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Overview

Viral and Host Attributes Underlying the Origins of Zoonotic Coronaviruses in Bats

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With a presumed origin in bats, the COVID-19 pandemic has been a major source of morbidity and mortality in the human population, and the causative agent, SARS-CoV-2, aligns most closely at the genome level with the bat coronaviruses RaBtCoV4991/RaTG13 and RmYN02. The ability of bats to provide reservoirs of numerous viruses in addition to coronaviruses remains an active area of research. Unique aspects of the physiology of the chiropteran immune system may contribute to the ability of bats to serve as viral reservoirs. The coronavirus spike protein plays important roles in viral pathogenesis and the immune response. Although much attention has focused on the spike receptor-binding domain, a unique aspect of SARS-CoV-2 as compared with its closest relatives is the presence of a furin cleavage site in the S1–S2 region of the spike protein. Proteolytic activation is likely an important feature that allows SARS-CoV-2—and other coronaviruses—to overcome the species barriers and thus cause human disease. The diversity of bat species limits the ability to draw broad conclusions about viral pathogenesis, but comparisons across species and with reference to humans and other susceptible mammals may guide future research in this regard.

Abbreviations: COVID-19, coronavirus disease 2019; FCoV, feline coronavirus; HCoV, human coronavirus; MERS-CoV, Middle East respiratory syndrome coronavirus; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

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The COVID-19 pandemic remains a global public health emergency. The virus causing the pandemic (SARS-CoV-2) was identified in late 2019, after an outbreak of respiratory disease in Wuhan, China.[87] Early sequencing aligned SARS-CoV-2 with the previously detected RaBtCoV/4991.[13,20] SARS-CoV-2 was then further sequenced to yield the full genome (reported as RaTG13[95]), defining SARV-CoV-2 as being a probable bat-origin zoonotic coronavirus. Severe acute respiratory syndrome coronavirus (SARS-CoV), which emerged in 2002, and Middle East respiratory syndrome coronavirus (MERS-CoV), which emerged in 2012, are also considered to be of bat origin.[46,51,71] At least 2 other commonly circulating coronaviruses in humans also likely originated in bats: HCoV-229E and HCoV-NL63.[15,33] However, the human coronaviruses (HCoV) OC43 and HKU1 have alternatively been suggested to have emerged from rodents via cattle and from rodents via an unknown intermediate, respectively.[47,73] Swine acute diarrhea syndrome coronavirus, a cause of significant mortality in piglets, emerged in 2016 in the Guangdong province of China from the bat virus Rhinolophus bat HKU2.[22,59,94] The swine enteric coronavirus porcine epidemic diarrhea virus is also considered to have bat origins.[31] Numerous other viral diseases of human concern, including Nipah and Hendra viruses and ebolavirus, moved into human populations from bats.[14,25,41] Bats are also a common reservoir for the lethal rabies virus.[9,64] The ability of bats to harbor and spread these viruses continues to be an active area of study, integrating surveillance, ecology, disease forecasting, and basic virology to protect human and animal populations.

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Coronaviruses and Bats

The family *Coronaviridae* includes a range of single-stranded, positive-sense, RNA viruses from the order Nidovirales.[40] They are one of the largest RNA viruses with a genome size of approximately 30 kb.[37] The coronaviruses are divided into 4 genera: *Alpha-*, *Beta-*, *Gamma-*, and *Deltacoronavirus*.[60] The alpha- and betacoronaviruses are primarily associated with mammalian infections, including human coronaviruses HCoV-NL63, HCoV-229E, and HCoV-OC43; feline coronavirus; canine coronavirus; bovine coronavirus; and equine coronavirus; however, these genera are highly diverse, and classification is challenging.[80] The deltacoronaviruses are primarily avian-associated, with a primary exception being porcine deltacoronavirus.[82] Likewise, the gammacoronaviruses are mainly found in avian species but include viruses found in beluga whales and bottlenose dolphins.[56,82,83] Surveillance studies have identified a wide range of alpha- and betacoronaviruses in bat species.[81] Phylogenetic analysis places SARS-CoV-2 in the *Sarbecovirus* subgenus (or lineage B) of *Betacoronavirus* in *Coronaviridae*, divergent from SARS-CoV.[51]

