5

BATS AND CORONAVIRUSES

5.1 INTRODUCTION

Many species of coronaviruses exist among humans and animals, including in bats, birds, cats, dogs, pigs, mice, livestock (horses, sheep, cattle, and camels), and whales, but no host-specific coronavirus (CoV) has been reported in monkeys or apes. Coronaviruses that have been reported to be associated with bats are found in Table 5.1. Coronaviruses cause mild to highly severe or fatal respiratory, enteric, hepatic, or neurological disease. The first two coronaviruses known to infect humans were HCoV-229E and HCoV-OC43, found in the 1960s to cause typically mild respiratory illnesses (reviewed in van Boheemen *et al*. 2012). Two other species, however, cause diseases with a high mortality rate in humans: severe acute respiratory syndrome coronavirus (SARS-CoV), discovered in 2003, and Middle East respiratory syndrome virus (MERS-CoV), found in 2012. The much less pathogenic HCoV-NL63 and HCoV-HKU1were characterized in 2004 and 2005, respectively (reviewed in van Boheemen *et al*. 2012).

Coronaviruses belong to the family Coronaviridae, subfamily Coronoavirinae of the order Nidovirales. There are four genera of coronaviruses – α, β, γ, and δ. Alpha- and betacoronaviruses have only been reported in mammals and members of both groups sicken humans to some extent. Coronaviruses are enveloped and spherical, with a ssRNA (+) genome. The genome is 27–32 kb and is the largest among that of all known RNA viruses. Its envelope is studded with spikes.

Evidence for exposure or infection with coronaviruses is present in eleven of the eighteen bat families from either frugivorous or insectivorous mega- and microbats and harbor alpha- or betacoronaviruses (reviewed by Drexler *et al*. 2014). The majority of

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TABLE 5.1 Coronaviruses associated with bats

Bat family	Bat common name	Bat species	Coronavirus species
Pteropodidae	Muluccan naked-backed fruit bat	*Dobsonia moluccensis*	Betacoronavirus sp.
Pteropodidae	Malagasy fruit bat	*Eidolon dupreanum*	Betacoronavirus sp.
Pteropodidae	Straw-colored fruit bat	*Eidolon helvum*	Alphacoronavirus sp.
Pteropodidae	Straw-colored fruit bat	*Eidolon helvum*	Betacoronavirus sp.
Vespertilionidae	Serotine bat	*Eptesicus serotinus*	Betacoronavirus, lineage c
Vespertilionidae	Big brown bat	*Eptesicus fuscus*	ARCoV, alphacoronavirus
Rhinolophidae	Intermediate roundleaf bat	*Hipposideros larvatus*	Betacoronavirus sp.
Rhinolophidae	Pomona roundleaf bat	*Hipposideros pomona*	HKU10 alphacoronavirus
Rhinolophidae	Pomona roundleaf bat	*Hipposideros pomona*	HpBtCoV/3740-2
Vespertilionidae	Savi's pipistrelle	*Hypsugo savii*	2c, betacoronavirus
Vespertilionidae	Japanese long-fingered bat	*Miniopterus fuliginosus*	HKU1 alphacoronavirus
Vespertilionidae	Schreiber's long-fingered bat	*Miniopterus schreibersi*	HKU8 alphacoronavirus
Vespertilionidae	Lesser mouse-eared bat	*Myotis blythii*	Alphacoronavirus sp.
Vespertilionidae	Pond bat	*Myotis dasycneme*	Alphacoronavirus sp.
Vespertilionidae	Cape bat	*Neoromicia capensis*	NeoCoV, MERS-like betacoronavirus
Vespertilionidae	Zulu serotine	*Neoromicia* cf. *zuluensis*	PML/2011, betacoronavirus
Vespertilionidae	Japanese pipistrelle	*Pipistrellus abramus*	HKU5, bat betacoronavirus, lineage c
Vespertilionidae	Kuhl's pipestrelle	*Pipiestrellus kuhlii*	Alphacoronavirus sp.
Vespertilionidae	Common pipistrelle	*Pipistrellus pipistrellus*	VM31, betacoronavirus
Pteropodidae	Madagascan flying fox	*Pteropus rufus*	Betacoronavirus sp.
Rhinolophidae	Intermediate horseshoe bat	*Rhinolophus affinis*	LYRa11, SARS-related betacoronavirus
Rhinolophidae	Greater horseshoe bat	*Rhinolophus ferrumequinum*	Rf1, SARS-like betacoronavirus, lineage b
Rhinolophidae	Great-eared horseshoe bat	*Rhinolophus macrotis*	Rm1, SARS-like betacoronavirus, lineage b
Rhinolophidae	Pearson's horseshoe bat	*Rhinolophus pearsonii*	SARS-like bat betacoronavirus
Rhinolophidae	Blyth's horseshoe bat	*Rhinolophus pusillus*	SARS-like bat betacoronavirus
Rhinolophidae	Chinese rufous horseshoe bat	*Rhinolophus sinicus*	SARS-like bat betacoronavirus, lineage b
Rhinolophidae	Chinese rufous horseshoe bat	*Rhinolophus sinicus*	RsSHC014, bat betacoronavirus
Rhinolophidae	Chinese rufous horseshoe bat	*Rhinolophus sinicus*	Rs3367, clade 1 bat betacoronavirus
Rhinolophidae	Chinese rufous horseshoe bat	*Rhinolophus sinicus*	Rp3, clade 1 bat betacoronavirus
Rhinolophidae	Chinese rufous horseshoe bat	*Rhinolophus sinicus*	HKU1 alphacoronavirus

(*Continued*)

TABLE 5.1 (Continued)

Bat family	Bat common name	Bat species	Coronavirus species
Rhinolophidae	Chinese rufous horseshoe bat	*Rhinolophus sinicus*	HKU2 alphacoronavirus
Rhinolophidae	Chinese rufous horseshoe bat	*Rhinolophus sinicus*	HKU8 alphacoronavirus
Rhinolophidae	Chinese rufous horseshoe bat	*Rhinolophus sinicus*	RaBtCoV/4991 SARS-like betacoronavirus
Rhinolophidae	Chinese rufous horseshoe bat	*Rhinolophus sinicus*	Rs806, clade 2 bat betacoronavirus
Rhinolophidae	Chinese rufous horseshoe bat	*Rhinolophus sinicus*	Rs672, bat betacoronavirus
Pteropodidae	Flying foxes	*Rousettus* sp.	HKU9, bat betacoronavirus, lineage d
Vespertilionidae	Lesser bamboo bats	*Tylonycteris pachypus*	HKU4, bat betacoronavirus, lineage c
Vespertilionidae	Asian parti-colored bat	*Vespertilio superans*	SC2013, bat betacoronavirus

bat coronaviruses, however, have been reported in insectivorous bats and only four species in frugivorous bats. The straw-colored fruit bat (*Eidolon helvum*) is linked to one unclassified alpha- and one unclassified beta-CoV. Interestingly, only two of the four frugivorous bat species are infected by a SARS-like coronavirus: the Malagasy fruit bats (*Eidolon dupreanum*) and the Madagascan flying fox (*Pteropus rufus*) (Razanajatovo *et al*. 2015). Both of these bats are found only in Madagascar, while the SARS epidemic originated in China and is believed by many to have passed from Chinese fruit bats to civit cats and raccoon dogs before infecting humans. Of note, all bat species known to harbor SARS-like coronaviruses in Asia or Southeast Asia are from the insectivorous Rhinolophidae horseshoe bat family (*Rhinolophus ferrumequinum, R. macrotis, R. pearsonii, R. sinicus*, and *R. pusillus*) and not from fruit bats. The bats most closely associated with human MERS-CoV are also insectivorous, but are found in Africa and the Middle East, in regions where MERS is also present. Interestingly, SARS-CoV-like and MERS-CoV-like bat cornonaviruses have recently been reported in Korea (Kim *et al*. 2016). The authors mentioned that Korea experienced a MERS outbreak, however, since the index case had just travelled to the Middle East, it is not likely that bats pose a threat for zoonotic transmission to humans in Korea.

Infection of people by human coronaviruses HCoV-NL63, HCov-229E, HCoV-OC43 (originating in cattle), and HCoV-HKU1 are self-limiting, common cold-like illnesses, however, as is the case for most microbial infections, more severe symptoms may occur in children, the elderly, and immunocompromised patients. Alphacoronaviruses have a broader host range and genetic diversity than betacoronaviruses in bats and have been reported in Asia and Southeast Asia, North America, Africa, and Australia (Ge *et al*. 2013; Drexler *et al*. 2014). Betacoronaviruses have, however, been reported in bats from Thailand, the Philippines, Mexico, Neotropical South America, China, the Philippines, Madagascar, Kenya, South Africa, and the Middle East (reviewed by Drexler *et al*. 2014; Razanajatovo *et al*. 2015). HCoV-229E and HCoV-NL63 are alphacoronaviruses, while SARS- and MERS-CoV are betacoronaviruses. Betacoronaviruses are

divided into four lineages (lineages a–d). The human HCoV-OC43 and HCoV-HKU1 belong to lineage a: SARS-CoV, civet SARS-related coronaviruses, and SARS-related *Rhinolophus* bat coronaviruses belong to lineage b; and HCoV-EMC/2012 (EMC/2012) and MERS-CoV belong to lineage c. Both betacoronavirus lineages c and d include viruses detected in bats, such as HKU4 bat CoV from the lesser club-footed bat (*Tylonycteris pachypus*) and HKU5 bat CoV from the Japanese pipistrelle (*Pipistrellus abramus*) (both lineage c beta-CoV) and the *Rousettus* bat CoV HKU9 from the frugivorous Leschenault's rousette (lineage d) (Lau *et al*. 2010b; reviewed by van Boheemen *et al*. 2012 and Woo *et al*. 2012).

