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Invited Minireview—

The Relationship of Severe Acute Respiratory Syndrome Coronavirus with Avian and Other Coronaviruses

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SUMMARY. In February 2003, a severe acute respiratory syndrome coronavirus (SARS-CoV) emerged in humans in Guangdong Province, China, and caused an epidemic that had severe impact on public health, travel, and economic trade. Coronaviruses are worldwide in distribution, highly infectious, and extremely difficult to control because they have extensive genetic diversity, a short generation time, and a high mutation rate. They can cause respiratory, enteric, and in some cases hepatic and neurological diseases in a wide variety of animals and humans. An enormous, previously unrecognized reservoir of coronaviruses exists among animals. Because coronaviruses have been shown, both experimentally and in nature, to undergo genetic mutations and recombination at a rate similar to that of influenza viruses, it is not surprising that zoonosis and host switching that leads to epidemic diseases have occurred among coronaviruses.

Analysis of coronavirus genomic sequence data indicates that SARS-CoV emerged from an animal reservoir. Scientists examining coronavirus isolates from a variety of animals in and around Guangdong Province reported that SARS-CoV has similarities with many different coronaviruses including avian coronaviruses and SARS-CoV–like viruses from a variety of mammals found in live-animal markets. Although a SARS-like coronavirus isolated from a bat is thought to be the progenitor of SARS-CoV, a lack of genomic sequences for the animal coronaviruses has prevented elucidation of the true origin of SARS-CoV. Sequence analysis of SARS-CoV shows that the 5′ polymerase gene has a mammalian ancestry; whereas the 3′ end structural genes (excluding the spike glycoprotein) have an avian origin. Spike glycoprotein, the host cell attachment viral surface protein, was shown to be a mosaic of feline coronavirus and avian coronavirus sequences resulting from a recombination event. Based on phylogenetic analysis designed to elucidate evolutionary links among viruses, SARS-CoV is believed to have branched from the modern Group 2 coronaviruses, suggesting that it evolved relatively rapidly. This is significant because SARS-CoV is likely still circulating in an animal reservoir (or reservoirs) and has the potential to quickly emerge and cause a new epidemic.

RESUMEN. *Estudio Recapitulativo por Invitación*—Relación del coronavirus causante del síndrome respiratorio agudo severo con coronavirus aviares y otros coronavirus.

En Febrero del año 2003, en la provincia de Guangdong, China emergió en humanos un coronavirus causante del síndrome respiratorio agudo severo (por sus siglas en Inglés SARS-CoV) que causó una epidemia con un impacto severo en la salud pública, los viajes y el intercambio comercial. Los coronavirus tienen distribución mundial, son altamente infecciosos y extremadamente difíciles de controlar porque poseen alta diversidad genética, periodos cortos de regeneración y una alta tasa de mutación. Pueden causar enfermedades respiratorias, entéricas y en algunos casos enfermedades hepáticas y neurológicas en una amplia variedad de animales y en humanos. Entre los animales existe un enorme reservorio de coronavirus no reconocido con anterioridad. Debido a que se ha demostrado experimentalmente y en la naturaleza que los coronavirus son capaces de sufrir mutaciones y recombinaciones genéticas a una tasa similar a la de los virus de influenza, no es sorprendente que el cambio de huésped y la zoonosis que conlleva a enfermedades epidémicas hayan ocurrido entre los coronavirus. El análisis de la secuencia del genoma del SARS-CoV indica que este emergió de un reservorio animal. Analizando los aislamientos de coronavirus provenientes de una variedad de animales dentro y en los alrededores de la provincia de Guandong, los científicos reportaron que SARS-CoV tiene similitudes con diferentes coronavirus incluyendo coronavirus aviares y virus parecidos a SARS-CoV provenientes de una variedad de mamíferos hallados en mercados de animales vivos. Aun cuando se piensa que un virus parecido al SARS-CoV aislado en un murciélago es el progenitor del SARS-CoV, la inexistencia de secuencias de los coronavirus de este animal ha impedido elucidar el verdadero origen del SARS-CoV. El análisis de la secuencia del SARS-CoV muestra que el gen 5′ de la polimerasa tiene un ancestro mamífero, mientras que los genes estructurales del extremo 3′ (excluyendo la glicoproteína de la espícula) tienen un origen aviar. Se ha demostrado que la glicoproteína de la espícula, que es la proteína viral de superficie que permite el contacto con el huésped, es un mosaico de secuencias de coronavirus felinos y coronavirus aviares resultante de un evento de recombinación. Basándose en análisis filogenéticos diseñados para elucidar las interrelaciones evolutivas entre virus, se cree que el SARS-CoV se separó del grupo 2 de coronavirus modernos, sugiriendo que evolucionó relativamente rápido. Esto es muy significativo porque es probable que el SARS-CoV aun esté circulando en un reservorio o reservorios animales y tiene el potencial de emerger rápidamente y causar una nueva epidemia.

