SARS-CoV interact with the receptor (ACE2) in a similar manner; even though MERS-CoV #S binds DPP4, it does so with a similar approach angle as observed between #S from SARS-CoV and SARS-CoV-2 and ACE2.
- In addition to depending on ACE2 for host cell entry, both SARS-CoV and

SARS-CoV-2 depend on entry activation by host cell proteases at the S1/S2 and S2' sites, regardless of whether entry occurs by fusion or endocytosis. In contrast to SARS-CoV, SARS-CoV-2 #S (as MERS-CoV) also has a Furin Cleavage Site (FCS).

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►► Host cell entry of Middle East respiratory syndrome coronavirus after two-step, furin-mediated activation of the spike protein. Proc. Natl. Acad. Sci. USA. 2014 Oct 21 -- PMID:25288733. PMCID:PMC4210292 DOI: 10.1073/pnas.1407087111

Significance

The emergence of Middle East respiratory syndrome coronavirus (MERS-CoV), a deadly human coronavirus, has triggered considerable interest in the biomedical community. Similar to other enveloped viruses, coronaviruses access host cells by membrane fusion, a process mediated by specific fusion or “spike” proteins on the virion, often activated by cellular proteases.

 We have identified unique features of the MERS-CoV spike (S) protein cleavage activation. Our findings suggest that S can be activated by furin, a broadly expressed protease, by a two-step cleavage mechanism, occurring at distinct sites, with cleavage events temporally separated. Such furin-mediated activation is unusual in that it occurs in part during virus entry. Our findings may explain the polytropic nature, pathogenicity, and life cycle of this zoonotic coronavirus.

Abstract

Middle East respiratory syndrome coronavirus (MERS-CoV) is a newly identified betacoronavirus causing high morbidity and mortality in humans. The coronavirus spike (S) protein is the main determinant of viral entry, and although it was previously shown that MERS-CoV S can be activated by various proteases, the details of the mechanisms of proteolytic activation of fusion are still incompletely characterized. Here, we have uncovered distinctive characteristics of MERS-CoV S.

 We identify, by bioinformatics and peptide cleavage assays, two cleavage sites for furin, a ubiquitously expressed protease, which are located at the S1/S2 interface and at the S2' position of the S protein. We show that although the S1/S2 site is proteolytically processed by furin during protein biosynthesis, the S2' site is cleaved upon viral entry. MERS-CoV pseudovirion infection was shown to be enhanced by elevated levels of furin expression, and entry could be decreased by furin siRNA silencing. Enhanced furin activity appeared to partially override the low

pH-dependent nature of MERS-CoV entry. Inhibition of furin activity was shown to decrease MERS-CoV S-mediated entry, as well as infection by the virus.
 Overall, we show that MERS-CoV has evolved an unusual two-step furin activation for fusion, suggestive of a role during the process of emergence into the human population. The ability of MERS-CoV to use furin in this manner, along with other proteases, may explain the polytropic nature of the virus.
https://www.pnas.org/content/111/42/15214
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►► The Emergence of the Spike Furin Cleavage Site in SARS-CoV-2. Molecular Biology and Evolution, Nov.2021. DOI:10.1093/molbev/msab327.
Abstract
Compared with other SARS-related coronaviruses (SARSr-CoVs), SARS-CoV-2 possesses a unique furin cleavage site (FCS) in its spike. This has stimulated discussion pertaining to the origin of SARS-CoV-2 because the FCS has been observed to be under strong selective pressure in humans and confers the enhanced ability to infect some cell types and induce cell–cell fusion. Furthermore, scientists have demonstrated interest in studying novel cleavage sites by introducing them into SARSr-CoVs. We review what is known about the SARS-CoV-2 FCS in the context of its pathogenesis, origin, and how future wildlife coronavirus sampling may alter the interpretation of existing data.
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The Discovery and Characterization of the Unique S1/S2 FCS in SARS-CoV-2
 In comparison to all known SARSr-CoVs, SARS-CoV-2 possesses a unique four-residue P-R-R-A (681–684) insertion at its spike S1/S2 junction, producing an FCS. Although the SARS-CoV-2 FCS (P-R-R-A-R) may sometimes be described as “non-canonical” (it is not an R-R-X-R-R), it is highly functional and similar to FCSs found in other CoVs such as MERS (P-R-S-V-R, which is one R short compared with that of SARS-CoV-2).
The SARS-CoV-2 S1/S2 FCS was identified in January and early February by Li et al. (2020) and Coutard et al. (2020) respectively. Li et al. (2020) claimed to be the first to report the FCS on January 21, 2020, and postulated that the “cleavage site may increase the efficiency of virus infection into cells, making 2019-nCoV has significantly stronger transmissibility than SARS coronavirus”. Coutard et al. (2020) suggested that the novel FCS could have “significant functional implications for virus entry”. Another group, Walls et al. observed in their pseudovirion production that, although the SARS-CoV spike remained largely uncleaved at the S1/S2 junction, the SARS-CoV-2 spike was found to have near-complete S1/S2

cleavage; they similarly hypothesized that the FCS could “expand its tropism and/or enhance its transmissibility, compared with SARS-CoV and SARSr-CoV isolates, due to the near-ubiquitous distribution of furin-like proteases and their reported effects on other viruses” (Walls et al. 2020).

 It was a straightforward deduction for independent groups of scientists that an S1/S2 FCS could confer functional advantages to a SARSr-CoV.

https://doi.org/10.1093/molbev/msab327

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►► The furin cleavage site in the SARS-CoV-2 spike protein is required for transmission in ferrets. Nat. Microbiol. Vol.6, 2021 -- DOI:10.1038/s41564-021-00908-w.

Abstract

SARS-CoV-2 entry requires sequential cleavage of the spike glycoprotein at the S1/S2 and the S2′ cleavage sites to mediate membrane fusion. SARS-CoV-2 has a polybasic insertion (PRRAR) at the S1/S2 cleavage site that can be cleaved by furin. Using lentiviral pseudotypes and a cell-culture-adapted SARS-CoV-2 virus with an S1/S2 deletion, we show that the polybasic insertion endows SARS-CoV-2 with a selective advantage in lung cells and primary human airway epithelial cells, but impairs replication in Vero E6, a cell line used for passaging SARS-CoV-2. Using engineered spike variants and live virus competition assays and by measuring growth kinetics, we find that the selective advantage in lung and primary human airway epithelial cells depends on the expression of the cell surface protease TMPRSS2, which enables endosome-independent virus entry by a route that avoids antiviral IFITM proteins.

 SARS-CoV-2 virus lacking the S1/S2 furin cleavage site was shed to lower titres from infected ferrets and was not transmitted to cohoused sentinel animals, unlike wild-type virus.

 Analysis of 100,000 SARS-CoV-2 sequences derived from patients and 24 human postmortem tissues showed low frequencies of naturally occurring mutants that harbour deletions at the polybasic site. Taken together, our findings reveal that the furin cleavage site is an important determinant of SARS-CoV-2 transmission.