Genetic diversity of coronaviruses is multifactorial, involving the infidelity of RNA-dependent RNA-polymerase (RdRp), which has a high frequency of homologous RNA recombination due to unique random template switching during replication, their unusually large genomes, gain and loss of domains, and interspecies jumping events, at least in betacoronaviruses (reviewed by van Boheemen *et al*. 2012 and Woo *et al*. 2012). The poor fidelity of the RdRp, however, is partially offset by the presence of an exonuclease replicase protein, absent in other positive-strand RNA viruses, that appears to serve as a proofreading mechanism (Denison *et al*. 2011). Nevertheless, the mean evolutionary rate due to RdRp in betacoronaviruses is estimated to be 2.37×10^{-4} nucleotide substitutions per site per year. This diversity may promote emergence of viruses with novel traits that adapt to different ecological niches and hosts, sometimes leading to spillover to humans or our domestic animals (reviewed in van Boheemen *et al*. 2012). An example of the former is the finding that HCoV-OC43 is a zoonotic virus of bovine origin that emerged around 1890, most likely from bovine-to-human transmission (reviewed in Woo *et al*. 2012).

5.2 SARS CORONAVIRUS

5.2.1 The history of SARS

The first known cases of SARS occurred in mid-November, 2002, in Guangdong Province, China, and presented as fever and respiratory symptoms, including atypical pneumonia. This was followed about a month later by an independent outbreak originating with a Chinese chef. Several other early clusters in Guangdong or Guangxi Provinces followed a pattern of spread to family members and health care workers and then disappearing after several rounds of human-to-human transmission. Contact with exotic or game animals, often in restaurants or "wet markets," was associated with outbreak initiation. Consumption of exotic animals is generally believed to have health-promoting benefits and is especially common during winter months, a time in which respiratory tract infections are prevalent. A SARS-like-CoV was isolated using nasal or fecal swabs of six masked palm civets (*Paguma larvata*) and one raccoon dog (*Nyctereutes procyonoides*) from a wet market in Shenzhen, China. Such markets bring together many species of animals from different geological locations, caged close to each other in crowded areas where they are exposed to a variety of fecal material. The isolate's full genome is 99.8% identical to the human epidemic strain SARS-CoV Urbani, differing by 18 amino acids in the S protein. Only civets from wet markets were found to be seropositive for SARS-CoV, not those coming from farms or wild-caught

animals (Ge *et al*. 2013). Ferret badgers from these markets in southern China also have a SARS-like CoV (reviewed in Raj *et al*. 2014a). Of note, bats are also commonly found and served in animal markets and restaurants in Guangdong, China (Lau *et al*. 2010a).

In late January 2003, the first "super-spreader" emerged. Such people transmitted disease to large numbers of others, triggering rapid spread of the disease into the community, including those with whom they had only casual contact, such as on public transportation. The disease spread via health care providers and their contacts to Hong Kong, Vietnam, Singapore, and Canada (Hilgenfeld & Peiris 2013). Eventually, over 8000 cases and 774 deaths were reported in 30 countries in five continents during 2002–2003 (Ge *et al*. 2013).

Heroic efforts on the part of health care providers, public health workers, and researchers working together with law enforcement and political bodies brought extremely rapid resolution to the SARS outbreak. By late March 2003, a novel CoV was linked to SARS infection. Within a month, the virus, SARS-CoV, was fully mapped and declared to be the causative agent of this disease. In early July of 2003, the outbreak ended. Two small outbreaks occurred in late 2003–early 2004, linked to either a laboratory or to a live animal market. No further human cases have been reported since then. Epidemiological studies indicate that zoonotic transmission of SARS-CoV has occurred at least twice in China: in Guangdong in November 2002, leading to a large outbreak, and in Guangzhou, in December 2003, in a small outbreak. Sequence analysis of viruses demonstrated that they were not derived from the preceding epidemic (Tan *et al*. 2006).

The process of disease control was aided by a peculiar feature of the infection in which virus numbers in the upper respiratory tract secretions were low early during infection and increased afterwards, becoming most infectious when people were very ill, during hospitalization, thus limiting community exposure. This may be due to the location of the SARS-CoV receptor, which is expressed on pneumocytes deep in the lung, but to a far lesser extent in the upper respiratory tract. The targeting of pneumocytes in the lower respiratory tract may lead to a severe clinical disease course with early onset of respiratory distress, hospitalization, and isolation of patients prior to them producing high virus levels in their respiratory secretions (reviewed in Müller *et al*. 2012). Unfortunately, SARS-CoV is more stable in the environment than most coronaviruses, surviving at lower temperature and lower humidity.

In the areas of the large markets that housed diverse groups of animals, a virus closely related to SARS-CoV was detected in some small mammalian species used as exotic food, such as Himalayan palm civets and raccoon dogs. Workers in those areas had a high prevalence of antibodies to SARS-CoV, even if they did not develop disease, while those workers in other areas of the markets lacked these antibodies. This suggests the existence of a high degree of prolonged exposure of humans to coronaviruses of other mammal species, providing many opportunities for spillover of precursors of SARS-CoV to occur. This is supported by the linkage between SARS acquisition and working in a restaurant that kept and killed these animals.

5.2.2 SARS pathology

The incubation period of SARS is generally 2–10 days, followed by fever, chills, rigor, headache, dizziness, malaise, and myalgia. The respiratory stage of SARS begins with a dry, nonproductive cough with mild nasal discharge. By the time of fever onset, most

patients have abnormal chest radiographs, beginning with subtle peripheral pulmonary infiltrates that progress to bilateral and generalized, with interstitial or confluent infiltrates, with air-space opacities eventually developing. Moderate to severe cases develop dyspnea and hypoxia. In 10–20% of hospitalized patients, mechanical ventilation is required due to progressive immune infiltration of the lungs with diffuse alveolar damage that, nevertheless, fails to clear the viral infection. This eventually culminates in acute respiratory distress syndrome in approximately 16% of SARS patients, associated with a mortality rate of 50%. In addition to damaging the respiratory (including alveoli) and immune systems (including T lymphocytes, monocyte/macrophages, lymph nodes, and spleen), the kidneys, brain, digestive tract, heart, liver, thyroid gland, and urogenital tract are affected (Guo *et al*. 2008). The greatest risk factor for severe disease is being older than 60 years, along with other prognostic factors, including the presence of comorbidities such as diabetes mellitus and cardiac disease, elevations of baseline LDH and ANC, and baseline hypoxemia.

Much of the pathology in SARS may be immune-mediated. Innate interferon (IFN) responses fail to function correctly during inflammatory responses in severe cases and unregulated expression of type I IFNs and the IFN-stimulated chemokines CXCL10 and CCL2 may result in widespread immune dysregulation. Elevated levels of the chemokines IL-8, CCL2, and CXCL10 are found during acute SARS infection and levels of the cytokines IFN-γ, IL-1, IL-6, and IL-12 remain elevated for at least 2 weeks. Increased amounts of CXCL10, CXCL9, and IL-8 early during the disease are associated with adverse outcome (reviewed by Cameron *et al*. 2008; Thiel & Weber 2008). Severe SARS patients also had higher levels of CXCL10 and CCL2 during the late phase of the disease, together with lower levels of IL-12p70 and TNF-α than was seen in patients with less severe illness (Cameron *et al*. 2008). The immune response to SARS-CoV infection is discussed in greater detail in Chapter 1.

5.2.3 Viral and cellular proteins and their role in entry into the host cells

As stated in Section 5.1, coronaviruses have the one of the largest reported positive single-stranded RNA genomes. The SARS-CoV genome is 27.8 kb and contains fourteen open reading frames (ORFs) that code for at least 28 proteins (reviewed in Hilgenfeld & Peiris 2013). Their spike (S) protein is a type I transmembrane protein that protrudes from the viral surface, giving it a crown-like ("corona") appearance. The S protein contains a distinctive N terminus (S1) in additional to a conserved C terminus (S2). S1 contains the receptor binding domain (RBD) that determines the virus's host specificity. S2 is responsible for viral fusion. Both S1 and S2 are produced as a single polyprotein that must be cleaved by host proteases before the coronaviruses can enter host cells. The ability of the S protein to be cleaved by a particular host's enzymes helps to determine viral host selection (reviewed by Y. Yang *et al*. 2014). SARS- and MERS-CoV use the human type 2 transmembrane serine protease (TMPRSS2). The host endosomal protease cathepsin L is also necessary for S protein cleavage. The angiotensin-converting enzyme-2 (ACE2) is the host cell receptor that binds to the RBD portion of human SARS-CoV. HCoV-NL63, an aminopeptidase N (APN), acts as the cellular receptor for HCoV-229E CoV. DPP4, a conserved ectopeptidase that cleaves dipeptides from hormones, chemokines, and cytokines, is the MERS-CoV receptor.

DPP4's enzymatic activity is not critical for cellular infection by MERS-CoV since inhibition of its enzymatic activity does not block infection (reviewed in Wang *et al*. 2013). Other CoV structural proteins include the nucleocapsid and matrix proteins and the envelope glycoprotein.

SARS-CoV is well-adapted to the human ACE2 receptor and is unable to infect bat cells (reviewed in Müller *et al*. 2012). Of note, human SARS-CoV and the closely related civet SARS-CoV S protein cannot use the Pearson's horseshoe bat (*Rhinolophus pearsoni*) ACE2 protein as a receptor. The crystal structure of the human SARS-CoV RBD complexed with human ACE2 suggests that this restriction is due to truncations in the RBD of bat SARS-like-CoV S protein (reviewed by Hou *et al*. 2010). By contrast, the ACE2 of the bats *Myotis daubentoni* and *Rhinolophus sinicus* do support SARS-CoV entry, suggesting that these bats might be susceptible to human SARS-CoV infection. It should be noted, however, that viral entry utilizing the bat ACE2 receptor differs in efficiency with that of human ACE2 protein due to the mutation of several key amino acids. Genetic diversity of bat ACE2 is also greater than that displayed by other known human SARS-CoV-susceptible mammals, suggesting that other bat species may or may not act as reservoirs for viruses similar to SARS-CoV (Hou *et al*. 2010). In addition to the inability of SARS-CoV to bind the ACE2 protein of most bats, bat SARS-like CoV S proteins expressed by an HIV-based pseudovirus are also not able to support infection of cell lines expressing human, civit, or the bat *R. pearsonii* ACE2, but replacement of amino acids 310–518 converts the SARS-like-CoV S to a form in which it is able to bind human ACE2 (Ren *et al*. 2008). Unfortunately, appropriate cell lines from *Rhinolophus* were not available for testing at the time of the study.