Coronavirus virions contain a prominent spike protein, which is a major factor in viral pathogenesis; this protein mediates both receptor binding and membrane fusion and is the notable antigenic component (Figure 1). The clinical disease spectrum is vast across coronaviruses, but typically comprises respiratory or gastrointestinal infection. In some cases, systemic infections

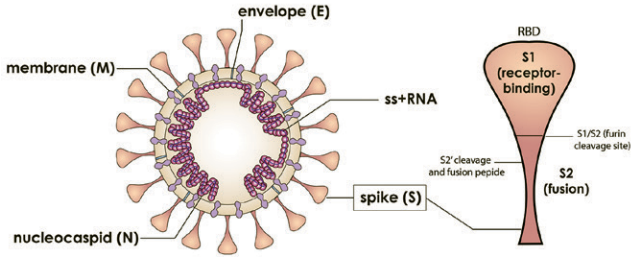

Figure 1. (A) The major structural components of a typical coronavirus. The virus is comprised of prominent spike (S), membrane (M), envelope (E), and nucleocapsid (N) proteins complexed with a positive-sense RNA strand. Some coronaviruses in the Embecovirus subgenus contain an additional surface glycoprotein (hemagglutinin–esterase, HE), which is involved in receptor binding (not shown). (B) Major domains of a typical coronavirus spike protein. The S1 (receptor-binding) domain contains the receptor-binding domain (RBD), and the S2 (fusion) domain contains the conserved S2′ cleavage site and fusion peptide. Some spike proteins contain an additional cleavage site (S1–S2) between the receptor-binding and fusion domains.

occur—feline coronavirus (FCoV), for instance, can result in mild to inapparent gastrointestinal disease or a lethal, systemic infection known as feline infectious peritonitis.[38]

The large genome and inherent replication mechanisms of coronaviruses make them error-prone, thus enabling mutations and recombination events between coronaviruses, despite proofreading ability due to RNA-dependent RNA polymerase and exoribonuclease activity from the nonstructural protein exoN (nsp14).[18] This error-prone nature is one component in understanding the bat origin of numerous human coronaviruses, including SARS-CoV-2. Recombination occurs during replication, increasing viral diversity.[68] Interviral family recombination events can also occur; for example, the Rousettus bat coronavirus GCCDC1 (Ro-BatCoV GCCDC1) contains a functional p10 gene that it potentially gained through recombination with a bat orthoreovirus.[30]

Bats belong to the order Chiroptera, the second largest order of mammals after rodents which comprises about a fifth of all known mammalian species. The order Chiroptera is highly diverse—encompassing more than 1300 species—but is united by member species' ability for sustained flight. Bats and bat-associated coronaviruses share an extensive global geographical distribution and exist on all continents except Antarctica.[81] Traditionally, bats were divided into 2 suborders— Microchiroptera (microbats) and Megachiroptera (megabats)—but more recently, a new scheme includes Yangochiroptera and Yinpterochiroptera, which are defined according to molecular and echolocation differences.[69] Despite being a source of numerous human viruses, including highly pathogenic coronaviruses, bats provide numerous ecologic benefits, including consuming insect pests, such as mosquitoes and those that affect agricultural crops, and carrying out pollination.[36] In addition, bat guano farms recycle nutrients; however, nearly 75% of samples harvested from insectivorous bats on a guano farm harbored a coronavirus.[32] The role that bats may play in disease emergence involves numerous species-dependent factors, including migration, dietary, and roosting patterns.[27] Despite the tendency of many to blame bats, disease emergence is also affected by anthropogenic activity, including deforestation.[1] Furthermore, although bats frequently are asymptomatic reservoirs of numerous viruses,[48] they are not exempt from succumbing to infectious diseases. For example, little brown bats (*Myotis lucifugus*) developed disease symptoms after inoculation with the gammacoronavirus infectious bronchitis virus, and tissue homogenates from the infected bats induced disease in chicks.[62] In another report, horseshoe bats (*Rhinolophus* spp.) harboring SARS-Rh-BatCoV tended to have lower body weights compared with those harboring Rh-BatCoV HKU2.[43] Passage of a betacoronavirus in Lechenault's rousette fruit bats (*Rousettus leschenaultii*) did not result in clinical disease, although virus was detected in feces and intestines after infection.[77] In contrast, Egyptian fruit bats (*Rousettus aegyptiacus*) inoculated with SARS-CoV-2 developed rhinitis, and viral RNA was detected in both upper and lower respiratory tissues.[65]