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Our study confirms TMPRSS2 as a potential drug target. Whilst inhibition of TMPRSS2 protease activity would not prevent infection via the endosome, using this pathway is detrimental to virus replication in airway cells. We have shown in this study that the protease inhibitor camostat is highly efficient at blocking SARS-CoV-2 replication in human airway cells and we note that clinical trials are ongoing

(ClinicalTrials.gov Identifier: NCT04455815). Our study also confirms the limitations of relying on Vero E6 cells as a system for developing classes of drugs such as entry inhibitors as they do not accurately reflect the preferred entry mechanism of SARS-CoV-2 into human airway cells51,52. Indeed, the data here explain why chloroquine is ineffective in clinic against SARS-CoV-2 (ref. 51), since during replication in the human airway WT SARS-CoV-2 has evolved to enter cells without the need for endosomal acidification.

 Presence of a furin CS at the S1/S2 junction is not uncommon in human coronaviruses; while half of human seasonal coronaviruses as well as MERS-CoV contain furin CSs, the remaining strains and SARS-CoV do not6,16. Thus, furin-mediated cleavage of spike is not an absolute requirement for efficient human respiratory transmission. Monitoring animal coronaviruses will probably be important in predicting and preventing future pandemics.

We suggest that gain of a furin CS in the wider SARS-related coronaviruses is a cause for concern. The polybasic insertion to the S1/S2 CS provides a significant fitness advantage in TMPRSS2-expressing cells and is probably essential for efficient human transmission. We also note that the SARS-CoV-2 CS remains suboptimal for furin cleavage.

 It is unclear if this is a trade-off (that is, with stability of spike) or whether further optimization of this site could result in higher transmissibility. In this regard, multiple SARS-CoV-2 variants have recently emerged and spread rapidly, including some, such as the B.1.1.7 'UK' variant, that have mutations proximal to the S1/S2 CS predicted to enhance furin cleavage. This further emphasizes the role of this site for virus transmission and the importance of continued monitoring as SARS-CoV-2 circulates in the human population53.

https://doi.org/10.1038/s41564-021-00908-w

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►► In vitro and computational analysis of the putative furin cleavage site (RRARS) in the divergent spike protein of the rodent coronavirus AcCoV-JC34 (sub-genus luchacovirus). BioRxiv preprint vers.Dec.20 2021. -- DOI:10.1101/2021.12.16.473025

Abstract

The Coronaviridae is a highly diverse virus family, with reservoir hosts in a variety of wildlife species that encompass bats, birds and small mammals, including rodents. Within the taxonomic group alphacoronavirus, certain sub-genera (including the luchacoviruses) have phylogenetically distinct spike proteins, which remain essentially uncharacterized. Using in vitro and computational techniques,

we analyzed the spike protein of the rodent coronavirus AcCoV-JC34 from the subgenus luchacovirus, previously identified in Apodemus chevrieri (Chevrier's field mouse).

We show that AcCoV-JC34—unlike the other luchacoviruses—has a putative furin cleavage site (FCS) within its spike S1 domain, close to the S1/S2 interface. The pattern of basic amino acids within the AcCoV-JC34 FCS (-RR-R-) is identical to that found in "pre-variant" SARS-CoV-2—which is in itself atypical for an FCS, and suboptimal for furin cleavage. Our analysis shows that, while containing an -RR-R-motif, the AcCoV-JC34 spike "FCS" is not cleaved by furin (unlike for SARS-CoV-2), suggesting the possible presence of a progenitor sequence for viral emergence from a distinct wildlife host.

Discussion

The "furin cleavage site" or FCS of SARS-CoV-2 has been at the center of the many discussions on the origin of the COVID-19 pandemic; see [11] for a recent summary. Despite being interpreted as "highly unusual", an FCS is—to the contrary—very common among the Coronaviridae [12], with sarbecoviruses and most alphacoronaviruses being the exception rather than the rule in lacking this important regulatory sequence.

In fact, many zoonotic coronaviruses and those in reservoir hosts appear to contain sequences and structural loops at the S1/S2 interface that are subthreshold for furin-mediated cleavage [13-16] and may be "poised" for spillover events.

Examples include "pre-variant" SARS-CoV-2, as well as the sarbecoviruses RmYN02, RacCS203, BANAL-20-116, BANAL-20-246 that have potential phylogenetic homology to the SARS-CoV-2 FCS [17]—and may include the luchacovirus AcCoV-JC34 analyzed here. It is noteworthy that AcCoV-JC34 is the only luchacovirus containing this -R-RR-motif.

While containing an -RR-R-motif, as found in SARS-CoV-2, the data presented here show that this AcCoV-JC34 sequence is not cleaved by furin. The reasons for this are currently unclear. One possibility is that the upstream proline found in SARS-CoV-2, as well as in other spike cleavage site sequences, may promote cleavage by creating a structural turn beneficial for furin activity. It is also possible that the additional downstream arginine residue in AcCoV-JC34 spike may be inhibitory for the tight active site binding pocket present in furin [18]. Alternatively, the structural loop present in AcCoV-JC34 spike may be cleaved by other proprotein convertases of the furin family that have less stringent cleavage requirements, or by trypsin-like enzymes or cathepsins. Notably, the -RR-R-motif is rare in furin substrates, and only other known example of this sequence motif in FurinDB (a database of furin substrates) is found in proaerolysin, a bacterial toxin

[19].

One notable aspect of the -RR-R-motif in AcCoV-JC34 is that is does not align precisely with the S1/S2 motif of most coronavirus spikes (see Figure 3) and is a structurally exposed location above the typical S1/S2 loop (see Figure 4). Analysis of the MERS-CoV spike also shows an addition putative FCS in the MERS-CoV spike (SRSTRS); while this contains a minimal furin motif this sequence shows low scores for furin cleavage with both Pitou and ProP, and FRET-based peptides were not cleaved by furin in biochemical cleavage assays—in contrast to the PRSVRS motif at the expected S1/S2 junction (J. K. Millet, unpublished results). Nevertheless, it is possible that, as with AcCoV-Jc34, this “secondary” MERS-CoV sequence comprises a “blocked” FCS due to flanking hydrophobic and charges residues in the downstream C-terminal positions (i.e., SRSTRSMLKRRDS). This putative secondary cleavage site also lacks an upstream proline/proline-rich region, as with many other S1/S2 regions that are known to be cleaved by furin.

For SARS-CoV-2, it is clear that selection is occurring to up-regulate the spike FCS, as seen with several of the highly transmissible variants that have emerged [20-24]. The FCS can also be readily down regulated upon Vero cell adaptation; for examples see refs [25, 26]. Likewise, some coronaviruses in animal reservoirs may be “poised” for proteolytic cleavage-activation at S1/S2, with selection occurring along with modifications to their receptor binding domain.