Bat ACE2 are identical in size to the human ACE2 (805 amino acids) and have an amino acid identity of 80–82% to human and civet ACE2. The amino acid identity of ACE2 varies among different bat families, ranging from 78 to 84% identity, and within the genus *Rhinolophus*, from 89 to 98%. The major sequence variation among bat ACE2s is within the N-terminal region, which contains the SARS-CoV-binding region (Hou *et al*. 2010). ACE2 from *M. daubentonii* and *R. sinicus* from the Hubei province of China (Rs-HB) permitted cellular infection by a pseudovirus bearing the human SARS-CoV S protein, but not the ACE2 protein of *R. sinicus* from the Chinese Guangxi province or the ACE2 of *R. ferrumequinum, Rhinolophus macrotis, R. pearsoni, Rhinolophus pusillus*, or *Hipposideros pratti* bats. Additionally, ACE2 of *R. sinicus* from the Hubei province contains structural features that make it a low affinity receptor for human SARS-CoV.

SARS-CoV has eight accessory proteins whose length varies greatly (39–274 amino acids). Accessory gene functions are not essential for replication in cell culture and thus most of them may not be under as great a level of selective pressure as other genes. In animal models, however, they help to determine virulence, block cell cycle progression, induce apoptosis, and block innate immune system signaling *in vivo* (Tan *et al*. 2006; reviewed by van Boheemen *et al*. 2012). Because of a low degree of selective pressure, several accessory genes undergo rapid evolution that may be critical for virulence. ORF8 of CoV from palm civits and from humans early during the SARS outbreak only encoded one protein, but by early 2003, the genome of human SARS-CoV lost 29 nucleotides and subsequently encoded two separate accessory proteins, 8a and 8b. This event may be at least in part responsible for the increased efficiency of human-to-human

transmission that initiated the epidemic stage of the SARS outbreak (Tan *et al*. 2006; reviewed by Hilgenfeld & Peiris 2013).

Another accessory protein, 3a, is an integral membrane protein expressed on the viral surface. Its external domain elicits strong antibody responses that allow removal of infected cells by the complement component of the host's innate immune response. The 3a protein is of particular interest since it interacts intracellularly with the S protein and may play a role in modulating S protein surface expression. The genes for both S and 3a proteins appear to be under positive selection during virus evolution (reviewed by Tan *et al*. 2006). Viral 3a may influence the up-regulation of fibrinogen seen in immune cells of infected individuals (reviewed in Tan *et al*. 2006). Excessive production of fibrinogen may increase cytokine production by the host's adaptive immune response and alter the pro-coagulant and fibrinolytic balance. This may result in the dysregulated coagulation and fibrin polymerization pathways seen in the lung pathogenesis of most SARS patients.

Viral ORF1 is approximately two-thirds of the SARS-CoV genome and encodes two huge polyproteins, pp1a (approximately 486 kDa) and pp1ab (approximately 790 kDa), which are cleaved into 15–16 nonstructural proteins by two cysteine proteases, a papain-like protease (PLpro) and the main protease (M_{pro} or 3CLpro). M_{pro} is the target of several anti-coronavirus drug candidates. The majority of the viral nonstructural proteins in conjunction with some host components assemble the viral replication and transcription complex in double-membrane vesicles as well as other unusual membrane structures derived from the endoplasmic reticulum membrane. Afterwards, a nested set of subgenomic mRNAs is produced and translated into the structural and accessory proteins which, together with newly synthesized genomic RNA, are assembled into progeny virions. These then bud through the membranes of the intermediate endoplasmic reticulum-to-Golgi compartment and leave the host cell by exocytosis (reviewed by Hilgenfeld & Peiris 2013). One of the conserved nonstructural proteins, the RNA-dependent RNA polymerase RdRp, RdRp, has been the target of much of the comparative sequencing efforts used to develop hypotheses concerning the relatedness of SARS- and MERS-CoV to a variety of coronaviruses from bats and other animals.

MERS-CoV generates less of a proinflammatory response in differentiated bronchial epithelial cells *in vitro* than SARS-CoV does, perhaps partially explaining why it replicates to a lesser extent in these tissues than SARS-CoV. MERS-CoV also targets type I and type II alveolar cells of the lungs. This may be significant in the disease pathology since type II cells are important for tissue repair. HCoV-229E, a milder human pathogen, does not replicate in lung tissue, while the highly pathogenic influenza A (H5N1) virus, associated with pneumonia, does (reviewed in Mackay & Arden 2015).

5.2.4 SARS in civits and raccoon dogs

RNA of coronaviruses that are very closely related to SARS-CoV was isolated from Himalayan palm civets, a raccoon dog, and humans in a live-animal market in Guangdong, China. When comparing healthy wild-animal traders, people involved in animal slaughter, and vegetable traders, seropositivity for SARS-CoV was 40, 20, and 5%, respectively. Full-genome sequencing of human and palm civit SARS-CoV isolates showed a 99.8% homology. Three isolates from palm civits (originally from different geological locations) were phylogenetically distinct, having up to 18 nucleotide differences.

Five human SARS-CoV isolates from separate geographical sites differed by 14 nucleotides. The S genes of three civits' and 1 raccoon dog's viruses had eight nucleotide differences and there were 20 differences among 11 human SARS-CoV isolates from Hong Kong, Guangdong, Canada, and Vietnam. Interestingly, while 70% of the polymorphisms among the human viruses were nonsynonymous mutations, only 25% were so in the animal viruses. Eleven consistent nucleotide signatures appear to have differentiated the animal and human viral isolates. All but one human isolate tested in this study lacked a 29-nucleotide sequence in ORF8 that was present in all animal isolates (Guan *et al*. 2003). The ORF8 of human strains from later stages of the epidemic increased viral replication and induced apoptosis via a mitochondria-dependent pathway, while that from civet and early human isolates was instead found in the endoplasmic reticulum (reviewed in Lau *et al*. 2010a).

Interestingly, a 2007 study found that pseudoviruses expressing four different civet-CoV S genes containing distinct RBDs infected cells expressing human ACEs and infected human cells with 90–95% less efficiency than those expressing S genes from human SARS-CoV. This has been suggested to be because these civet coronaviruses contain either one or the other of the critical RBD residues 479 N and 487 T, but not both (Liu *et al*. 2007). Since 479 N was found in eight civet coronaviruses, the additional mutation 487 T may be important for adapting to entry into human cells. Three human SARS-CoV isolates lack 487 T and only caused mild human infections with low transmissibility, suggesting an independent cross-species event (Liu *et al*. 2007).

Sheahan *et al*. (2008), however, reported that the SARS human epidemic Urbani viral isolate grew similarly in cells expressing either human or civit ACE2, while a recombinant human SARS-CoV virus expressing the S protein from the civit-CoV SZ16 isolate only grew in cells expressing the civit ACE2. Civit and human ACE2 differ by only two amino acids. Recombinant SZ16-S mutant viruses K479N and D22, bearing mutations at three specific sites, however, grew well in cells expressing human ACE2 but not civit ACE2. This suggests that the evolutionary pathway that promoted efficient human ACE2 binding simultaneously abolished efficient civit ACE2 interaction. Since the human epidemic Urbani SARS-CoV strain had dual species tropism, the virus may have evolved high affinity for civit and human ACE2 receptors by repeated passages between human and civet hosts (Sheahan *et al*. 2008). This report also supports the contention that the civit-CoV SZ16 strain is closely related to at least some human SARS-CoV isolates. Interestingly, civets infected with human-tropic SARS-CoV develop disease that is similar to that seen in infected humans (Sheahan *et al*. 2008). Taken together, these findings suggest that human CoV infection likely originated from coronaviruses of palm civits.

5.2.5 Relatedness of bat SARS-like CoV to SARS-CoV

Great diversity of SARS-like coronaviruses is present in *R. sincus*. Yuan *et al*. (2010) isolated a strain from *R. sinicus* that contains the distinctive 579-nucleotide deletion in the nsp3 region that is a characteristic of human SARS-CoV from the late-phase epidemic, but is not present in most bat isolates. Phylogenetic analysis of ORF1 suggests that the SARS-like CoV of *R. sincus* is more closely related to SARS-CoV than isolates from other *Rhinolophus* species. Importantly, *R. sincus* is an extremely common species of this genus in China. The SARS-like CoV sequences from *R. sincus* contain two

topologically distinct clusters: Rp3, HKU3, and Rs806 in clade 1 and Rs672 in clade 2 throughout southern China. Orf1a and Orf1b of Rs672 are more similar to that of the human SARS-CoV than to that of other bat SARS-like coronaviruses, however, a different region is more similar to bat SARS-like CoV than to that of human SARS-CoV, suggesting a possible recombination between bat and human SARS-CoV, as had been previously reported for the Rp3 isolate. Two different analyses suggest that the potential recombinatorial breakpoint is immediately after the start codon of the spike gene at the same position as that found in Rs806. The genome regions upstream and downstream of this point are designated the major and minor parental regions. The major parental region of RS672 is phylogenetically closer to human SARS-CoV than to bat viruses and the minor parental region of Rs672 clusters with the bat SARS-like CoV lineage. Both Rs672 and Rp3 may have evolved from a common ancestor, however, Rs672 and Rp3 and their hosts may have diverged a relatively long time ago. The potential direct or indirect interspecies transmission between bats and the onset of the SARS epidemic is estimated to be 4.29 years (Yuan *et al*. 2010).