Key words: severe acute respiratory syndrome, avian coronavirus, phylogenetic relationship, molecular evolution, mutation, recombination, animal reservoir, emergence, SARS-CoV, infectious bronchitis virus, turkey coronavirus

Abbreviations: BCoV = bovine coronavirus; E = envelope; FIPV = feline infectious peritonitis virus; HCoV = human coronavirus; HE = hemagglutinin-esterase; IBV = infectious bronchitis virus; M = membrane; MHV = mouse hepatitis virus; N = nucleocapsid; PEDV = porcine epidemic diarrhea virus; RBD = receptor binding domain; RdRp = RNA-dependent RNA-polymerase; S = spike; SARS = severe acute respiratory syndrome; SARS-CoV = severe acute respiratory syndrome coronavirus; TCoV = turkey coronavirus; TGEV = transmissible gastroenteritis virus

Intensive animal agriculture practices, human population growth, and cultural habits and customs have put humans in close contact with animal reservoirs of viruses that have the potential to cause zoonotic diseases. In February 2003, severe acute respiratory syndrome (SARS) in humans was reported in Guangdong Province, the People's Republic of China. The etiological agent of SARS was quickly identified as a newly emerged coronavirus, but not before the disease spread to over 24 countries in only a few months, infecting 8098 people worldwide and killing 774 (World Health Organization, http://www.who.int/csr/sars/en/; 10,30,36). It is widely believed that the SARS coronavirus (SARS-CoV) originated from an animal reservoir, but the true origin of the virus is still unknown (23,28). There is a newly recognized reservoir of coronaviruses that exists among animals. Because coronaviruses have been shown, both experimentally and in nature, to undergo genetic recombination by a genomic template-switching mechanism and to generate genetic point mutations at a rate similar to that of other RNA viruses including influenza A viruses, it is not surprising that zoonosis and host switching leading to epidemic diseases occur among coronaviruses. It should be noted that coronaviruses among wildlife and domestic animals can also pose a threat to the health of commercial poultry.

Many questions regarding coronavirus origin, evolution, and genetic diversity can be answered in part from phylogenetic and evolutionary analysis of genome sequence data. Since the first report of SARS-CoV, scientists have been examining coronavirus isolates from a variety of animals in and around Guangdong Province in an attempt to identify natural host reservoirs and determine their roles in propagation, evolution, and transmission of these viruses. Genomic sequence analysis of SARS-CoV shows similarities with many different coronaviruses including avian coronaviruses and SARS-CoV-like viruses from a variety of mammals found in live animal markets in China (22,39). However, a lack of genomic sequences for animal coronaviruses has hindered efforts to identify the true origin of the SARS-CoV. That information is essential if we are to prevent future coronavirus outbreaks.

Coronaviral diseases. Coronaviruses are worldwide in distribution and highly infectious by nature. They cause respiratory, enteric, and in some cases hepatic and neurological diseases in a wide variety of animals and humans (25). The diseases can be acute or chronic and can be transmitted by respiratory or enteric routes. Most coronaviruses replicate in the epithelial cells of the upper respiratory tract and the enteric tract causing respiratory disease and diarrhea. Other sites of infection in the host can include the kidney, reproductive tract, liver, spleen, thymus, and brain (18,25).