- One interesting example of this may exemplified by the MERS-like bat-CoVs HKU-4 and HKU-5, with HKU-4 binding human DPP4, but having no identifiable FCS, and with HKU-5 not able to bind hDPP4 and having a robust FCS [27].
- Our studies highlight the possible presence of a distinct proteolytic cleavage loop in the coronavirus spike protein and the specific features of the luchacovirus spike—which along with that found in the rhinacoviruses (e.g., SADS-CoV) appears to represent an evolutionary disparate spike protein with apparent similarities to a betacoronavirus spike protein (see Figure 1), despite the taxonomic designation of these viruses as alphacoronaviruses.

https://doi.org/10.1101/2021.12.16.473025

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►► SARS-CoV-2 and MERS-CoV Share the Furin Site CGG-CGG Genetic Footprint. -- Preprints 2021, 2021100080 DOI:10.20944/preprints202110.0080.v2.

Abstract

The SARS-CoV-2 polybasic furin cleavage site is still a missing link.

- Remarkably, the two arginine residues of this protease recognition site are encoded by the CGG codon, which is rare in Betacoronavirus.

 However, the arginine pair is common at viral furin cleavage sites, but are not CGG-CGG encoded.
 The question is: Is this genetic footprint unique to the SARS-CoV-2?
To address the issue, using Perl scripts, here I dissect in detail the NCBI Virus database in order to report the arginine dimers of the Betacoronavirus proteins.
 The main result reveals that a group of Middle East respiratory syndrome-related coronavirus (MERS-CoV) (isolates: camel/Nigeria/NVx/2016, host: Camelus dromedarius) also have the CGG-CGG arginine pair in the spike protein polybasic furin cleavage region.
 In addition, CGG-CGG encoded arginine pairs were found in the orf1ab polyprotein from HKU9 and HKU14 Betacoronavirus, as well as, in the nucleocapsid phosphoprotein from few SARS-CoV-2 isolates. To quantify the probability of finding the arginine CGG-CGG codon pair in Betacoronavirus, the likelihood ratio (LR) and a Markov model were defined.
 In conclusion, it is highly unlikely to find this genetic marker in betacoronaviruses wildlife, but they are there.
 Collectively, results shed light on recombination as origin of the virus CGG-CGG arginine pair in the S1/S2 cleavage site.
https://www.preprints.org/manuscript/202110.0080/v2
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►► Furin cleavage sites naturally occur in coronaviruses. Stem Cell Research, Vol.50, Jan.2021. DOI:10.1016/j.scr.2020.102115.
Highlights.
•Phylogenetic tree of spike proteins reveals major groups of coronaviruses.
 •Furin cleavage sites at spike S1/S2 are common in coronaviruses.
 •Furin cleavage sites at spike S1/S2 naturally occurred independently for multiple times in coronaviruses.
https://ars.els-cdn.com/content/image/1-s2.0-S1873506120304165-ga1_lrg.jpg
Abstract.
The spike protein is a focused target of COVID-19, a pandemic caused by SARS-CoV-2. A 12-nt insertion at S1/S2 in the spike coding sequence yields a furin cleavage site, which raised controversy views on origin of the virus. Here we analyzed the phylogenetic relationships of coronavirus spike proteins and mapped furin recognition motif on the tree.
 Furin cleavage sites occurred independently for multiple times in the evolution of the coronavirus family, supporting the natural occurring hypothesis of SARS-CoV-2.

- Furin cleavage sites are common in Betacoronavirus.
- Our mapping results showed that the furin recognition motif is more common in Merbecovirus and Embecovirus (Figs. 4 and S2, S3).
- In Merbecovirus, furin sites at spike S1/S2 occur in three clades:
- MERS-CoV strains,
- the bat coronavirus HKU5 strains, and
- Coronavirus Neoromicia/PML-PHE1/RSA/2011 with its relatives (Figs. 4A and S2).
- Besides, MERS-CoV and bat coronavirus HKU5 are the only clades in Merbecovirus having furin cleavage site at S2'.

In Embecovirus, furin recognition motif at spike S1/S2 is universal: All strains but a few exceptions have furin cleavage sites at spike S1/S2 (Figs. 4B and S3). Interestingly, the Longquan Aa mouse coronavirus (Wang et al., 2015) loses this furin site, while its close relatives (e.g. China Rattus coronavirus HKU24, sequence identity 96.0%) maintains the furin cleavage site. This provides an example of naturally occurred sequence variation at spike S1/S2 among closely related coronaviruses. Besides, for spike S2', only several single strains have furin recognition motif (Fig. S3).

2.4. Furin cleavage sites also occur in other genera of coronavirus

- Our mapping results showed furin cleavage sites are widely present in the whole coronavirus family (Fig. 5). For spike S1/S2, furin recognition motif is universal in Gammacoronavirus, and also occurs in two clades of Alphacorovanvirus: feline coronavirus and relatives, and Chevrier's field mouse coronavirus. For spike S2', furin recognition motif occurs in several independent clades, covering all the three genera.
- Notably, in the two human coronaviruses in Alphacoronavirus causing common cold, HCoV NL63 has furin cleavage site at spike S2', while the HCoV 229E (protein sequence identity 63.8%) lacks such feature.
- Furin cleavages sites occurred independently for six times in Betacoronavirus.

The alignment of linking regions of spike S1 and S2 domains in representative Betacoronavirus (Fig. 6A) shows this region is less conserved than the neighboring folded S1 and S2 segments. Within a subgenus the sequences are well aligned, but among subgenera the similarity is low.

- The furin cleavage site of SARS-CoV-2 spike S1/S2 is formed by a insertion of PRRA in comparison to other Sarbecovirus including close relative RaTG13, showing it occurred very recently and independently. Similarly, Hipposideros bat coronavirus (Zhejiang 2013) in Hibecovirus has furin site of independent origin, though the occurring time is hard to decide for in this subgenera only two sequences were published.

Merbecovirus and Embecovirus both have multiple coronavirus species with furin cleavage sites at spike S1/S2, but their situations are different: In Merbecovirus, furin cleavage sites prevail in three non-sister clades (Figs. 4A and 6A). Moreover, the positions of furin recognition motifs in the linking regions are unique to each clade, as exhibited in alignments of both protein sequences (Fig. 6A) and nucleotide sequences (Fig. S4A). These indicated for of the three clades in Merbecovirus, furin cleavage sites have an independent origin. In Embecovirus, to the contrast, all the furin cleavage sites are variations based on a 5-residue region with consensus sequence RRXRR. The region is well aligned in both protein and nucleotide sequences (Figs. 6A and S4B). This suggested the furin cleavage sites of Embecovirus share a common ancestor.

In addition, in Alphacoronavirus and Gammacoronavirus, S1/S2 cleavage sites reside at a different loop comparing to the site in Betacoronavirus (Fig. 6B), therefore furin cleavage sites at spike S1/S2 in these two genera occurred independently from those in Betacoronavirus in evolution.

Discussion.

Furin cleavage is critical to many viral diseases, including HIV, Ebola, and influenza H5 and H7 (Becker et al., 2012). Furin is a ubiquitously expressed protease. In human body, it has a wider distribution range than the major protease responsible for cleaving spike, TMPRSS2 (Fig. S5). Therefore, coronaviruses with spike containing furin cleavage site may have advantage in spreading. Deletions of furin cleavage site in SARS-CoV-2 attenuates replication on respiratory cells (Johnson et al., 2020) and pathogenesis in hamster (Johnson et al., 2020, Lau et al., 2020). Furin inhibitors suppress virus production and cytopathic effects in kidney cells (Cheng et al., 2020). Natural polymorphisms losing furin recognition motif in SARS-CoV-2 spike S1/S2 are observed, but very rare (Xing et al., 2020).