Between 2004 and 2008, 9.4 and 6.3% of the insectivorous *R. sincus* bats from Hong Kong and Guangdong, China, respectively, contained SARS-like CoV in their digestive samples. These bats can migrate from 1.86 to 17 km. The positive bats appear to be healthy, but have lower body weights than bats without signs of infection. Viruses are cleared by the bat immune system within 2 weeks to 4 months. Frequent recombination occurs between Rp3 from Guangxi, China, and Rf1 from Hubei, China, with the breakpoint at the ORF1/S junction. Molecular clock analysis indicated that the bat strains diverged in 1972, followed by the divergence of civet and bat strains in 1995. This supports the hypothesis that *Rhinolophus* bats act as reservoirs for recombination between SARS-like CoV strains from different geographical locations that are within reachable foraging range and that civet SARS-like CoV, such as strain SZ3, may have arose by recombination similar to that occurring between bat Rp3 and Rf1 (Lau *et al*. 2010a).

At least five *Rhinolophus* species in mainland China and Hong Kong host SARS-like coronaviruses (betacoronaviruses of lineage b): *R. sincus*, *R. pearsonii, R. ferrumequinum, R. macrotis*, and *R. pusillus*. These SARS-like CoV isolates are HKU3-1, HKU3-2, Rp3, Rf1, and Rm1 (reviewed by Ren *et al*. 2006). Bat beta-CoV Rf1 and Rm1 isolates were sequenced from *R. ferrumequinum* and *R. macrotis* bats and have an overall genome sequence identity of 88–92% between themselves and human/civet isolates. The greatest variation exists in the genes encoding ORF1, ORF3a, S, and ORF8 (Ren *et al*. 2006). Bat CoV Rf1 may be an evolutionary intermediate between bat lineage b betacoronaviruses and those from humans and civets. The latter two coronaviruses have an ORF3b of 154 amino acids that is absent from most bat SARS-like CoV, while in the corresponding region of the Rf1 genome, there were two ORFs of 113 and 32 amino acids (Ren *et al*. 2006). The sequence identity of the S genes of bat and human or civit isolates is 76–78%, while that of the S1 domain is 63–64%. Bat isolates additionally have a 6 amino acid insertion and three deletions of various lengths in the S1. Two of the deletion sites are in the RBD and overlap with the RBM (Ren *et al*. 2006), calling into question the ability of these bat betacoronaviruses to serve as the predecessors of SARS-CoV since these regions are vital for the binding of host cells.

Upon the discovery of a beta-CoV, lineage b, in *Hipposideros larvatus* bats from Southeast Asia, it has been hypothesized that the presence of beta-CoV in *Rhinolophus*

bats was the result of a spillover from those infecting *Hipposideros*, its sister taxon (Gouilh *et al*. 2011). Unlike the short time periods of persistence in *Rhinolophus* bats, the novel bat beta-CoV persisted for 18 months in a *Hipposideros* bat colony. The latter colonies might be more tolerant of betacoronaviruses over long periods of time or the betacoronaviruses of *Rhinolophus* bats may have acquired factors that limit their virulence. Studies of bat ancestors of civit and human SARS-CoV should perhaps be expanded to the *Hipposideros* genera as well, especially since studies have not focused as heavily upon this bat group (Gouihl *et al*. 2011). It should be noted that beta-CoV infection may be confined to only a few *Hipposideros* species since *Hipposideros armiger* dwelling in a separate site in the same cave were not infected. Alternatively, more direct contact between the bat groups may be required for interspecies transmission.

Molecular clock analysis suggests that bat and civet/human strains diverged 4–17 years before the large human outbreak (reviewed in Lau *et al*. 2010a). SARS-related coronaviruses appear to have been transmitted from civets to humans, with horseshoe bats being perhaps the primary host. Civet SARS-related CoV may have also arisen from recombination of different strains of SARS-like bat CoV from different locations in China (reviewed in Woo *et al*. 2012). Analysis of nonsynonymous and synonymous substitution rates (K_a and K_s, respectively) suggest that SARS-like coronaviruses in bats are not under positive-selection pressure and have evolved independently for a relatively long time. Human and civet isolates appear to have undergone a strong positive selection, suggesting recent interspecies transition (Ren *et al*. 2006).

Whole-genome sequencing detected two novel bat coronaviruses (RsSHC014 and Rs3367) from the Rhinolophidae family of horseshoe bats in Yunnan, China (Ge 2013). Their genes have a high degree of homology in the RBD of the S protein from SARS-CoV. RsSHC01014 has 99.9% nucleotide homology with the WiVi isolate from bat feces, which utilizes the ACE2 of horseshoe bats, civits, and humans during entry into its target cells. Rs3367 is also able to use human ACE2 for cell entry (Ge *et al*. 2013). A novel SARS-like beta-CoV (LYRa11) was found in *Rhinolophus affinis* in Yunnan, China, which has spherical, enveloped virus-like particles with surface spikes, but nevertheless does not have the typical petal-shaped CoV morphology. Infectious viruses were not able to be isolated from rectal samples and only a few CoV-like particles with the unusual spike morphology were found (He *et al*. 2014). LYRa11 has a 98.4% nucleotide identity with the conserved *RdRp* gene of bat coronavirus Rs3367. Full genome sequencing of LYRa11 indicates 91% nucleotide identity with SARS-CoV, with the variable *S* gene having 99% identity. The genome contains 29 805 nucleotides (slightly larger than SARS-CoV) with 40.7% G+C content and 13 ORFs. It has 83.3–84.0% amino acid identity with S1 of human and civet SARS and bat Rs3367 and a low degree of identity with other bat SARS-like CoV. The RBD has 92.5–94.6% amino acid identity to human and civet SARS-CoV and 95.1% identity to Rs3367, while other bat SARS-like CoV has only 58.7–61.3% amino acid identity to human and civit SARS-CoV. LYRa11, however, lacks ORF4 of the human SARS-CoV isolate Tor2 and bat CoV Rs3367 (He *et al*. 2014).

Within the RBD (319–518 amino acids) lies the receptor binding motif (RBM) (426–518 amino acids), the most variable region and that which determines host selection. Another bat isolate, BM48, from the Bulgarian *Rhinolophus blasii* bat has a four amino acid deletion in this critical RBM region, as well as a greater amino acid difference

to human and civet viruses in comparison with LYRa11. Other bat SARS-like coronaviruses have a 17–18 amino acid deletion in the RBM. By contrast, LYRa11 and Rs3367 have no deletion in the RBM (He *et al*. 2014). The RBM contains two critical amino acids involved in receptor recognition and binding enhancement. Substitution of both of these amino acids (but not either one alone) stops binding to the ACE2 receptor. Since both bat Rs3337 and LYRa11 have mutations at only one of those critical amino acids, both isolates are still able to bind to human ACE2. The two viral isolates are distinct since Rs3367 contains ORF 4 and was isolated over 350 km from the location of LYRa11(Kumming). Of interest, coinfection of host cells with two distinct coronaviruses may lead to genomic recombination. This may have been involved in the origins of RS3367, LYRa11, and human SARS-CoV – the "Gap-Filling virus" hypothesis (Kumming).

In order to further explore the relatedness of the bat and human beta-CoV, their mechanisms of avoiding the host innate immune response was compared, with particular interest in IFN, since this host cytokine is among the most powerful means of controlling or eliminating viral infections. Human SARS-CoV contains at least five proteins (products of ORF3b, ORF6, the nucleocapsid protein and several products of ORF1) that act as antagonists of either IFN production or signaling pathways. Homologs of SARS-CoV ORF3b in the bat SARS-like coronaviruses Rf1, Rm1 and Rpl contain different C-terminal truncations (Zhou *et al*. 2012). The three bat-derived ORF3b proteins vary in their ability to suppress IFN. ORF3b of bat CoV Rf1 is toxic to human cells without inducing apoptosis, while that of bat CoV Rp1 does not antagonize IFN, and that of bat CoV Rm1 is a potent IFN antagonist in human cells that acts by blocking IRF3 nuclear translocation and preventing activation of the IFN-β gene promoter. This is the same mechanism of action used by the ORF3b protein of human SARS-CoV (Zhou *et al*. 2012). The nucleocapsid protein of bat CoV Rm1 is also a functional IFN antagonist.

5.3 MERS CORONAVIRUS

5.3.1 MERS pathology

MERS emerged in 2012 in Saudi Arabia. The mean incubation period is approximately 5 days, and 95% of cases become symptomatic within 13 days, although subclinical or asymptomatic infection may occur and one health care worker shed virus for 42 days in the absence of overt illness (reviewed by Mackay & Arden 2015). The most common symptoms are fever, fever with chills or rigors, cough, shortness of breath, and myalgia. MERS can, however, cause severe lower respiratory tract infection and renal failure and has a much higher fatality rate than SARS (approximately 30%). Gastrointestinal symptoms, such as diarrhea, vomiting, and abdominal pain, may also occur. In experimentally infected dromedary camels, lesions are present in the epithelium of both upper and lower respiratory tracts, with viable virus recoverable from both locations (reviewed by Khalafalla *et al*. 2015). MERS-CoV replicates efficiently in human respiratory tissues and also targets alveolar epithelial cells and the endothelium of lung blood vessels. In the lungs of experimentally infected macaques, MERS-CoV was found primarily in type I and type II pneumocytes (reviewed in Hilgenfeld & Peiris 2013; van Doremalen *et al*. 2014b).