Ultimately, protecting human and animal populations from disease threats requires a 'One Health' approach. The concept of One Health recognizes the interconnectedness of human health, animal health, and the environment. Protecting bat habitats, including by minimizing land encroachment, may help to protect both bat and human health. Furthermore, the epidemiologic triad combines factors from the host, agent, and environment. For coronaviruses in bats, this approach requires additional investigation of viral isolates circulating in chiropteran species as well as assessing unique physiologic and immunologic adaptations in the context of bats' ecological niches, ranging from migration patterns to roosting habitats. The emergence of novel viruses, including those with pandemic potential, is multifactorial, but host and viral factors both contribute.

Surveillance for Coronaviruses in Bats

Alpha- and betacoronaviruses are detected frequently in bat species, and continual surveillance is ongoing. Surveillance in the Yunnan province of China has found coronaviruses in numerous bat species, including *Rhinolophus sinicus*, *R. affinis*, *Hipposideros pomona*, *Miniopterus schreibersii*, *M. fuliginosus*, and *M. fuscus*.[20] During this surveillance, both HKU2 and a SARS-related virus designated as RaBtCoV/4991 were frequently detected in *R. affinis*.[20] Bat-CoV-2-HKU2 was first described in *R. sinicus* during surveillance that also found bat-SARS-CoV in more than 17% (21 of 118) of *R. sinicus* bats that were sampled.[84] SARS-like viruses have also been identified in other bat species, including *Rhinolophus pusillus* and *Chaerophon plicata*.[89] These surveillance efforts have helped to provide a comprehensive picture of coronavirus circulation in bat species. Despite a focus on the Rhinolophidae as a primary source of SARS-like viruses, the Hipposideridae may also be an important contributor to the emergence of these viruses.[23] A recent study conducted in China confirmed that both Rhinolophidae and Hipposideridae contributed to the evolution of betacoronaviruses.[42] The authors noted that Rhinolophidae bat species shared roosts with other genera of bats, implying potentially enhanced transmission of coronaviruses among various bat species. Another study demonstrated on a macroevolutionary level that the basal phylogeny of betacoronaviruses paralleled the phylogenetic relationships between their hosts, with a clear demarcation between Yinpterochiroptera and Yangochiroptera.[42] The initial sequencing of SARS-CoV-2 revealed its close relationships with the bat virus RaTG13, which shares 96% sequence identity with SARS-CoV,[95] the novel bat coronavirus designated RmYN02 (93% genomic nucleotide identity) and several other bat coronaviruses, including bat-SL-CoVZC45 and bat-SL-CoVZXC21, with increasing evidence for recombination events as key contributors to viral evolution.[50,52,53,91,92]

The Coronavirus Spike Protein

The coronavirus spike protein is the primary driver of viral tropism and pathogenesis.[49,77] Early investigation demonstrated angiotensin converting enzyme 2 as the primary receptor for SARS-CoV-2.[29] SARS-CoV-2 can naturally and experimentally