Variations in this region are more common in viruses cultured in vitro than viruses isolated from clinical samples, suggesting this cleavage site is under selection pressure in human body (Lau et al., 2020, Liu et al., 2020).

Our analysis exhibits furin cleavage sites at spike S1/S2 occurred independently for several times in coronavirus.

Consequently, natural occurring of the site in SARS-CoV-2 is highly possible. This is further supported by other observed natural variations at the linking region of S1 and S2: A natural insertion in SARS-CoV spike though not related to furin recognition motif was reported (Zhou et al., 2020). In Embecovirus; Longquan Aa mouse coronavirus (Wang et al., 2015) has a frameshift mutation led to the loss of furin recognition motif (Fig. S4B); Some strains of murine coronavirus lose furin recognition motif through substitution mutations (Fig. S3), e.g. in MHV-2 (Yamada et al., 1997). Further study of losing the furin cleavage site in Embecovirus would

help to interpret the S1/S2 cleavage of Betacoronaviruses. Besides, independent occurrences of furin cleavage sites in surface glycoproteins are not unique to coronavirus: for the hemagglutinin of influenza, only H5 and H7 have furin cleavage sites (Bottcher-Friebertshauser et al., 2013); and these subtypes are distant in phylogenetic tree (Fig. S6).

Conclusion.

Furin cleavage sites in spike proteins naturally occurred independently for multiple times in coronaviruses. Such feature of SARS-CoV-2 spike protein is not necessarily a product of manual intervention, though our observation does not rule out the lab-engineered scenario.

https://doi.org/10.1016/j.scr.2020.102115

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►► Global Diversification and Distribution of Coronaviruses With Furin Cleavage Sites. Front. Microbiol., 2021, DOI:10.3389/fmicb.2021.649314.

ABSTRACT.

 Knowledge about coronaviruses (CoVs) with furin cleavage sites is extremely limited, although these sites mediate the hydrolysis of glycoproteins in plasma membranes required for MERS-CoV or SARS-CoV-2 to enter cells and infect humans.

 Thus, we have examined the global epidemiology and evolutionary history of SARS-CoV-2 and 248 other CoVs with 86 diversified furin cleavage sites that have been detected in 24 animal hosts in 28 countries since 1954.

 Besides MERS-CoV and SARS-CoV-2, two of five other CoVs known to infect humans (HCoV-OC43 and HCoV-HKU1) also have furin cleavage sites.

 In addition, human enteric coronavirus (HECV-4408) has a furin cleavage site and has been detected in humans (first in Germany in 1988), probably via spillover events from bovine sources.

 In conclusion, the presence of furin cleavage sites might explain the polytropic nature of SARS-CoV-2- and SARS-CoV-2-like CoVs, which would be helpful for ending the COVID-19 pandemic and preventing outbreaks of novel CoVs.

 IBV CoV, FCS: GTRRSRR↓SI, from Gallus (United States, 1954).

 IBV CoV, GTRRSRR↓SI & GTRRSRR↓SV, from Gallus (North America, 1954-1964).

 IBV CoV, GTRRFRR↓SI, from Gallus (USA, 1972).

 IBV CoV, GVHRSRR↓SI, from Gallus (USA, 1976).

 IBV CoV, GTRRSRR↓SV, from Gallus (USA, Belgium, China, 1976-1981).

 NIBV CoV, STRRSRR↓SV, from Gallus (Belgium, 1984).

- IBV CoV, SSHRSRR↓ST, from Gallus (China Guangxiwas, 1985).
- ECV-4408 CoV, TKRRSRR↓AI, from Human (Germany, 1988).
- CoV-OC43 CoV, KNRRSRR↓AI, from Human (USA, 1991-1996).
- amba deer CoV, FCS, from samba deer genera Ruse (USA, 1993-1994).
- amma-CoVs, 11 FCS, FROM Gallus, Meleagris (China, S.Korea, Italy, Poland, USA, 1987–1997).
- amma-CoVs, FCS, from avians (Australia, China, India, South Korea, France, Poland, USA, 1998–2008).
- CoV, HSRRSRR↓ST, from Felis (USA, 1998).
- CoV, 11 FCS, from Felis (Netherlands, 2007-2008).
- eta-CoVs, FCS, from Human, Oryctolagus, Sus, Bos (various countries, 1998–2008).
- CoV-HKU1, SSRRKRR↓SI, from Human (China, 2004; France, 2005).
- amma-CoV, FCS, (Australia, Brazil, China, Egypt, Italy, Malaysia, Poland, S.Africa, S.Korea, Sudan, USA, Ukraine, Uruguay, 2009–2014).
- lpha-CoVs, FCS, (Belgium, 2013).
- eta-CoVs, FCS, from Bubalus, Rattus, Canis, Bos, Camelus, Mus musculus, Humans (2009–2014).
- eta-CoVs, FCS, from Bats (Myotis, Hipposideros, Pipistrellus: China; Neoromicia: S.Africa; 2009–2014).
- amma-CoVs, FCS, from Gallus (wide diffusion, 2015–2019), Branta (Canada, 2017).
- lpha-CoVs, 4 FCS, from Felis (Brazil, Belgium, Denmark, 2015; China, 2016-2018).
- 11 Beta-CoVs, FCS, from different species (wide diffusion, 2015–2019).
- ERS-CoVs, FCS, from Neoromicia (South Africa, 2015–2019).
- ERS-CoVs, FCS, from Camelus (Kenya, UAE, 2015–2019).
- ERS-CoVs-like named “Neo-CoV”, FCS (Genbank Acc.N.AGY29650), from Neoromicia (in South Africa, 2011).
- ARS-CoV-2, NSPRRAR↓SV, from Human (Pandemic, 2019).

Conclusion

Thorough structural understanding of SARS-CoV-2 is crucial to control the global outbreak of the virus and prevent outbreaks of related viruses. However, the furin cleavage site’s role did not receive sustained attention following the discovery of coronaviruses until the COVID-19 outbreak in 2019.

- ur results show that 86 types of furin cleavage sites have been detected in strains of three coronavirus genera detected in 24 animal hosts in 28 countries since 1954, including at least 25 types in Beta-CoVs recorded in the years 1988–2019 in 14 countries (Vietnam, Bangladesh, China, South Korea, Saudi Arabia, the

United Arab Emirates, Cote d'Ivoire, Uganda, Kenya, South Africa, France, Germany, the Netherlands, and United States).

- Most of them could cause unexpected threats to human beings or other mammals. Four of seven CoVs known to infect humans carry furin cleavage sites, including two with low pathogenicity (HCoV-OC43 and HCoV-HKU1) and two highly pathogenic zoonotic viruses (MERS-CoV and SARS-CoV-2).
- Moreover, evidence of frequent interchange of furin cleavage site motifs among the three coronavirus genera indicates that frequencies of recombination of CoVs' furin cleavage sites may have been underestimated (Supplementary Figure 1). The presence of furin cleavage sites associated with changes in pathogenicity might also explain the polytropic nature of SARS-CoV-2 and SARS-CoV-2-like CoVs.