The disease caused by the SARS-CoV is characterized by a lower respiratory tract infection accompanied by high fever, headache, loss of appetite, and diarrhea in about 10%–20% percent of patients (1,34,35). At the onset, a mild respiratory disease is observed with a dry cough that usually develops into pneumonia after 7 days (19). The SARS-CoV is primarily transmitted by respiratory aerosol but the virus is also shed in the feces, making fecal–oral transmission a possibility (48). The virus can infect nonhuman primates, civet cats, domestic cats, ferrets, mice, and golden Syrian hamsters, whereas pigs and chickens are refractory to infection (42,47,48).

The avian coronaviruses, infectious bronchitis virus (IBV) and turkey coronavirus (TCoV), are known to cause a respiratory disease in chickens and enteric disease in turkeys, respectively (8,15). Infectious bronchitis in chickens is a mild disease characterized by watery eyes, catarrhal tracheitis, swollen sinuses, sneezing, coughing, and tracheal rales. Turkey coronavirus causes a severe diarrhea in poults less than 4 wk of age and has been associated with poult enteritis and mortality syndrome (16). The clinical signs can include depression, anorexia, and dehydration as a result of a watery diarrhea, which can contain urates and mucus. The lower intestines including the ceca are often thin-walled and pale (14). Chickens are the only animals naturally infected by IBV, and TCoV infects turkeys of all ages and possibly chickens, but only young turkeys develop clinical disease (6,14).

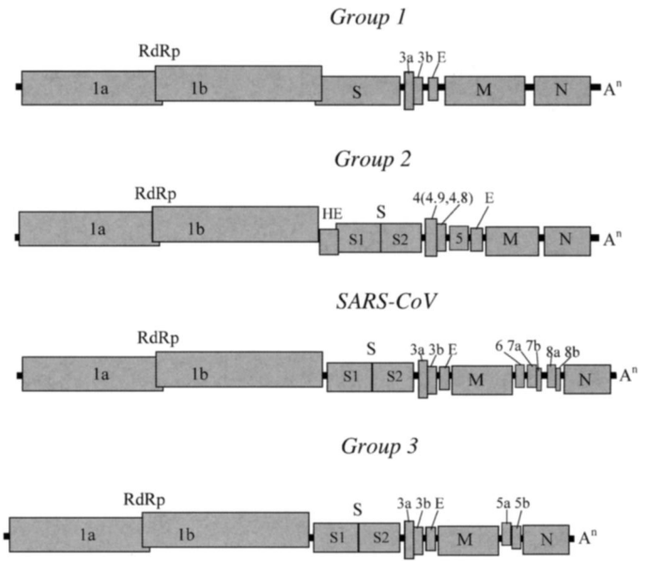

Fig. 1. Gene organization for the three groups of coronaviruses and for SARS-CoV. The gray boxes depict open reading frames coding for known viral proteins and are showing gene order only, they are not to scale. A^n = polyA tail.

Coronaviruses. Coronaviruses are enveloped viruses containing the largest (~28 to 30 kb) single-stranded positive-sense RNA genome known (18). The shape of the virion is pleomorphic and thus varies in diameter from 80 nm to 100 nm. The major proteins encoded by the viral genome are, in order, starting at the 5′ end, the viral polymerase (open reading frames 1a and 1b), hemagglutinin-esterase (HE, in some viruses), the spike (S) glycoprotein located on the surface of the virus, a small envelope protein (E), an integral membrane glycoprotein (M), and the nucleocapsid protein (N), which is closely associated with the viral RNA. Several nonstructural and regulatory proteins are also encoded by the viral genome (see Fig. 1).