infect numerous other species,[11,58,67] and in vitro work and modeling efforts have delineated the potential for the virus to bind the angiotensin converting enzyme 2 of numerous species.[17] In addition to receptor binding, proteolytic cleavage of the spike protein is important in determining viral tropism,[57] with the presence of an acceptable protease being considered important in zoonotic coronavirus spillovers and the ability of various proteases to minimize the barriers to crossing species.[55] The spike protein contains 2 domains, with S1 contributing to receptor binding and S2 contributing to membrane fusion (Figure 1). For SARS-CoV-2, an indel mutation in the spike gene S1–S2 junction creates a cleavage site for a furin-like protease that is not found in other sarbecoviruses and that contributes to virus transmission.[75,76,86] This S1–S2 furin motif indel mutation has also been reported in alphacoronaviruses.[80] The introduction of the furin-like cleavage site might have occurred due to the 'breakpoint sequence hypothesis' and been facilitated through recombination events.[19] In vitro work has shown that the SARS-CoV-2 ΔPRRA mutant virus replicates more efficiently than wildtype virus in Vero E6 cells.[35] However, replication of the ΔPRRA or equivalent mutant viruses was less efficient in other cells lines, including Calu3 and human airway epithelial cells, paralleling observations in 2 rodent models and a ferret model.[35,61] Beyond furin, other proteases, including trypsin, matriptase, and cathepsins B and L, contribute to spike protein activation at the S1–S2 site.[34,70] Like the S1–S2 cleavage site, the S2 domain is proteolytically activated at the S2′ site,[57] and the protease TMPRSS-2 is particularly important for SARS-CoV-2 spike activation at the S2′ site under laboratory conditions.[8,29] Common proteases functioning in spike protein activation include furin and furin-like enzymes, TMPRSS-2, other type II transmembrane serine proteases, and cathepsins, important for the initiation of membrane fusion and thus cellular entry.[28] The combined complexity and plasticity of spike cleavage activation is likely a powerful force in the emergence of novel coronaviruses.[79]

Zoonotic coronaviruses are likely to originate through recombination. One possibility is that ancestral viruses that have a robust furin cleavage site but cannot bind human receptors undergo downregulation of furin cleavage, opening the path for human infection, similar to MERS-CoV. In the *Merbecovirus* subgenus, the bat viruses BatCoV-HKU4 and BatCoV-HKU5 are closely related to MERS-CoV,[44] with NeoCoV subsequently identified as the bat virus with highest homology to MERS-CoV.[17] BatCoV-HKU4 has previously been identified in lesser Bamboo bats, whereas BatCoV-HKU5 is associated with Japanese pipistrelles.[84] The MERS-CoV spike protein is unusual in that it can be proteolytically activated by furin at both S1–S2 and S2′.[57] Although the MERS-CoV spike is genetically more similar to BatCoV-HKU4,[44] BatCoV-HKU5 is predicted to be efficiently cleaved by furin, even more so than MERS-CoV. However, BatCoV-HKU5 cannot bind human dipeptidyl peptidase 4, the receptor for MERS-CoV, whereas BatCoV-HKU4 has retained that ability.[89] This difference raises the question of whether the furin activation sites across zoonotic coronaviruses are gain-of-function or loss-of-function mutations or whether they instead are due to unsampled coronaviruses that are circulating in bats but are yet to be identified, given that evolutionary analysis supports the historical circulation of a SARS-like coronavirus in bat species.[11] The coronaviruses BatCoV-RmYN02 and BatCoV-RacCS203 contain a defined S1–S2 cleavage loop, although they lack a furin cleavage site,[74,91] thus suggesting these or equivalent viruses may have contributed to SARS-CoV-2 emergence.

Ultimately, improved understanding of SARS-CoV-2 pathogenesis requires both in vitro and in vivo investigation. SARS-CoV-2 can replicate in cell lines derived from *R. sinicus* (brain and lung) and *P. abramus* (kidney) although viral loads in Vero E6 cells were notably higher.[45] SARS-CoV-2 has been isolated successfully by using *R. sinicus* small intestinal organoids,[93] which may provide a tool for use in future studies. However, isolation of other naturally occurring coronaviruses using primary cells from Leschenault's rousette bats, *Myotis* kidney cells, various cell lines (including BKT-1, Tb-1 Lu, Vero-E6, MDCK, and A549) and other common, established cell lines) is frequently unsuccessful.[30,77,85]