Perspectives

- The last outbreak of a human coronaviruses with a furin cleavage site before the current pandemic was the MERS-CoV outbreak in 2014. At the end of January 2020, over 2,500 laboratory-confirmed cases of MERS with more than 800 deaths (case-fatality rate: 34.3%) were reported worldwide (WHO, 2020).

The reported cases of MERS were mainly in Saudi Arabia, and the outbreak did not attract global attention due to its small spread compared with the current COVID-19 pandemic. The latter poses massive global challenges, numbers of deaths due to the novel virus are still increasing, and the end of the pandemic is still unpredictable.

The origin of the novel coronavirus has also been strongly debated, and tracing SARS-CoV-2's source is important for controlling its spread. In early stages of the outbreak, some people argued that the novel coronavirus with a furin cleavage site was an artificial virus.

- However, previous findings (Wu and Zhao, 2020) and our results show that CoVs with furin cleavage sites have existed since at least 1954. The global host ranges and geographical distributions of these viruses and their history of at least 60 years show that diversified furin cleavage sites do not have synthetic origins and might provide CoVs multiple pathways to infect human beings or other animals. Besides transmission from animals to humans, SARS-CoV-2 can also spread through cold food supply chains (Zhou and Shi, 2021).

Recently, genetic variants of SARS-CoV-2 have been emerging worldwide. Up to June 2021, several variants of concern for which there is evidence of an increase in transmissibility and reduced effectiveness of treatments or vaccines have been reported. These include B.1.1.7 (alpha variant), B.1.351 (beta variant), P.1 (gamma variant), and B.1.617.2 (delta variant), first identified in the United Kingdom, South Africa, Japan/Brazil, and India, respectively (CDC, 2021; Saito et al., 2021).

Mutations of concern include the P681R mutation in the S protein of B.1.617.2, close to the furin cleavage site, which may increase the rate of S1/S2 cleavage and enhance viral fusion (Cherian et al., 2021; Saito et al., 2021). Fortunately, a reverse genetic system for SARS-CoV-2 has been developed to generate mutants of the virus, which could be used to examine effects of the furin cleavage site's deletion on virus replication and facilitate analyses of the replication and pathogenicity of the virus (Xie et al., 2020; Johnson et al., 2021).

The evolution of multiple CoVs with furin cleavage sites during the last 60 years clearly highlights the need to understand roles of the site and other functional elements of SARS-CoV-2 in order to identify therapeutic targets and facilitate vaccine development. Global collaborative efforts are needed to meet these goals and help efforts to prevent further spread of SARS-CoV-2 and improve therapeutic interventions. To aid such efforts, we make the following suggestions.

1. Vaccination is the first option to counter the COVID-19 pandemic. Evidence indicated that vaccines can reduce the risk of household transmission by 40–50% (Harris et al., 2021). Vaccine development is accelerating all over the world, but there are urgent needs for more rapid production of effective COVID-19 vaccines and therapeutic agents. Moreover, even a highly effective vaccination program may not be sufficient to end the COVID-19 epidemic. People must remain vigilant and equitable distribution of vaccines around the world is crucial.

2. It is important to identify potential intermediate hosts. Intermediate hosts of SARS-CoV and MERS-CoV are palm civet (Paradoxurus hermaphroditus) and camels (C. dromedarius), respectively, but the intermediate host of SARS-CoV-2 is still unknown. The virus can reportedly be transmitted from animals to humans, so discovery of potential intermediate hosts is essential for cutting the transmission between animals and humans via timely and effective intervention. In addition, transmission via cold food supply chains cannot be neglected as SARS-CoV-2 can survive on surfaces of cold food packages up to 3 weeks (Zhou and Shi, 2021). So, countries should take urgent measures to control the spread of SARS-CoV-2 through supply chains. It also helps to prevent this pandemic from further deteriorating and could decrease loss of life and property.

 3. Genetic recombination of both DNA and RNA viruses is a common phenomenon. The ability of SARS-COV-2- and SARS-COV-2-like CoVs to mutate may have been vastly underestimated, and mutations affect strains' lethality. Thus, there are urgent needs to comprehensively clarify the pathogenic mechanism of SARS-COV-2, which is poorly understood at present.

4. To prevent or control the future spread of novel CoVs, global efforts are needed to construct a comprehensive global CoV database and timely warning system by collecting samples from humans and potential animal hosts (including bat species,

which are both potentially natural hosts of CoVs and globally distributed). https://doi.org/10.3389/fmicb.2021.649314

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►► A metagenomic viral discovery approach identifies potential zoonotic and novel mammalian viruses in Neoromicia bats within South Africa. PLOS, Mar.26, 2018. DOI:10.1371/journal.pone.0194527.

Abstract

Species within the Neoromicia bat genus are abundant and widely distributed in Africa. It is common for these insectivorous bats to roost in anthropogenic structures in urban regions. Additionally, Neoromicia capensis have previously been identified as potential hosts for Middle East respiratory syndrome (MERS)-related coronaviruses. This study aimed to ascertain the gastrointestinal virome of these bats, as viruses excreted in fecal material or which may be replicating in rectal or intestinal tissues have the greatest opportunities of coming into contact with other hosts. Samples were collected in five regions of South Africa over eight years. Initial virome composition was determined by viral metagenomic sequencing by pooling samples and enriching for viral particles. Libraries were sequenced on the Illumina MiSeq and NextSeq500 platforms, producing a combined 37 million reads. Bioinformatics analysis of the high throughput sequencing data detected the full genome of a novel species of the Circoviridae family, and also identified sequence data from the Adenoviridae, Coronaviridae, Herpesviridae, Parvoviridae, Papillomaviridae, Phenuiviridae, and Picornaviridae families. Metagenomic sequencing data was insufficient to determine the viral diversity of certain families due to the fragmented coverage of genomes and lack of suitable sequencing depth, as some viruses were detected from the analysis of reads-data only. Follow up conventional PCR assays targeting conserved gene regions for the Adenoviridae, Coronaviridae, and Herpesviridae families were used to confirm metagenomic data and generate additional sequences to determine genetic diversity. The complete coding genome of a MERS-related coronavirus was recovered with additional amplicon sequencing on the MiSeq platform. The new genome shared 97.2% overall nucleotide identity to a previous Neoromicia-associated MERS-related virus, also from South Africa. Conventional PCR analysis detected diverse adenovirus and herpesvirus sequences that were widespread throughout Neoromicia populations in South Africa. Furthermore, similar adenovirus sequences were detected within these populations throughout several years. With the exception of the coronaviruses, the study represents the first report of sequence data from several viral families within a Southern African

insectivorous bat genus; highlighting the need for continued investigations in this regard.