Approximately 75% of human patients have one or more underlying medical condition, such as diabetes; chronic kidney, heart, or lung disease; hypertension; asthma; obesity; smoking; steroid use; malignancy; recent surgery; or co-infection with influenza A virus, parainfluenza virus, herpes simplex virus, or pneumococcus (Abdel-Moneim 2014). Outbreak index cases have been traced to Saudi Arabia, Jordan, Qatar, and the United Arab Emirates, and travel-associated cases have been found in an ever-expanding number of locations, including France, Germany, Italy, Tunisia, the UK, the US, South Korea, and Thailand. Fatal cases of MERS tend to occur in those having underlying illnesses, especially those who are immunocompromised. Secondary transmission has become a major means of transmission to healthy family members and in hospitals to health care providers, to other patients, and even to those paying brief visits to a ward with an undiagnosed MERS patient. The ability to undergo human-to-human transmission appears to be increasing over time and was the sole factor operating in the large outbreak in South Korea.

5.3.2 Viral and cellular proteins and their role in entry into the host cells

The mammalian host cell receptor for the MERS-CoV S protein is dipeptidyl peptidase IV (DPP4 or CD26), a type II transmembrane protein expressed in the human respiratory tract, kidneys, small intestine, liver, parotid gland, spleen, testes, prostate, and activated immune cells. It is conserved among many animal species, including nonhuman primates, dromedaries, sheep, cows, and bats (reviewed in Hilgenfeld & Peiris 2013). The MERS-CoV S protein's S1 core domain is responsible for DPP4 recognition and high affinity binding to host cells. The S2 domain serves as a C-terminal 240-amino acid RBD composed of amino acids 367–606. The external subdomain portion of viral S2 binds to the host DPP4 receptor and has thus been designated the RBM (Lu *et al*. 2013; Wang *et al*. 2013). Several of the amino acids involved in binding the MERS-CoV S protein are also crucial in binding to the human enzyme adenosine deaminase (ADA), a natural DPP4 ligand. Recombinant forms of ADA are able to compete with the MERS-CoV S1 region for DPP4 binding to cell lines *in vitro* and inhibit their infection. ADA's normal functions include differentiation and maturation of lymphoid cells of the adaptive immune system by stimulating dendritic cells, costimulating T helper lymphocytes, and increasing production of proinflammatory cytokines that may be involved in MERS pathogenesis (reviewed in Raj *et al*. 2014a). The ability of recombinant ADA to limit *in vitro* infection of cells may aid in the development of other antagonists for DPP4-mediated entry of MERS-CoV, thus limiting disease severity. Five human MERS-CoV accessory proteins share homology only with those from bat HKU4 and HKU5 coronaviruses (Raj *et al*. 2014b). As with SARS-CoV, MERS-CoV has mechanisms to avoid triggering the host's interferon response, but unlike SARS-CoV, it remains sensitive to any interferon that is produced (reviewed in Hilgenfeld & Peiris 2013).

MERS-CoV has been subdivided into several clades. Clade A is only known to contain variants derived from African green monkey kidney Vero cells, cell-culture passaged EMC/2012 variants, two Jordan-N3 variants, but no camel-derived MERS-CoV variants. Clade B contains Bisha 1, directly sequenced from the upper respiratory tract of a human primary MERS case, having a 115 nucleotide difference from the EMC/2012 variants produced after culturing MERS-CoV from this patient *in vitro*

(reviewed in Mackay 2015). Clade C contains a very divergent variant derived from an Egyptian dromedary, NRCE-HKU205|Nile|2013, most likely imported from Sudan. An additional virus from a *Neoromicia capensis* bat, NeoCoV, is more closely related to MERS-CoV than previous bat sequences and may link camel and bat viruses as members of the same CoV species (described in more detail below; reviewed in Mackay & Arden 2015). Nine or more of the human MERS-CoV genomes contain amino acid substitutions in the RBD and several of the substitutions appear to be markers of adaptive change. An *in vitro* analysis did not, however, demonstrate differences in viral shedding, replication, or immune escape among the tested MERS-CoV variants (reviewed in Mackay & Arden 2015).

5.3.3 MERS-CoV and spillover from domestic livestock

MERS-CoV transmission to humans as a zoonotic spillover has been convincingly traced to exposure to live dromedaries or their raw milk or urine. In addition to the presence of high neutralizing antibody titers to MERS-CoV in many dromedaries throughout the Middle East, viral genomes identical to that of human MERS-CoV have been isolated from these animals. In one instance, a human isolate was identical to that obtained by a nasal swab from a sick dromedary for which the patient had cared (Haagmans *et al*. 2014).

Cows, goats, sheep, and dromedary camels are the primary sources of meat and milk in Jordan, Saudi Arabia, and United Arab Emirates. Two species of camels exist: one-hump dromedaries (*C. dromedarius*) and two-hump Bactrian camels (*C. bactrianus*). Dromedaries are found in hot desert regions of the Arabian Peninsula, Middle East, Afghanistan, central Asia, India, and parts of Africa. Dromedary density is highest in and around the Greater Horn of Africa (Ethiopia, Somalia, Kenya, Sudan, and South Sudan) and these camels are exported to other regions. In the Arabian Peninsula, Yemen has the highest dromedary density, particularly the Ha'il region, however known cases of human MERS are less common in this region than in Saudi Arabia. Human-dromedary contact occurs at festivals, races, sales, and parades (Mackay & Arden 2015). MERS-CoV infection in dromedaries is asymptomatic or results in only mild respiratory symptoms, so its presence may be undetected (reviewed in Gossner *et al*. 2014). Bactrian camels inhabit the colder steppes of Mongolia, Central Asia, Pakistan, and Iran.

Experimental infection of dromedaries with MERS-CoV leads to a mild (nasal discharge and slight fever), transient, primarily upper respiratory tract infection (Adney *et al*. 2014). The camels shed large amounts of infectious virus and RNA in their nasal secretions until 7 days after infection and viral RNAs were detectable for up to four additional weeks. Despite the detection of small levels of MERS-CoV RNA by PCR in exhaled breath, no infectious virus was found at that time (reviewed in Khalafalla *et al*. 2015).

Very little virus is present in oral samples and may result from nasal drainage. No RNA was detected in fecal, urine, serum, or blood samples. Infectious virus was detected in several tissues from a camel euthanized on day 5 post-infection, but not from camels euthanized at days 28 or 42. No infectious virus was present in the digestive tract (abomasum, forestomachs, duodenum, jejunum, colon, or rectum), liver, spleen, kidney, bladder, or heart of these animals. Infectious virus was confined to tissues of the upper respiratory tract (primarily the nasal turbinates, but also the olfactory epithelium,

pharynx, and larynx), lower respiratory tract (trachea and in one of the lung lobes), and lymph nodes (retropharyngeal, mediastinal, mesenteric, and tracheobronchial). Mild to moderate inflammatory lesions, comparable with that caused by the common cold among humans, were present in the pseudostratified columnar epithelial cells lining the upper and lower respiratory tract, but not in the alveoli. The location of MERS-CoV in the upper respiratory tract may at least partially explain the lack of systemic illness in naturally infected camels as well as the means of camel-to-camel and camel-to-human transmission (Adney *et al*. 2014).

In a large study of sera from these domestic livestock, all sera from camels from Oman ($n=50$) contained neutralizing antibodies against the S1 region of the MERS-CoV spike protein, while only 14% from the Canary Islands contained these antibodies ($n=105$). Dutch or Spanish sheep, goats, cattle, and other camelids (2 Dutch Bactrian camels, 2 llamas, 6 alpacas, and as well as 2 Bactrian camels, 5 llamas, 18 alpacas, and 2 guanacos from Chile) were seronegative (Chan *et al*. 2015). Antibody titers ranged from 1/320 to 1/2560 for Omani camels, but were only 1/20 to 1/320 for those from the Canary Islands (Reusken *et al*. 2014). Unfortunately, this study did not examine sera from sheep, goats, or cattle from MERS-endemic regions. Studies published in 2013 and 2014 failed to detect MERS-CoV-specific antibodies in sheep, goats or cattle in Jordan or Saudi Arabia (reviewed in Gossner *et al*. 2014).

Antibodies to MERS-CoV in dromedaries have also been detected in Jordan, Egypt, United Arab Emirates, Saudi Arabia, Qatar, Nigeria, Tunisia, Ethiopia, Kenya, Sudan, South Sudan, and the Canary Islands (Perera *et al*. 2013; Reusken *et al*. 2013; Corman *et al*. 2014; Gossner *et al*. 2014; Reusken *et al*. 2014). The virus appears to have been circulating in dromedaries by 1992 in Saudi Arabia and 2003 in the United Arab Emirates (reviewed in Gossner *et al*. 2014). Many of these animals were also seropositive for the bovine coronavirus, known to widely circulate among camel populations, but they lacked antibodies against SARS-CoV. Some of these samples were collected in 2009 or as early as 2003, indicating that the virus was wide-spread in dromedary populations before the MERS-CoV outbreak in humans (Reusken *et al*. 2014). In a separate study, 80% or more of dromedaries in Somalia and Sudan were seropositive for MERS-CoV in 1983 and similar results were found in Egypt in 1997 (Müller *et al*. 2014). Due to the high levels of civil unrest and war in the former countries, it is possible that human MERS cases have been present in the region and undetected for several decades (Müller *et al*. 2014).

RNA from two to three MERS-CoV genes was detected in nasal swabs from 6 of 14 dromedaries from a farm in Oman. There was 100% identity between a tested S protein fragment from three camels and S protein from several human MERS-CoV isolates, including that of a patient related to that farm, but some sequence differences were found in ORF1 and a MERS-CoV EMC isolate. No viral RNA was found in rectal swabs and fecal samples. All animals had antibodies to MERS-CoV antigen, but not to SARS-CoV or human coronavirus HCoV-OC43 (Haagmans *et al*. 2014).