Host Responses in Bats

The host response to coronavirus infection contributes to disease outcome.[63] Probing the differences across the human and chiropteran immune systems and metabolic processes may improve our understanding of the potential for zoonotic transfer and the ability of bats to serve as a primary reservoir for coronaviruses with pandemic potential. Despite the need for improved understanding of bat immunology, the diversity of bats poses challenges for reagent development and for drawing broad conclusions. In a significant contribution to the field, one group recently showed that bats mount dampened inflammatory responses during infection with several viruses, including MERS-CoV.[2] Specifically, the study demonstrated reduced activation of NLRP3 (inflammasome sensor NLR family pyrin domain containing 3) in bat primary cells as compared with cells derived from mice or humans, a finding that has implications for understanding the mechanisms behind some of the unique immunologic and physiologic characteristics of bats, including their lifespan and metabolism. This study underscores the distinct ability that bats have evolved to tolerate viral infections while minimizing overt disease manifestations and excessive inflammation.[26] In addition, mutations in STING (stimulator of interferon genes) that diminish the type I IFN response provide evidence of the unique chiropteran ability to manage aberrant DNA from either a DNA virus infection or the effects of metabolic stress,[88] whereas the loss of absent in melanoma 2 (AIM2) has been associated with decreased inflammasome activation.[21] Although bat cells can mount antiviral responses, the expression of proinflammatory cytokines, such as TNF, is typically restricted.[6] Numerous other distinctions between human and chiropteran immunology have been noted, including differences in TLR8, inflammatory pathways, the type III IFN response, and antibody responses including affinity maturation, constitutive IFN activation and features of NK cells and the Treg response in bats.[5,66] The unique immunologic features of bats contribute to their ability to serve as viral reservoirs.[66]

Despite initial infection in the respiratory tract, COVID-19 also causes endotheliitis and vasculitis, resulting in disease manifestations across numerous organ systems,[24,72] not too unlike feline infectious peritonitis.[4] Bats have unique cardiovascular and respiratory adaptations, reflecting their capacity for flight and ability to remain inverted while roosting. For example, in addition to a thin barrier for blood–gas exchange, lung volume and heart size in bats are greater than those of other mammals of similar size.[12,54] In addition, at least one chiropteran species, straw-colored fruit bats (*Eidolon helvum*), appears susceptible to cardiac injury after periods of inverted roosting but may have a unique ability for cardiac myocyte repair and regeneration.[3] Whether these adaptations influence the ability of chiropterans