Conclusions

Multi-pathogen surveillance approaches are integral to pathogen discovery programs that aim to identify potential public health risks, and also increase our knowledge of virus diversity and evolution [82]. The sequence-independent manner utilized by metagenomic high throughput sequencing methodologies enables detection of both known and unknown viral species that may not have been detected with conventional nucleic acid methods. Marked limitations of the metagenomic approach implemented here were highlighted by inadequate sequencing of several viral families, which could subsequently be detected with conventional PCR assays; such as the adenoviruses, herpesviruses and coronaviruses. In spite of the lack of coronavirus contigs produced from the metagenomic data, the complete coding genome of a MERS-related betacoronavirus was still recovered with additional amplicon sequencing. Since the experimental portion of the study was conducted, alternative methods have been suggested that may reduce these biases and improve sequencing results from nonclinical samples with low viral nucleic acid concentrations–such as utilization of the ScriptSeq library kit (Illumina) directly after extraction of RNA [66].

Despite limitations with the metagenomic sequencing output, the study identified a novel Cyclovirus species, confirmed MERS-related virus circulation within this host genus, detected diverse adenoviruses and herpesviruses which are widespread among Neoromicia populations in South Africa, and determined that adenoviruses seemingly persist within these populations throughout several years. Follow up longitudinal studies can be implemented to confirm this finding and establish the total duration of the viral persistence. The Neoromicia adenovirus sequences shared high similarity to those identified in European bats, whereas the Neoromicia herpesviruses were much more diverse than previously identified bat-associated viruses. This observation may reflect differences in sampling efforts applied to each viral family. Further investigation of the identified viral families are required to sequence complete genes involved in receptor recognition and attachment to host cells. In the absence of viral isolates, this would allow functional assessment of the receptors utilized for cell entry, enable estimations of their potential to spread to new species, and assess the risks they pose to public or veterinary health. Lastly, the novel sequence data generated from the Neoromicia virome can be utilized in assay development for additional nucleic acid detection surveillance activities, to determine the prevalence rates of selected novel viruses.

At present, metagenomic high throughput sequencing may be unsuited for routine viral surveillance practices, as it may be restrictive in terms of sensitivity, incapable

of detecting of the complete viral diversity, slow in turn-over time due to extensive bioinformatics data analysis or limited as a result of the high cost of large sequencing volumes. Future improvements to sample preparation and data analysis techniques would be invaluable, and enable these methodologies to be used routinely in strategies for pathogen discovery programs, with the ultimate goal of being aware of high-risk viral species that may be present in wildlife populations.
https://doi.org/10.1371/journal.pone.0194527
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►► Covid-19: Do many people have pre-existing immunity? BMJ 2020. bmj, Sept.17, 2020. DOI:https://doi.org/10.1136/bmj.m3563.
It seemed a truth universally acknowledged that the human population had no pre-existing immunity to SARS-CoV-2, but is that actually the case? Peter Doshi explores the emerging research on immunological responses
Even in local areas that have experienced some of the greatest rises in excess deaths during the covid-19 pandemic, serological surveys since the peak indicate that at most only around a fifth of people have antibodies to SARS-CoV-2: 23% in New York, 18% in London, 11% in Madrid.123 Among the general population the numbers are substantially lower, with many national surveys reporting in single digits.
With public health responses around the world predicated on the assumption that the virus entered the human population with no pre-existing immunity before the pandemic,4 serosurvey data are leading many to conclude that the virus has, as Mike Ryan, WHO's head of emergencies, put it, "a long way to burn."
Yet a stream of studies that have documented SARS-CoV-2 reactive T cells in people without exposure to the virus are raising questions about just how new the pandemic virus really is, with many implications.
Not so novel coronavirus?
At least six studies have reported T cell reactivity against SARS-CoV-2 in 20% to 50% of people with no known exposure to the virus.5678910
- In a study of donor blood specimens obtained in the US between 2015 and 2018, 50% displayed various forms of T cell reactivity to SARS-CoV-2.511
- A similar study that used specimens from the Netherlands reported T cell reactivity in two of 10 people who had not been exposed to the virus.7
- In Germany reactive T cells were detected in a third of SARS-CoV-2 seronegative healthy donors (23 of 68).
- In Singapore a team analysed specimens taken from people with no contact or

personal history of SARS or covid-19; 12 of 26 specimens taken before July 2019 showed reactivity to SARS-CoV-2, as did seven of 11 from people who were seronegative against the virus.8

- Reactivity was also discovered in the UK and Sweden.6910

Though these studies are small and do not yet provide precise estimates of pre-existing immunological responses to SARS-CoV-2, they are hard to dismiss, with several being published in Cell and Nature. Alessandro Sette, an immunologist from La Jolla Institute for Immunology in California and an author of several of the studies (box 1), told The BMJ, “At this point there are a number of studies that are seeing this reactivity in different continents, different labs. As a scientist you know that is a hallmark of something that has a very strong footing.”

Box 1

Swine flu déjà vu

In late 2009, months after the World Health Organization declared the H1N1 “swine flu” virus to be a global pandemic, Alessandro Sette was part of a team working to explain why the so called “novel” virus did not seem to be causing more severe infections than seasonal flu.12

Their answer was pre-existing immunological responses in the adult population: B cells and, in particular, T cells, which “are known to blunt disease severity.”12 Other studies came to the same conclusion: people with pre-existing reactive T cells had less severe H1N1 disease.1314 In addition, a study carried out during the 2009 outbreak by the US Centers for Disease Control and Prevention reported that 33% of people over 60 years old had cross reactive antibodies to the 2009 H1N1 virus, leading the CDC to conclude that “some degree of pre-existing immunity” to the new H1N1 strains existed, especially among adults over age 60.15

The data forced a change in views at WHO and CDC, from an assumption before 2009 that most people “will have no immunity to the pandemic virus”16 to one that acknowledged that “the vulnerability of a population to a pandemic virus is related in part to the level of pre-existing immunity to the virus.”17 But by 2020 it seems that lesson had been forgotten.

Researchers are also confident that they have made solid inroads into ascertaining the origins of the immune responses. “Our hypothesis, of course, was that it’s so called ‘common cold’ coronaviruses, because they’re closely related,” said Daniela Weiskopf, senior author of a paper in Science that confirmed this hypothesis.18 “We have really shown that this is a true immune memory and it is derived in part from common cold viruses.” Separately, researchers in Singapore came to similar conclusions about the role of common cold coronaviruses but noted that some of the T cell reactivity may also come from other unknown coronaviruses, even of

animal origin.8

Taken together, this growing body of research documenting pre-existing immunological responses to SARS-CoV-2 may force pandemic planners to revisit some of their foundational assumptions about how to measure population susceptibility and monitor the extent of epidemic spread.

Population immunity: underestimated?

Seroprevalence surveys measuring antibodies have been the preferred method for gauging the proportion of people in a given population who have been infected by SARS-CoV-2 (and have some degree of immunity to it), with estimates of herd immunity thresholds providing a sense of where we are in this pandemic. Whether we overcome it through naturally derived immunity or vaccination, the sense is that it won't be over until we reach a level of herd immunity.