Coronaviruses belong to the family *Coronaviridae* in the *Nidovirales* order (11). Other families in the *Nidovirales* order are *Arteviridae* and *Roniviridae*, which include viruses that infect swine and equine, and viruses that infect invertebrates, respectively. Coronaviruses are divided into three groups based on antigenicity and genetic structure (see Fig. 1) (25). Group I viruses include, among others, transmissible gastroenteritis virus (TGEV) in pigs, feline infectious peritonitis virus (FIPV), canine coronavirus, human coronavirus (HCoV) strain 229E, and porcine epidemic diarrhea virus (PEDV). The viruses in Group I do not have an HE protein; the M protein is N-glycosylated and the S-glycoprotein is not cleaved. Some examples of Group II coronaviruses are mouse hepatitis virus (MHV), bovine coronavirus (BCoV), and HCoV strain OC43. Most of the coronavirus in Group II have an HE protein; the M protein is O-glycosylated and the S-glycoprotein is cleaved into two subunits. The Group III coronaviruses include the avian coronaviruses IBV, TCoV, and pheasant coronavirus. Viruses in Group III do not have an HE protein; the M protein is N-glycosylated and the S-glycoprotein is cleaved. Based on sequence analysis of the polymerase protein, the SARS-CoV is currently

placed as a distant member of the Group II coronaviruses. However, like Group III avian coronaviruses, it does not have an HE protein, and the SARS-CoV membrane protein is N-glycosylated and the S-glycoprotein was shown to be cleaved *in vitro* (44,51). It is also interesting to note that the gene organization of the 3′ end of the SARS-CoV genome is most similar to the Group III avian viruses (Fig. 1).

The most studied coronavirus structural protein is the S-glycoprotein. The S-glycoprotein forms club-shaped projections on the surface of the virus particles. It is anchored in the viral envelope, and in Group II and III coronaviruses, it is posttranslationally cleaved by host cell serine proteases into two subunits designated S1 and S2. The S-glycoprotein mediates host cell attachment and entry. Generally, coronaviruses bind to specific host cell receptors, and several have been identified. The SARS-CoV binds to angiotensin-converting enzyme 2, whereas the avian coronaviruses utilize sialic acid alpha 2,3 linked to galactose and possibly a secondary receptor protein, aminopeptidase N, or other protein, to attach and enter cells (25,27,31,49,50). When the S-glycoprotein binds to its specific cell receptor it undergoes a conformation change involving two heptad repeat regions that brings the S-glycoprotein fusion peptide in close proximity to the viral transmembrane segment, which facilitates membrane fusion and entry into the cell (2).

The S-glycoprotein plays a role in the pathogenesis of the virus (3,25,48). A receptor-binding domain (RBD) within the amino terminus of the S-glycoprotein forms part of the globular head of the mature protein. The RBD of SARS-CoV (residues 318–510), maps to the hypervariable region III in IBV (residues 274–387), which is associated with neutralizing epitopes on the S-glycoprotein (32,50). Changes in the S-glycoprotein and specifically in the RBD can alter host cell specificity and mediate shifts in viral pathogenesis (17,24,37). Replacing the S-glycoprotein gene of an attenuated respiratory strain of TGEV with the S-glycoprotein from a pathogenic enteric strain changed the respiratory strain into a pathogenic enterotropic virus in pigs (37). Changes in the S-glycoprotein can also lead to host switching. Using targeted recombination, the MHV S-glycoprotein gene was substituted for the FIPV S-glycoprotein and the recombinant feline MHV containing the feline S-glycoprotein was now capable of infecting feline cells and lost its ability to infect murine cells (24). A host shift was also observed when TCoV, which is closely related to IBV, acquired a S-glycoprotein gene of unknown origin allowing it to emerge and cause enteric disease in turkeys (20). However, pathogenicity does not appear to be solely related to the S-glycoprotein in some coronaviruses. For example, when the ectodomain of the S-glycoprotein gene in the nonpathogenic Beaudette strain of IBV was replaced with the S-glycoprotein from a pathogenic strain of the same serotype (Mass 41), no difference in pathogenicity was observed between the recombinant and the parental strain (17).