The owner of a small herd in Saudi Arabia developed a fatal case of MERS after contact with mucus secretions from an ill dromedary. Three of his other eight animals were also ill. Viruses isolated from patient and camel nasal swabs were grown in culture. Full genome sequencing of the cultured patient and human MERS-CoV RNA were identical. No MERS-CoV RNA was recovered from the camel's nasal swabs 28 days later, suggesting a transient, acute infection since all of the ill camels were healthy

several weeks later. RNA was not recovered from milk, urine, or rectal samples from any of the camels in this study (Azhar *et al*. 2014), however, there have been several reports of MERS-CoV in camel feces in Saudi Arabia and in feces and milk in Qatar (reviewed by Gossner *et al*. 2014).

Dromedary infection in Saudi Arabia in 2013–2014 varied by season, with RNA present during the cooler months (November–January) and decreasing with warming weather, reaching a low point in May (Khalafalla *et al*. 2015). Cooler temperatures enhance survival of coronaviruses outside of the host. The cool season is the time of greatest circulation of human respiratory viruses as well as corresponding to the peak of dromedary calving season in Saudi Arabia (Khalafalla *et al*. 2015). Gossner *et al*. (2014), however, found a different seasonal pattern in human case incidence: the first primary case was detected in April 2012, an increase in new human cases occurred around April and May 2013, and a third increase in April 2014. Interestingly, calves are first weaned in March–April at the beginning of the hot season. The calves are very susceptible to diarrhea at this time and infected calves can excrete MERS-CoV in their feces. Milking is usually performed manually and, if teats are not properly cleaned, infected feces from calves may enter into milk consumed by humans (reviewed by Gossner *et al*. 2014).

A large study of more than 750 dromedaries in Dubai demonstrated that more than 96% of adult dromedaries (over 4 years old) were seropositive, as were 85% of calves (less than 1 year old). MERS-CoV RNA was detected in only in nasal swab specimens from dromedaries less than 4 years of age, primarily in calves. Viral isolation from animals in Dubai and Saudi Arabia showed similar age discrimination, suggesting that calves are much more likely to become transiently infected than older animals (Khalafalla *et al*. 2015; Wernery *et al*. 2015). Slaughtering of camels usually involves adults (over 5 yearsold), perhaps accounting for the relative lack of MERS risk for slaughter-house workers (MacKay & Arden 2015). MERS-CoV RNA was detected in 29% of nasal swab samples from live dromedaries and 62% of lung tissue samples from carcasses of healthy animals (Khalafalla *et al*. 2015). MERS-CoV detection is enhanced in human lower respiratory tract samples and is found there for approximately 1 month. During that time, oronasal swab samples tested negative (reviewed in Khalafalla *et al*. 2015). Testing only nasal swabs may therefore fail to detect infected persons or animals.

Cell lines from goats and camels are able to support infection and efficient replication of MERS-CoV (Eckerle *et al*. 2014). A 2013 search of a number of different animal species in Oman, Egypt, and the Canary Islands found MERS-CoV neutralizing antibodies in dromedary camels (Perera *et al*. 2013; Reusken *et al*. 2013). Human kidney cancer, human alveolar adenocarcinoma, bat and goat kidney and lung, and dromedary umbilical cord supported MERS-CoV replication. Viral nucleoprotein was also produced by many experimentally infected mammalian cells, including human *ex vivo* bronchial and lung tissue and embryonic lung fibroblasts, gastrointestinal, liver, and histiocytoma cells (reviewed by Mackay & Arden 2015).

5.3.4 Relatedness of bat-CoV to MERS-CoV

In June 2012, a lineage c beta-CoV, HCoV-EMC/2012 (with variants known as England-Qatar, Jordan-N3 and England 1 and, currently, as MERS-CoV), was isolated from a patient from Saudi Arabia with a fatal case of acute respiratory distress syndrome and

multiple organ dysfunction syndrome with renal failure (Ge *et al*. 2013). A second human case observed 3 months later involved a hospitalized patient from Qatar. The MERS-CoV genome contains between 30 106 and 30 119 nucleotides. It has at least ten predicted ORFs, nine of which appear to be expressed from a nested set of seven subgenomic mRNAs. It has a G+C content of 41% (Woo *et al*. 2012).

At the time, MERS-CoV appeared to be most closely related to the bat coronaviruses HKU4 and HKU5, isolated from *T. pachypus* and *P. abramus*, respectively, in Hong Kong. The latter bat species is widely distributed, not only in China, but also Russia, the Korean peninsula, Japan, Vietnam, Burma, India, and Saudi Arabia and neighboring countries in the Middle East (reviewed in Lau *et al*. 2010b). HKU4 has 30 286–30 316 nucleotides and HKU5 has 30 482–30 488: their G+C contents are 38 and 43%, respectively (Woo *et al*. 2012). MERS-CoV has only 66.3% nucleotide and 66.1% amino acid identity and 63.8% nucleotide and 63.5% amino acid identity with the S proteins of HKU4 and HKU5, respectively (van B 2012). The major difference between human MERS-CoV and bat HKU4 and HKU5 lies in the region between the *S* and *E* genes: MERS-CoV has five ORFs, rather than four found in the bat coronaviruses (Woo 2012). The *RtRp* gene is generally much highly more conserved among coronaviruses and human MERS-CoV has amino acid identities of 89% and 92% with bat HKU4 and HKU5, respectively (van Boheemen *et al*. 2012). Molecular clock analysis indicates that HKU4 and HKU5 diverged from a common ancestor with MERS-CoV hundreds of years ago. Furthermore, complete sequencing of *RdRp, S*, and nucleocapsid genes of 13 HKU4 and 15 HKU5 strains showed that these viruses are stably evolving in each of their bat host species (Lau *et al*. 2010b). Another beta-CoV, VM314, was isolated in 2008 from a *Pipistrellus pipistrellus* bat in the Netherlands. This bat virus has an 88% identity with MERS-CoV in a *RdRp* 332-nucleotide fragment (reviewed in van Boheemen *et al*. 2012). It should be noted that this bat species is also found in Saudi Arabia.

Considerable amino acid variance also exists between bat HKU4 and HKU5 coronaviruses and human MERS-CoV in the RBD region, crucial to host cell binding and tropism (54.4 and 52.9% identity, respectively) (Lau *et al*. 2013). HKU5 additionally has two deletions in the RBM, an especially critical region of the RBD, thus making it even less likely be a progenitor for MERS-CoV (Wang *et al*. 2013). Even the more closely related HKU4 has only 40.8% amino acid identity in the RBM and contains an insertion not present in MERS-CoV. Nevertheless, HKU4's RBD, but not that of HKU5, is able to bind the human DPP4 cellular receptor. The K_D of binding is 35.7 mM, however, about three orders of magnitude lower binding affinity than that of the MERS-CoV RBD. HKU4 binds slightly better to a bat DPP4 than does MERS-CoV, but it should be noted that the bat DPP4 used in the study was from a different bat genus than that from which HKU4 was isolated (Y. Yang *et al*. 2014). Additionally, unlike the MERS-CoV S protein, pseudoviruses containing the HKU4 S protein are able to infect a human cell line via DPP4, but only in the presence of exogenous trypsin, and to a lesser extent than pseudoviruses containing the MERS-CoV S protein. This is due to an inability of the human enzymes TMPRSS2 or endosomal proteases to cleave the bat HKU4 S proteins, although these host proteases effectively cleave the human MERS-CoV S protein (Wang *et al*. 2013; Y. Yang *et al*. 2014). By contrast, MERS-CoV is able to infect established bat cell lines expressing human DPP4 either endogenously or that are engineered to express it. Antibodies against human DPP4 were able to block viral cell entry

(Cai *et al*. 2014). Importantly, the cell lines used in this study were established from bats found in western Asia and northern Africa. Those cell lines able to be infected were from bat embryos, fetal lung and kidney, or adult kidney, but not from adult bat lung (Cai *et al*. 2014). This suggests that if human or dromedary MERS-CoV was indeed of bat origin, it may have been transmitted via the urinary, rather than the respiratory, route. Lung cells from *Rhinolophus landeri*, however, as well as kidney cells from *Roussetus aegyptiacus, P. pipistrellus, Myotis daubentonii*, and *Carollia perspicillata* bats are able to replicate MERS-CoV. These bat species represent four major chiropteran families from both bat suborders (Müller *et al*. 2012).

MERS-CoV can also infect cell lines from nonhuman primates, camels, civets, rabbits, goats, cattle, sheep, chickens, and pigs, but not cell lines of cat, dog, hamster, mouse, ferret, chicken, or insect origin (reviewed in Cai *et al*. 2014). Five amino acid variations in the MERS-CoV-binding domain of DPP4 from different species play a role in whether the host is susceptible or resistant to MERS-CoV infection (van Doremalen *et al*. 2014a). MERS-CoV-like antibodies have reported in dromedary camels in several countries having human MERS cases, but not in goats, sheep, cattle, horses, donkeys, or mules from the UAE and Spain (reviewed in van Doremalen *et al*. 2014b; Mackay & Arden 2015). The DPP4 protein from goats, cattle, and sheep are nevertheless still able to function as receptors for MERS-CoV, but with lower efficiency than for DPP4 from camels.

The complex structure by which bat HKU4's viral RBD binds DPP4 is similar to the binding mode used by human MERS-CoV (Wang *et al*. 2013), however, it lacks a helix and two small strands (b2 and b11) in the core subdomain as well as utilizing a 310 helix instead of the α-helix found in MERS-RBD (Wang *et al*. 2013). These key differences between HKU4 and MERS-CoV suggest that that the bat and human coronaviruses are quite distinct in their binding to the MERS-CoV receptor as well as their means of cleavage of the viral S protein. This suggests that changes in both of these processes need to occur before the bat HKU4 CoV can utilize human cells.