The fact that only a minority of people, even in the hardest hit areas, display antibodies against SARS-CoV-2 has led most planners to assume the pandemic is far from over. In New York City, where just over a fifth of people surveyed had antibodies, the health department concluded that "as this remains below herd immunity thresholds, monitoring, testing, and contact tracing remain essential public health strategies."19 "Whatever that number is, we're nowhere near close to it," said WHO's Ryan in late July, referring to the herd immunity threshold (box 2).

Box 2

Calculating the herd immunity threshold

In theory, outbreaks of contagious disease follow a certain trajectory. In a population that lacks immunity new infections grow rapidly. At some point an inflection in this growth should occur, and the incidence will begin to fall.

The 1970s gave rise to a theory that defined this inflection point as the herd immunity threshold (HIT) and offered a straightforward formula for estimating its size: $HIT=1-1/R0$ (where R0 is the disease's basic reproduction number, or the average number of secondary cases generated by an infectious individual among susceptible people). This simple calculation has guided—and continues to guide—many vaccination campaigns, often used to define target levels of vaccination.20

The formula rests on two assumptions: that, in a given population, immunity is distributed evenly and members mix at random. While vaccines may be deliverable in a near random fashion, from the earliest days questions were raised about the random mixing assumption. Apart from certain small closed populations such as "orphanages, boarding schools, or companies of military recruits," Fox and colleagues wrote in 1971,21 truly random mixing is the exception, not the rule. "We could hardly assume even a small town to be a single homogeneously mixing unit. Each individual is normally in close contact with only a small number of individuals, perhaps of the order of 10-50."

Nearly 50 years later, Gabriela Gomes, an infectious disease modeller at the University of Strathclyde, is reviving concerns that the theory's basic assumptions do not hold. Not only do people not mix randomly, infections (and subsequent immunity) do not happen randomly either, her team says. "More susceptible and more connected individuals have a higher propensity to be infected and thus are likely to become immune earlier. Due to this selective immunization by natural infection, heterogeneous populations require less infections to cross their herd immunity threshold," they wrote.22 While most experts have taken the R0 for SARS-CoV-2 (generally estimated to be between 2 and 3) and concluded that at least 50% of people need to be immune before herd immunity is reached, Gomes and colleagues calculate the threshold at 10% to 20%.2223

Ulrich Keil, professor emeritus of epidemiology from the University of Münster in Germany, says the notion of randomly distributed immunity is a "very naive assumption" that ignores the large disparities in health in populations and "also ignores completely that social conditions might be more important than the virus itself." He added, "Tuberculosis here is the best example. We all know that the immune system is very much dependent on the living conditions of a person, and this depends very much on education and social conditions."

Another group led by Sunetra Gupta at the University of Oxford has arrived at similar conclusions of lower herd immunity thresholds by considering the issue of pre-existing immunity in the population. When a population has people with pre-existing immunity, as the T cell studies may be indicating is the case, the herd immunity threshold based on an R0 of 2.5 can be reduced from 60% of a population getting infected right down to 10%, depending on the quantity and distribution of pre-existing immunity among people, Gupta's group calculated.24

But memory T cells are known for their ability to affect the clinical severity and susceptibility to future infection,25 and the T cell studies documenting pre-existing reactivity to SARS-CoV-2 in 20-50% of people suggest that antibodies are not the full story.

"Maybe we were a little naive to take measurements such as serology testing to look at how many people were infected with the virus," the Karolinska Institute immunologist Marcus Buggert told The BMJ. "Maybe there is more immunity out there."

The research offers a powerful reminder that very little in immunology is cut and dried. Physiological responses may have fewer sharp distinctions than in the popular imagination: exposure does not necessarily lead to infection, infection does not necessarily lead to disease, and disease does not necessarily produce detectable antibodies. And within the body, the roles of various immune system components are complex and interconnected. B cells produce antibodies, but B

cells are regulated by T cells, and while T cells and antibodies both respond to viruses in the body, T cells do so on infected cells, whereas antibodies help prevent cells from being infected.

An unexpected twist of the curve

Buggert's home country has been at the forefront of the herd immunity debate, with Sweden's light touch strategy against the virus resulting in much scrutiny and scepticism.26 The epidemic in Sweden does seem to be declining, Buggert said in August. "We have much fewer cases right now. We have around 50 people hospitalised with covid-19 in a city of two million people." At the peak of the epidemic there were thousands of cases. Something must have happened, said Buggert, particularly considering that social distancing was "always poorly followed, and it's only become worse."

Understanding this "something" is a core question for Sunetra Gupta, an Oxford University epidemiologist who developed a way to calculate herd immunity thresholds that incorporates a variable for pre-existing innate resistance and cross protection.24 Her group argues that herd immunity thresholds "may be greatly reduced if a fraction of the population is unable to transmit the virus."

"The conventional wisdom is that lockdown occurred as the epidemic curve was rising," Gupta explained. "So once you remove lockdown that curve should continue to rise." But that is not happening in places like New York, London, and Stockholm. The question is why.

"If it were the case that in London the disease hadn't disseminated too widely, and only 15% have experienced the virus [as serology tests indicate] . . . under those circumstances, if you lift lockdown, you should see an immediate and commensurate increase in cases, as we have observed in many other settings," Gupta told The BMJ, "But that hasn't happened. That is just a fact. The question is why."

Possible answers are many, she says. One is that social distancing is in place, and people are keeping the spread down. Another possibility is that a lot of people are immune because of T cell responses or something else. "Whatever it is," Gupta added, "if there is a significant fraction of the population that is not permissive to the infection, then that all makes sense, given how infectious SARS-CoV-2 is."

Buggert's study in Sweden seems to support this position. Investigating close family members of patients with confirmed covid-19, he found T cell responses in those who were seronegative or asymptomatic.10 While around 60% of family members produced antibodies, 90% had T cell responses. (Other studies have reported similar results.27) "So many people got infected and didn't create antibodies," concludes Buggert.

Deeper discussion

T cell studies have received scant media attention, in contrast to research on antibodies, which seem to dominate the news (probably, says Buggert, because antibodies are easier, faster, and cheaper to study than T cells). Two recent studies reported that naturally acquired antibodies to SARS-CoV-2 begin to wane after just 2-3 months, fuelling speculation in the lay press about repeat infections.282930

But T cell studies allow for a substantially different, more optimistic, interpretation. In the Singapore study, for example, SARS-CoV-1 reactive T cells were found in SARS patients 17 years after infection. “Our findings also raise the possibility that long lasting T cells generated after infection with related viruses may be able to protect against, or modify the pathology caused by, infection with SARS-CoV-2,”8 the investigators wrote.

T cell studies may also help shed light on other mysteries of covid-19, such as why children have been surprisingly spared the brunt of the pandemic, why it affects people differently, and the high rate of asymptomatic infections in children and young adults.

The immunologists I spoke to agreed that T cells could be a key factor that explains why places like New York, London, and Stockholm seem to have experienced a wave of infections and no subsequent resurgence. This would be because protective levels of immunity, not measurable through serology alone but instead the result of a combination of pre-existing and newly formed immune responses, could now exist in the population, preventing an epidemic rise in new infections.