Viral replication and genetic diversity. Coronaviruses have a short generation time and a high mutation rate, which provides the virus with extensive genetic diversity making it extremely difficult to control. To understand how genetic diversity is achieved in coronaviruses it is necessary to know how the virus replicates. After attachment and entry into the cell, the positive-sense viral genome acts as a messenger RNA (mRNA) for the transcription of the viral RNA-dependent RNA-polymerase (RdRp). Then, using a leader-primed mRNA synthesis mechanism, the polymerase generates a 3′ coterminal nested set of subgenomic-sized mRNAs that code for the other viral proteins. The polymerase also replicates the full-length viral genome by the same mechanism. During this process, the polymerase can generate genetic diversity by two means. First, the RdRp does not have proofreading capabilities, so it cannot fix mistakes made while copying the viral genome. The RdRp is estimated to generate mutations at a rate of 5.7×10^{-6} nucleotide substitutions per site per day or 0.17 mutations per genome per day, which is similar to rates reported for avian influenza viruses (40,43). Second, the RdRp can also produce genetic diversity by a template-switching mechanism (5). When two or more different strains of coronavirus enter the same cell, recombination can occur as a result of switching templates from one viral genome to another. In this way, whole genes or genome segments can be exchanged between viruses. This was shown to occur in vaccine strains in the field (21) and in a natural outbreak of IBV in Texas where a "hot spot" for recombination was identified in the S1 gene (45,46). A recent example of template switching occurred in the avian coronaviruses and led to the emergence of TCoV. Analysis of the TCoV S-glycoprotein gene showed the S1 portion to be genetically unique with a putative recombination crossover site identified in the 5′ end of the S2 gene (20). The other genes (3ab, M, 5ab, and N) downstream of the crossover site, including the majority of the S2 gene, were nearly identical to IBV (4,7,14,20). The ancestor of TCoV is clearly IBV, but the origin of the TCoV S-glycoprotein gene, which allowed that virus to emerge and cause disease in turkeys, is not known.

SARS-CoV genetic similarity with IBV, TCoV, and other coronaviruses. Recombination events contribute to the evolution and emergence of coronaviruses by creating mosaic viruses. A BLAST analysis (www.ncbi.nlm.nih.gov/BLAST) as well as phylogenetic and recombination studies of the SARS-CoV genome showed similarities with IBV, BCoV, HCoV (229E), MHV, PEDV, and TGEV (52). Stavrinides and Guttman (39), using Bayesian, neighbor-joining and split decomposition phylogenetic analysis of the full-length SARS-CoV genome showed that the polymerase protein (5′ region of the genome) had a mammalian ancestry and was most closely related to the Group II coronaviruses. In addition, they found evidence that the M and N proteins (3′ region of the genome) had an avian coronavirus origin, which was also shown by Marra *et al.* (30). Because the S-glycoprotein mediates cell attachment and is responsible for host specificity, it was interesting that the SARS-CoV S gene was found to be a mosaic containing both avian infectious bronchitis virus (Group III) and feline infectious peritonitis virus (Group I) sequences (39). The feline sequences, evident in the first 600 residues, are interesting because palm civet cats appear to play a role in the epidemiology of this disease. The IBV-related sequences were evident between residues 601 and 667, suggesting that a recombination event occurred in the middle of the S-glycoprotein gene, which may have contributed to the observed shift in host range. It is unclear which animal may have been the intermediary host.

Phylogenetic studies on TCoV conducted in our laboratory showed that sequences in the TCoV S-glycoprotein were also found in the SARS-CoV (20). Similarities with TCoV appear to be relatively distant and are located in the heptad repeat regions in the S2 subunit. Two heptad repeat regions are commonly found in many class I virus fusion proteins such as avian influenza hemagglutinin, paramyxovirus fusion protein, and coronavirus S-glycoprotein. Those regions are conserved structural features involved in protein folding that facilitates viral and cellular membrane fusion, and similarities among them likely do not indicate a direct parental link (9). Thus, sequence similarities between SARS-CoV and other coronaviruses, including avian coronaviruses indicative of virus origin, need to be carefully considered in the context of functionally conserved regions within the viral genome.