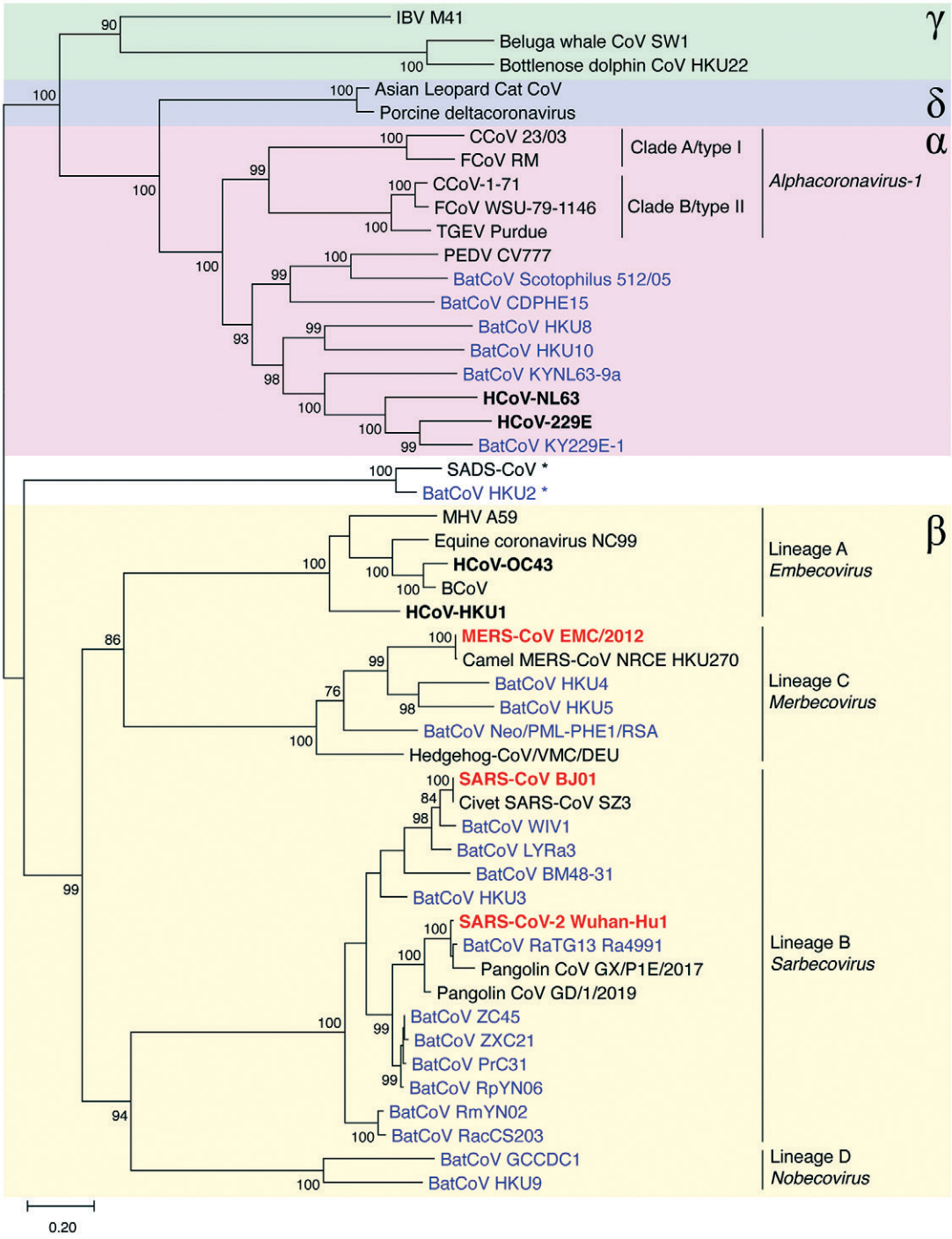

Figure 2. Phylogenetic analysis of coronavirus spike protein sequences. The amino acid sequences of 50 coronavirus spike proteins were aligned by using MUSCLE, and a maximum-likelihood (ML, LG model) phylogenetic tree was generated by using MEGA X. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Bootstrap values above 70% are shown at branch nodes and were calculated from 1000 replicates. The lack of strong bootstrap support for a number of branches in lineage B *Sarbecovirus* is likely a result of the numerous reported recombination events occurring in spike among members of this lineage. Human coronaviruses are in bold text, with recently emerged and highly pathogenic viruses in red. Bat coronaviruses are indicated in purple, and their over-representation among members of the *Betacoronavirus* genus is due to the choice of sequences used in the analysis and does not reflect natural abundance and diversity. Asterisks (*) denote that although SADS-CoV and BatCoV HKU2 are classified in the genus *Alphacoronavirus*, their spike proteins are more closely related to that of betacoronaviruses. The accession numbers of the sequences used in the analysis are in Table 1. Greek letters refer to the 4 genera of coronaviruses. IBV, infectious bronchitis virus; CCoV, canine coronavirus; FCoV, feline coronavirus; TGEV, transmissible gastroenteritis virus; PEDV, porcine epidemic coronavirus; BatCoV, bat coronavirus; HCoV, human coronavirus; SADS-CoV, swine acute diarrhea syndrome coronavirus; MHV, murine hepatitis virus; BCoV, bovine coronavirus; MERS-CoV, Middle East respiratory syndrome coronavirus; SARS-CoV, severe acute respiratory syndrome coronavirus, SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

to harbor coronaviruses remains unknown, and questions also remain with regard to endothelial disruption by SARS-CoV-2.[7] One group drew a parallel with Nipah virus, noting that they would not expect robust viral replication in primary pteropid endothelial cells, although hamster endothelial cells can be infected.[66] Further understanding of these adaptations and of