But they were all quick to note that this is speculation. Formally, the clinical implications of the pre-existing T cell reactivity remain an open question. “People say you don’t have proof, and they’re right,” says Buggert, adding that the historical blood donor specimens in his study were all anonymised, precluding longitudinal follow-up.

There is the notion that perhaps T cell responses are detrimental and predispose to more severe disease. “I don’t see that as a likely possibility,” Sette said, while emphasising that we still need to acknowledge the possibility. “It’s also possible that this absolutely makes no difference. The cross reactivity is too small or weak to affect the virus. The other outcome is that this does make a difference, that it makes you respond better.”

Weiskopf added, “Right now, I think everything is a possibility; we just don’t know. The reason we’re optimistic is we have seen with other viruses where [the T cell response] actually helps you.” One example is swine flu, where research has shown that people with pre-existing reactive T cells had clinically milder disease (box 1).121314

Weiskopf and Sette maintain that compelling evidence could come through a

properly designed prospective study that follows a cohort of people who were enrolled before exposure to SARS-CoV-2, comparing the clinical course of those with and without pre-existing T cell responses.

Understanding the protective value of pre-existing SARS-CoV-2 T cell reactivity “is identical to the situation on vaccines,” said Antonio Bertoletti, professor of infectious disease at Duke-NUS Medical School in Singapore. “Through vaccination we aim to stimulate antibodies and T cell production, and we hope that such induction of immunity will protect … but we need a phase III clinical study to really demonstrate the effect.”

German investigators came to the same conclusion, arguing that their T cell findings represented a “decisive rationale to initiate worldwide prospective studies” mapping pre-existing reactivity to clinical outcomes.31 Other groups have called for the same thing.6

“At the start of the pandemic, a key mantra was that we needed the game changer of antibody data to understand who had been infected and how many were protected,” two immunologists from Imperial College London wrote in a mid-July commentary in Science Immunology. “As we have learned more about this challenging infection, it is time to admit that we really need the T cell data too.”32

Theoretically, the placebo arm of a covid-19 vaccine trial could provide a straightforward way to carry out such a study, by comparing the clinical outcomes of people with versus those without pre-existing T cell reactivity to SARS-CoV-2. A review by The BMJ of all primary and secondary outcome measures being studied in the two large ongoing, placebo controlled phase III trials, however, suggests that no such analysis is being done.3334

Could pre-existing immunity be more protective than future vaccines? Without studying the question, we won’t know.

https://doi.org/10.1136/bmj.m3563

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►► Cross-Reactive Antibodies to SARS-CoV-2 and MERS-CoV in Pre-COVID-19 Blood Samples from Sierra Leoneans. Viruses 2021, 13(11). DOI:10.3390/v13112325.

Abstract

Many countries in sub-Saharan Africa have experienced lower COVID-19 caseloads and fewer deaths than countries in other regions worldwide. Under-reporting of cases and a younger population could partly account for these differences, but pre-existing immunity to coronaviruses is another potential factor. Blood samples from Sierra Leonean Lassa fever and Ebola survivors and their

contacts collected before the first reported COVID-19 cases were assessed using enzyme-linked immunosorbent assays for the presence of antibodies binding to proteins of coronaviruses that infect humans. Results were compared to COVID-19 subjects and healthy blood donors from the United States. Prior to the pandemic, Sierra Leoneans had more frequent exposures than Americans to coronaviruses with epitopes that cross-react with severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), SARS-CoV, and Middle Eastern respiratory syndrome coronavirus (MERS-CoV). The percentage of Sierra Leoneans with antibodies reacting to seasonal coronaviruses was also higher than for American blood donors. Serological responses to coronaviruses by Sierra Leoneans did not differ by age or sex. Approximately a quarter of Sierra Leonian pre-pandemic blood samples had neutralizing antibodies against SARS-CoV-2 pseudovirus, while about a third neutralized MERS-CoV pseudovirus. Prior exposures to coronaviruses that induce cross-protective immunity may contribute to reduced COVID-19 cases and deaths in Sierra Leone.

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 A recent study (10.1016/j.ijid.2020.10.104: year samples, 2017-2019) compared SARS-CoV-2 cross-reactive antibodies in pre-pandemic blood samples from residents of Tanzania, Zambia, and the United States [14]. The prevalence of SARS-CoV-2 cross-reactive antibodies was significantly higher in samples from people living in these sub-Saharan African countries compared with samples from people living in America. Although blood samples obtained before the COVID-19 pandemic are scarce, it is essential to determine pre-pandemic anti-coronavirus seroprevalence rates in other regions of Africa and elsewhere .....

..... Sierra Leone (September 2016 and April 2019) from blood samples. .....

Conclusions

Pre-existing immunity to coronavirus antigens should be further investigated as a potential factor contributing to reduced caseloads and deaths from COVID-19 in Sierra Leone. It is likely that humans in Sierra Leone are frequently exposed to SARS-related and MERS-related viruses. Studies should be conducted to fully characterize immune responses directed against coronaviruses by Sierra Leoneans. Several studies have reported T cell reactivity against SARS-CoV-2 in people with no known exposure to the virus, which may in part be related to prior exposure to seasonal coronaviruses. It is possible that cellular immunity to endemic coronaviruses also has a protective role. Studies to access the prevalence of humoral and cellular immunity to coronaviruses are needed in other African countries with low reported incidence of SARS-CoV-2 infections. Equitable COVID-19 vaccine distribution should continue even in countries with currently low numbers of cases and deaths. The role of both natural immunity and vaccine-

induced immunity should be investigated in these populations. Another priority for future research will be to define the diversity of coronaviruses that circulate in humans or frequently spillover from animals to humans living in Sierra Leone and other West African countries.
https://doi.org/10.3390/v13112325
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►► UniProtKB - A0A023VYK5 (A0A023VYK5_MERS) -- Human betacoronavirus 2c Jordan-N3/2012.
Post-translational modification
Specific enzymatic cleavages in vivo yield mature proteins.
 The precursor is processed into S1 and S2 by host cell furin or another cellular protease to yield the mature S1 and S2 proteins.
Additionally, a second cleavage leads to the release of a fusion peptide after viral attachment to host cell receptor.
https://www.uniprot.org/uniprot/A0A023VYK5
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►► UniProtKB - M1RNL5 (M1RNL5_MERS) -- Human betacoronavirus 2c England-Qatar/2012.
Post-translational modification
Specific enzymatic cleavages in vivo yield mature proteins.
 The precursor is processed into S1 and S2 by host cell furin or another cellular protease to yield the mature S1 and S2 proteins.
Additionally, a second cleavage leads to the release of a fusion peptide after viral attachment to host cell receptor.
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►► UniProtKB - K0BRG7 (K0BRG7_MERS) -- Human betacoronavirus 2c EMC/2012.
Post-translational modification
Specific enzymatic cleavages in vivo yield mature proteins.
 The precursor is processed into S1 and S2 by host cell furin or another cellular protease to yield the mature S1 and S2 proteins.
Additionally, a second cleavage leads to the release of a fusion peptide after viral

attachment to host cell receptor.
https://www.uniprot.org/uniprot/K0BRG7