MERS-CoV is much more closely related to other bat coronaviruses than to HKU4 or HKU5. One of these is NeoCoV, the RNA of which was obtained directly from fecal material from a South African *N. capensis* bat (Corman *et al*. 2014). The genome consists of 30 100 nucleotides, with a G+C content of 40%, comparable with various MERS-CoV strains, whose genome is 30 100–30 107 nucleotides, with a G+C content of 41%. Amino acid sequence identity between NeoCoV and MERS-CoV strains in seven nested nonstructural protein domains was 97.2–97.4%, exceeding the 90% threshold used by the International Committee on Taxonomy of Viruses to define separate CoV species. Based upon taxonomic and other structural criteria, NeoCoV and MERS-CoV belong to a single viral species. Their S1 units are genetically divergent, suggesting that intraspike recombination events may have occurred during the emergence of MERS-CoV. NeoCoV is a sister taxon of MERS-CoV rooted between a novel African virus camel and all other viruses, suggesting that a higher level of viral diversity exists in camels than in humans and that camels were the source of virus in humans rather than vice versa. The majority of camels in the Arabian Peninsula are imported from the Greater Horn of Africa (Ethiopia, Sudan, Somalia, and Kenya), where several *Neoromicia* bat species also are found. This is an important point, since bats have only limited contact with humans in the Arabian Peninsula (noted in Khalafalla *et al*. 2015). The camels may have thus acquired MERS-CoV from these bats in Sub-Saharan Africa.

Dromedaries may thus have served as mixing vessels for MERS-CoV and other mammalian coronaviruses (Corman *et al*. 2014).

Another candidate for a MERS-CoV precursor from bats was found in a fecal pellet from a female *Neoromicia* cf. *zuluensis* collected in 2011. This beta-CoV (PML/2011) is closely related to MERS-CoV in a conserved 816-nucleotide fragment (1 amino acid difference; 0.3%). This is more closely related than a Ghana virus from *Nycteris* bats and the Chinese HKU4 and HKU5 bat coronaviruses previously discussed (5.5–7.7% amino acid difference). It is also more closely related to MERS-CoV than a 2c beta-CoV *RdRp* gene fragment from a Spanish *Hypsugo savii* bat, from a gene fragment from Thailand bat guano, and from a Mexican *Nyctinomops* bat in another, shorter, *RdRp* gene fragment (3.5–8.0% amino acid sequence difference). In fact, PML/2011 is as closely related by Bayesian phylogenetic analysis to MERS-CoV as bat CoV Rs672 is to SARS-CoV in this 816-nucleotide fragment. When a 269-nucleotide fragment from the 3'-terminus of the more variable *S* gene was studied, however, a 10.9% amino acid sequence distance was found between PML/2011 and MERS-CoV. A 13.3% difference in this region was also found between MERS-CoV and a European *Pipistrellus* CoV and a 20.5–27.3% difference between MERS-CoV and bat CoV HUK5 or HUK4 (Ithete *et al*. 2013). Coronaviruses from these bats, therefore, are not as closely related to MERS-CoV as NeoCoV.

The search for MERS-like coronaviruses is continuing in many areas of the world, with mixed results that are dependent, at least in part, upon whether or not the complete genomes are examined and, if not, which genes or gene products are tested and the length of the tested gene fragment. One should also keep in mind that some of the tested genes are highly conserved (*RdRp*), while others are more species-specific and are more relevant to host species tropism and ability to infect host target cells. One of these studies (Memish *et al*. 2013) collected feces and multiple tissue samples from 96 bats of 7 species with roosting sites in date palm orchards in close proximity to the index MERS case in Saudi Arabia. One of 29 tested *Taphozous perforatus* fecal pellets (3.5% infection rate) had 100% nucleotide sequence identity in a conserved *RdRp*190-nucleotide sequence to that of human beta-CoV RNA taken from the MERS index patient. MERS-related CoV RNA sequences have been amplified from members of the bat families Vespertillionidae, Molosidae, Nyteridae, and Emballonuridae (sheath-tailed bats) from Africa, the Americas, Asia, and Europe. It should be noted, however, that MERS itself in humans occurs, however, in very restricted areas of the world despite the detection of MERS-like viruses in bats over wide-spread regions.

In 2013, full-length genomic sequencing was performed on anal swab samples from *Vespertilio superans* from southwestern China (designated BtVs-BetaCoV/SC2013) The genome contains 30 143 nucleotides and has 75.7% nucleotide identity with human MERS-CoV. This is the greatest identity seen using full-length genomic analysis of bat sequences. This bat isolate also had 69.9% nucleotide identity with HKU4-1 and 70.1% identity with HKU5-1. Its S protein clusters in a clade with HKU5 and forms a superclade with HKU5, HKU4, and hCoV-MERS (Yang *et al*. 2014).

It has been suggested by several researchers (Guan *et al*. 2003; Ge *et al*. 2013; Reusken *et al*. 2013; Haagmans *et al*. 2014) that betacoronaviruses circulating in bats "jumped" to an intermediate host (civets and dromedary camels, in the cases of SARS-CoV and MERS-CoV, respectively) from which human infection occurred. If this is the case, it would be useful to determine the relationship between bat and civit or camel

isolates, particularly in the RMB, in order to test this hypothesis. Many other animal species also are infected with betacoronaviruses. It would be of value to also compare their complete RBD sequences with that of pathogenic human viruses in civits and camels, focusing efforts on those bats and other mammal species abundant in the region in which the disease originated.

5.3.5 Transmission of MERS-CoV

The patterns of spread of MERS-CoV among humans suggest that transmission occurs through droplets or contact. The DPP4 receptor expression differs in upper and lower respiratory tracts of humans. This may help to explain the observed human-to-human transmission which occurs more often in those who are immunocompromised or have comorbidities, such as diabetes (reviewed in Raj *et al*. 2014a). Interestingly, detailed population analysis demonstrates multiple MERS-CoV variants within single samples (quasispecies) may be present in individual dromedaries. In individual humans, however, only clonal genomic sequences have been found, suggesting that camel-to-human transmission may permit only specific genotypes capable of by-passing bottleneck selection (Briese *et al*. 2014). Increasing numbers of dromedaries and a recent trend towards locating herding operations near larger population areas may also increase human–camel contact (reviewed by Gossner *et al*. 2014).

Only a relatively small proportion of primary human cases, however, have had direct contact with dromedaries. Other routes of transmission include consumption of unpasteurized camel milk or raw meat or medicinal consumption of camel urine (Gossner *et al*. 2014). Camel milk consumption is becoming increasingly popular in the Arabian Peninsula, where cheese production is difficult and limited. In Saudi Arabia, 78% of the camel milk is unpasteurized, fresh, or fermented when sold to consumers. MERS-CoV has been isolated from camel milk samples, but it not known whether the virus is excreted in milk or if it was contaminated during milking or by an infected suckling calf (reviewed by Gossner *et al*. 2014). MERS-CoV injected into raw camel milk is stable upon refrigeration and infectious virus may be recovered even after storage for 2 days at room temperature, but is destroyed by heating to 63 °C for 30 min (van Doremalen *et al*. 2014a).

MERS-CoV has been detected in low concentration in human urine, so consumption of camel urine may be a risk factor, especially for those with underlying illness or immune deficiencies. Camel urine is customarily used to wash the hands, face, and hair among Bedouins and other camel-herding peoples in parts of the Middle East. Camel urine is also used in some traditional medical practices, such as treatment of gastrointestinal illness, to reduce blood clotting, as an anti-cancer agent, to strengthen the immune response, and to keep parasites out of the hair (reviewed in Abdel-Moneim 2014 and Mackay & Arden 2015). Fresh urine is drunk alone or combined with camel milk and is a component of some ointments and skin creams (reviewed by Gossner *et al*. 2014). Transmission via the eating of raw, contaminated meat is less likely, since normally meat is well-cooked, slaughtering is conducted hygienically, and the meat is chilled when sold commercially (reviewed in Abdel-Moneim 2014).

Distribution of primary cases of MERS is skewed towards older men in the Middle East, while it is fairly balanced among age and gender for secondary cases. This skewing of primary cases may be due to differential human exposure since camel rearing is

an exclusively male activity popular among middle-aged and retired men (Gossner *et al*. 2014). Greater susceptibility to and higher disease severity among those with comorbidities, including those in older age groups, may also be a factor.

MERS-CoV and SARS CoV remain viable for relatively long periods of time on surfaces. On plastic or steel, MERS-CoV remained viable for 8 h at 30 °C and 80% relative humidity, and for 24 h at 30 °C and 30% relative humidity. In aerosols, MERS-CoV viability decreased 89% at 70% relative humidity but only 7% at 40% relative humidity at 20 °C. MERS-CoV survival is less than that of SARS-CoV, however it may thoroughly contaminate a room occupied by a symptomatic patient (reviewed by Mackay & Arden 2015). This should call attention to the risks associated with transmission of MERS-CoV and SARS-CoV by bioaerosols in settings such as waiting, treatment, and patient rooms; emergency departments; and open intensive care facilities. The quality of air exchange, circulation, and filtration; use of proper infection control procedures; and personnel protective care are important factors in risk reduction, particularly in light of the growing numbers of human-to-human hospital-based transmissions in Saudi Arabia and that which occurred in South Korea.

Bats may also indirectly transmit infection to humans. One index case lived and worked in close proximity to an abandoned date palm orchard. Roosting bats and guano was present in abandoned wells and ruins of the area. Food or water of domestic animals, including dromedaries, in areas containing palm orchards may be contaminated with bat guano, saliva, and/or urine, infecting the camels, and leading to human infection (reviewed in Abdel-Moneim 2014). This hypothesis bears testing in areas in where bats and dromedaries cohabit.