SARS origin and evolution. Initially it was reported that SARS-CoV resulted from a zoonotic shift in a coronavirus from the palm civet cat (*Parguma larvata*) and/or the raccoon dog (*Nyctereutes procyonoides*) (22). But, phylogenetic analysis of recent sequence data

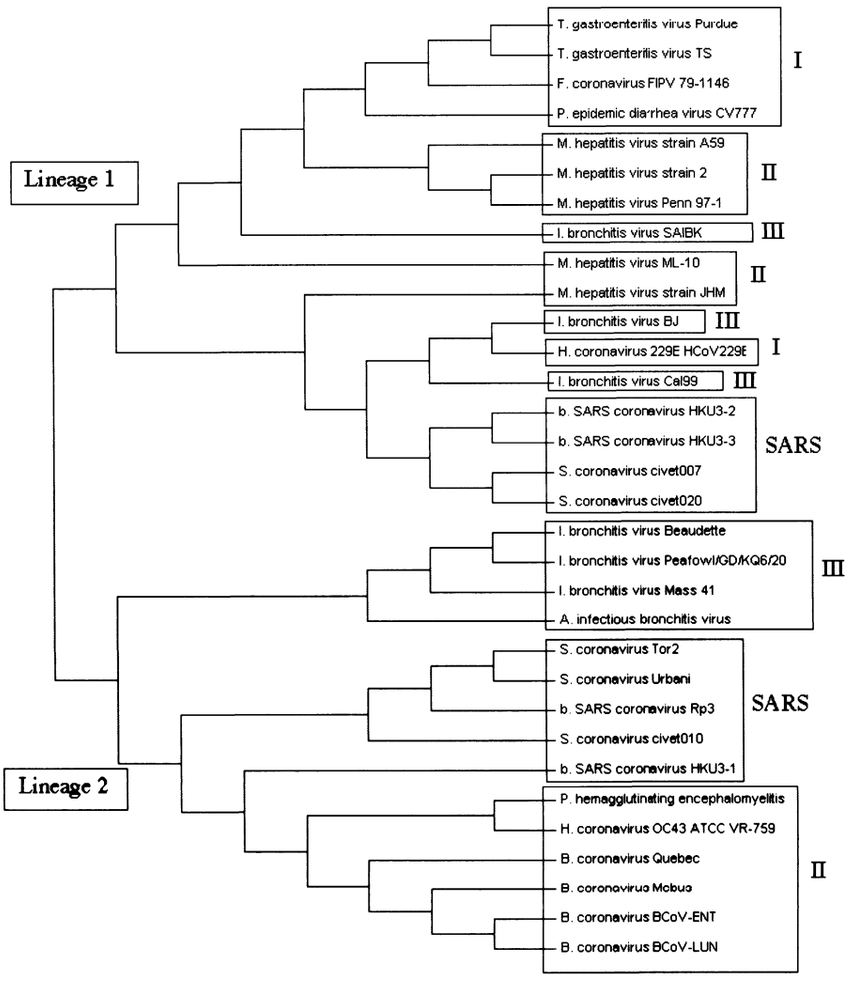

Fig. 2. Phylogeny reconstruction using the Nei-Gojobori (p-distance) analysis (MEGA 3.1, www.megasoftware.net) for all the available animal coronavirus whole genomic sequences listed by the PathoSystems Resource Integration Center (https://patric.vbi.vt.edu/) and available in GenBank (www.ncbi.nlm.nih.gov) as well as selected SARS-CoV isolates. The different coronavirus groups and SARS are boxed and indicated on the right side of the figure. Based on the available data, two lineages are observed and all of the coronaviruses were shown to be undergoing positive Darwinian selection (Z-test of selection, $P < 0.05$). Accession numbers for the sequences analyzed from the top of the figure to the bottom are as follows: NC002306, DQ201447, NC007025, NC003436, NC001846, AF201929, AF208066, DQ288927, AF208067, NC006852, AY319651, NC002645, AY514485, DQ084199, DQ084200, AY572034, AY572038, NC001451, AY641576, AY851295, AY646283, NC004718, AY278741, DQ071615, AY572035, DQ022305, NC007732, NC005147, AF220295, U00735, NC003045, and AF391542.