Table 1. Accession numbers of spike protein sequences

Virus	Database	Accession no.
Asian Leopard Cat CoV	N	ABQ39958
BatCoV BM48-31	N	YP_003858584
BatCoV CDPHE15	N	AGT21333
BatCoV GCCDC1	N	YP_009273005
BatCoV HKU2	N	ABQ57208
BatCoV HKU3	N	AAY88866
BatCoV HKU4	N	ABN10839
BatCoV HKU5	N	ABN10875
BatCoV HKU8	N	ACA52171
BatCoV HKU9	N	YP_001039971
BatCoV HKU10	N	AFU92104
BatCoV KY229E-1	N	APD51499
BatCoV KYNL63-9a	N	YP_009328935
BatCoV LYRa3	N	KF569997
BatCoV Neo/PML-PHE1/RSA	N	AGY29650
BatCoV PrC31	G	EPI_ISL_1098866
BatCoV RacCS203	N	QQM18864
BatCoV RaTG13 Ra4991	G	EPI_ISL_402131
BatCoV RmYN02	G	EPI_ISL_412977
BatCoV RpYN06	G	EPI_ISL_1699446
BatCoV Scotophilus 512/05	N	YP_001351684
BatCoV WIV1	N	KC881007
BatCoV ZC45	N	MG772933
BatCoV ZXC21	N	MG772934
BCoV	N	AAL40400
Beluga whale CoV SW1	N	ABW87820
Bottlenose dolphin CoV HKU22	N	AHB63481
Camel MERS-CoV NRCE HKU270	N	QDI73610
CCoV 23/03	N	AAP72150
CCoV-1-71	N	AAV65515
Civet SARS-CoV SZ3	N	P59594
Equine coronavirus NC99	N	AAQ67205
FCoV RM	N	ACT10854
FCoV WSU-79-1146	N	YP_004070194
HCoV-229E	N	BAL45637
HCoV-HKU1	N	AAT98580
HCoV-NL63	N	AAS58177
HCoV-OC43	N	AIX10763
Hedgehog-CoV/VMC/DEU	N	AGX27799
IBV M41	N	AAW33786
MERS-CoV EMC/2012	N	AFS88936
MHV A59	N	AAA46455
Pangolin CoV GD/1/2019	G	EPI_ISL_410721
Pangolin CoV GX/P1E/2017	G	EPI_ISL_410539
PEDV CV777	N	AAK38656
Porcine deltacoronavirus	N	AKC54428
SADS-CoV	N	AVM41569
SARS-CoV-2 Wuhan-Hu1	N	QHD43416
SARS-CoV BJ01	N	AY278488
TGEV Purdue	N	ABG89335

G, Global Initiative on Sharing All Influenza Data (GISAID); N, National Center for Biotechnology Information GenBank.

coagulation in bats may help to provide insight into the devastating effects of SARS-CoV-2 in human patients with COVID-19.

Coagulation after vascular injury, including from viral infection, is a concern in COVID-19 patients.[39] In species that undergo hibernation or torpor, including some chiropterans, blood stasis does not result in thromboses; this feature also may guide further understanding. Virchow's triad involves 3 factors—including blood stasis— that contribute to thromboses. However, hibernating animals, including bats, are able physiologically to tolerate blood stasis, and comparative studies may help to elucidate mechanisms to prevent thromboses in human patients.

Relationships between the Spike Proteins of Bat Coronaviruses and Other Members of Coronaviridae

As noted earlier, the coronavirus spike protein (S) is a major factor in viral pathogenesis, and comparative studies reveal that spike sequences obtained from bats are found across the *Coronaviridae*. A summary of coronaviruses, including those described herein, can be illustrated as a phylogenetic tree of spike protein sequences (Figure 2). Bat viruses are widespread in both the *Alphacoronavirus* and *Betacoronavirus* genera, with the representation of the *Betacoronavirus* genus reflecting the amount of research performed and not necessarily the abundance in bat reservoirs; as such, Figure 2 is not a complete representation of natural abundance and diversity. Instead, Figure 2 is based on the spike protein sequence; consequently, the relative similarity depicted there may differ from that of conventional phylogenetic trees that are based on whole genomes or more conserved viral proteins, such as the RNA-dependent RNA polymerase that is typically used for taxonomic organization. Notably, bat coronaviruses are absent from the *Gammacoronavirus* and *Deltacoronavirus* genera and *Betacoronavirus* lineage A (Embecovirus).

Conclusions and Perspectives

Understanding zoonotic disease emergence requires consideration of the viral features that allow movement across species and an understanding of host factors that may differ across species. Chiroptera is a diverse order, with species found across the globe in many ecological niches, and although bats are often collectively grouped together, adaptations of one particular species of bat may be different in other species. Nonetheless, unique physiologic and metabolic adaptations of bats allow them to withstand infection by coronaviruses without developing obvious disease, thus positioning them as reservoir hosts for spread of coronaviruses to humans. Along with other potential reservoir hosts in small terrestrial mammals such as the Rodentia and Eulipotyphla, wildlife surveillance and experimental studies will continue to be critical components in our responses to zoonotic disease threats in years to come.

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