5.4 OTHER CORONAVIRUSES OF BATS

The contention that bats may act as a major reservoir of alpha- and betacoronaviruses is supported by the fact that their genetic diversity is greater in bats than is currently known for any other host (Drexler *et al*. 2014). Even though coronaviruses are found in bat feces or urine, they cause no apparent gastrointestinal or other disease symptoms in these hosts, perhaps due to their high level of anti-CoV antibody generation (Drexler *et al*. 2014). Persistence of viruses in bat populations appears to rely on massive amplification during bat reproductive cycles, possibly due to fecal–oral transmission, as seen with other viruses, such as filoviruses, henipaviruses, astroviruses, and lyssaviruses in bat populations (Drexler *et al*. 2014).

In addition to those CoV species discussed previously, human coronavirus HCoV-229E and coronaviruses from Ghanaian *Hipposideros* bats share common ancestry (reviewed in Reusken *et al*. 2013). Further work to examine the extent of diversity of coronaviruses in other groups of mammals, especially in China and the Arabian Peninsula, is required, especially since coronaviruses infect mice and mice are known reservoirs for another severe respiratory illness caused by hantaviruses. A distinct lineage c beta-CoV (EriCoV) has been identified in hedgehogs. Human CoV HCoV-OC43 also has recent common ancestry with bovine CoV (reviewed in Reusken *et al*. 2013).

A 2013 study amplified regions of RNA encoding the helicase, S, and capsid or envelope proteins from 96 bats of 7 species with roosting sites in date palm orchards in close proximity to the index MERS case in Saudi Arabia. Of note, the chosen RNA

regions detected both alpha-CoV and beta-CoV RNA sequences even though this test was believed to be a MERS-CoV-specific assay and MERS-CoV is a beta-CoV. In this study, both alpha-CoV and beta-CoV RNA were amplified from insectivorous *T. perforatus* and *R. hardwickii* and the frugivorous *E. helvum* bats, but only alpha-CoV RNA was amplified from the insectivorous *P. kuhlii*. CoV RNA was present in bat rectal swabs and 23% of the fecal pellets and roost feces, with alpha-CoV detected more often than the beta-CoV group to which human SARS-CoV and MERS-CoV belong. No CoV RNA was found in throat swabs or urine or serum samples, suggesting that transmission between animals occurs via contact with infected fecal material (Memish *et al*. 2013).

Samples were collected from anal swabs of 75 insectivorous Italian Vespertillionidae bats (*Myotis myotis, M. blythii, Eptesicus serotinus*) in northern Italy after bat reproduction during the summers of 2008–2012. Two novel alphacoronaviruses were detected from *M. blythii* as well as two new lineage c betacoronaviruses from *E. serotinus* (ITA31/384/2012). Using nested RT-PCR, the betacoronaviruses were found to have 96.9% predicted amino acid sequence homology in a 816-nucleotide fragment of the conserved *RdRp* gene and to cluster with bat CoV from Spanish *E. isabellinus* (also found in the northern Sahara). The new alpha-CoV clusters with Spanish bat CoV from *M. blythii* and *Miniopterus schreibersi*, as well as *Myotis dasycneme* from the Netherlands (De Benedictis *et al*. 2014). Five distinct alpha-CoV clades were isolated from rectal swabs of *Rhinolophus* and *Myotis* species from Yunnan, China, as well (He *et al*. 2014). Several other studies found SARS-like CoV in several insectivorous bat species in China, Europe, and Africa that have 76–78% nucleotide identity in variable *S* gene and a 19-amino acid deletion in the RBD. Previous reports found coronaviruses in 20 bat species from four families throughout China and Hong Kong: 10 species from Vespertillionidae, 8 from Rhinolophidae, 1 from Molossidae, and 1 from Pteropodidae (reviewed in He *et al*. 2014). Muluccan naked-backed fruit bat (*Dobsonia moluccensisi*) from Indonesia harbors a beta-CoV RNA in 4.1% of tested fecal samples ($n=74$) (Anindita *et al*. 2015). This virus is most closely related to BatCoV HKU9 from China and BatCoV KY06 from Kenya. The large number and diversity of beta-CoV isolates from bats, the relative lack of knowledge of CoV diversity in other mammal species, the lack of some ORF, presence of nucleotide deletions in critical regions, and the wide range of nucleotide and amino acid identity in the RBD make it difficult to know which bat CoV served as a predecessor to either SARS-CoV or MERS-CoV or whether the CoV predecessor originated in a different group of mammals. Further research should help to uncover the history of the pathogenic human coronaviruses and perhaps the likelihood that bat-to-human zoonotic transfer will happen again.

A study of feces from multiple bat species inhabiting an abandoned mineshaft in China ($n=256$) found CoV RNA in feces from all of the following species: *R. sinicus, R. affinis, Hipposideros pomona, M. schreibersi, Miniopterus fuliginosus*, and *Miniopterus fuscus* (Ge *et al*. 2016). Prevalence of infection among the bat species ranged from 45 to 74%. Almost all of the viral sequences were related to previously known alphaviruses: HKU1 was present in *R. sinicus, M. schreibersi, M. fuliginosus*; HKU2 in *R. sinicus* and *R. affinis*; HKU7 in *M. schreibersi*; HKU8 in *R. sinicus, R. affinis, M. schreibersi*, and *M. fuscus*; and HKU10 in *H. pomona*. A novel SARS-like beta-CoV (RaBtCoV/4991) was also detected in *R. affinis* in addition to a novel beta-CoV (HpBtCoV/3740-2) in *H. pomona*. Co-infection with several CoV species occurred in all six of these bat species, a situation that increases the chance of recombination (Ge *et al*. 2016).

5.5 CONCLUSIONS

Coronaviruses are large, enveloped, ssRNA (+) viruses that infect many mammals and birds. Alpha- and betacoronaviruses contain members that cause mild to life-threatening respiratory, enteric, hepatic, or neurological disease in humans. HCoV-229E, HCoV-OC43, HCoV-NL63, and HCoV-HKU typically cause mild cold-like symptoms in immunocompetent humans, however, the SARS-CoV and MERS-CoV betacoronaviruses cause severe respiratory disease with high mortality rates. SARS-CoV, civet SARS-related coronaviruses, and SARS-related *Rhinolophus* bat coronaviruses are from beta-CoV lineage b, while MERS-CoV, and HKU4 and HKU5 bat coronaviruses are from lineage c. The following discussion will focus on betacoronaviruses from lineages b and c.

Genetic diversity in coronaviruses is partially due to the infidelity of its polymerase and their atypically large genome. This diversity may allow accumulation of novel traits which equip viral progeny to exploit different ecological niches and hosts, leading to interspecies transmission as may have occurred with HCoV-OC43, a cattle CoV that may have entered humans via zoonotic transmission.

Eleven bat families (the vast majority being insectivorous) contain species that either been exposed to or infected by alpha- or betacoronaviruses. Two of the four frugivorous bat species associated with a SARS-like CoV are restricted to Madagascar, while SARS originated in China. SARS-CoV is known to be transmitted to humans by close contact with several species of live animals from Chinese wet-markets, including palm civits. Civits are claimed to been infected by Chinese fruit bats, however, only insectivorous bat species harbor SARS-like coronaviruses in Asia or Southeast Asia.

Host species and host cellular targets result, to a large degree, from interactions between the viral S protein, responsible for receptor binding and fusion, and the host cell receptor, ACE-2 for SARS-CoV. Sequence identity of the *S* genes of bat and human or civit isolates is 76–78%, and that of the critical S1 domain is only 63–64%. Of note, bat isolates also have a six amino acid insertion and three deletions in S1, several of these found in the RBD.

SARS-CoV is well-adapted to the human ACE2 receptor and is unable to infect bat cells or bind ACE2 from most bats. Bat ACE2 and human ACE2 have amino acid identity of 80–82%, which may contribute to the failure of SARS-CoV to infect bat cells. By contrast, civit and human ACE2 differ by only two amino acids. A human SARS-CoV isolate grew similarly in cells expressing either human or civit ACE2.

Whole-genome sequencing discovered two novel bat coronaviruses (RsSHC014 and Rs3367) whose genes have a high degree of homology with the RBD of SARS-CoV's S protein. One or both of these isolates can use human ACE2 for cell entry, making them better candidates for a predecessor to SARS-CoV than other bat coronaviruses. Full-genome sequencing of human and palm civit SARS-CoV isolates, however, revealed 99.8% homology, much higher than that seen for bat SARS-like CoV.

MERS originated in and is confined primary to the Middle East. The host cell receptor for the MERS-CoV S protein is DPP4, which is conserved among many animal species, including human and nonhuman primates, dromedaries, sheep, cows, and bats. Zoonotic transmission of MERS-CoV to humans is via nasal secretions of dromedaries, drinking their raw milk or urine, and human-to-human. One human MERS-CoV isolate

was identical to that of a sick dromedary with which the human had close contact, further strengthening the ties between human and dromedary MERS-CoV.

Two bat MERS-like coronaviruses, HKU4 and HKU5, have been suggested to be linked to human infections. However, they have very low (40–55%) identity to the human MERS-CoV RBD and HKU5 also contains deletions in this region. This evidence strongly suggests that these bat viruses are unlikely to be responsible for transmission to humans. Since bat kidneys and urine are infected with these coronaviruses, transmission to humans, if it were to occur, would be via bat urine.

MERS-CoV is much more closely related to NeoCoV from fecal material of a South African bat (Corman *et al*. 2014). Amino acid identity between the bat and human viruses for seven proteins was approximately 97% and taxonomic criteria suggest that NeoCoV and MERS-CoV are a single viral species. It should be noted that the presence of viral RNA or proteins in feces does not necessarily mean that the bats were infected, since the viruses may instead have merely passed through the animals' digestive tracts. A number of other studies found varying degrees of nucleotide homology or identity between human MERS-CoV and various bat coronaviruses using relatively small fragments of conserved genes. The fact that these bats were from locations throughout the world and that human MERS is acquired in very restricted areas of the world would suggest that there is little risk of zoonotic transmission from bats and that research efforts perhaps should focus to a greater degree on dromedaries, which are known to transmit MERS-CoV to humans.

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