indicates that a SARS-like bat coronavirus could be the progenitor of SARS-CoV (28). Animals such as palm civet cats, raccoon dogs, and fruit bats, commonly found in the market place in China, are in close contact with people and are likely the source of the SARS-CoV. Unfortunately, the true origin of SARS-CoV is still not clear. Serology, epidemiology, and pathogenicity studies along with phylogenetic analysis of genomic sequence data for a wide variety of animal coronaviruses will be needed before the evolutionary origin of SARS-CoV can be identified.

A tremendous amount of work has recently been conducted on generating, organizing, and analyzing the sequence data for the SARS-CoV, but similar analysis have not been conducted for the animal coronaviruses. Using the Molecular Evolutionary Genetics Analysis program (MEGA 3.1, www.megasoftware.net), we generated a phylogenetic tree (Fig. 2) from alignments for all the available animal coronavirus whole genomic sequences listed by the PathoSystems Resource Integration Center (https://patric.vbi.vt.edu/) and available in GenBank (www.ncbi.nlm.nih.gov) as well as selected SARS-CoV isolates. The Nei-Gojobori (p-distance) analysis shows evolutionary trends among those viruses. Two major lineages are generated with human SARS-CoV isolates branching from the Group II coronaviruses as described in the literature (38). In addition, the bat SARS-like coronaviruses (HKU3-1 and Rp3) and a civet cat (010) isolate groups with two early human SARS-CoV isolates (Tor2 and Urbani) in lineage 2. The other animal-origin SARS-like viruses are in lineage 1 indicating that they are likely not progenitors of SARS-CoV.

As discussed above, the SARS-CoV has sequence similarities to a number of coronaviruses indicating that recombination played a role in its origin; however, the SARS-CoV likely did not emerge from a recombination event between mammalian and avian coronaviruses. It appears that it emerged because of genetic drift, which is the accumulation of genetic mutations over time. A detailed analysis of signature variation residues within the S-glycoprotein from a number of SARS-CoV isolates obtained from palm civets at the live-animal market where the SARS-CoV emerged showed an

accumulation of mutations over a 2-yr period leading to the epidemic group of viruses that caused disease in humans (22). Signature variation residues are unique changes in a gene that can be used to trace virus sources and track virus evolution within a defined environment. It is likely that the palm civet is an intermediate host (if it is assumed that bats are the reservoir for the virus), which contributed to rapid evolution of the virus and subsequent jump to humans. Accelerated positive Darwinian selection following a shift to an intermediate host is well known for avian influenza A viruses (13,26,33,40,41) and most likely also occurs in coronaviruses.

Phylogenetic analysis of the RdRp gene showed that the SARS-CoV is a close relative of the Group II coronaviruses (23,29,38). And, it is now widely believed that SARS-CoV branched from the modern Group 2 coronaviruses, and does not represent a new coronavirus group, which would suggest a recent ancestor for the virus (12). This is significant because it indicates that the virus evolved relatively rapidly to cause disease in humans and could continue to evolve in an animal reservoir, potentially causing another SARS epidemic in the future.

Based on information from the World Health Organization (www.who.int/csr/sars/postoutbreak/en/) the SARS epidemic was officially controlled on July 5, 2003. Although the SARS-CoV does not appear to be currently circulating in people, it is likely being maintained in an animal reservoir(or reservoirs) and could potentially emerge to cause a new epidemic. As in avian influenza virus, genetic mutations and exchange of genetic information among coronaviruses leads to the emergence of new viruses capable of infecting and causing disease in animals and humans. The potential of animal coronaviruses to serve as a genetic reservoir for human disease makes it extremely important to identify the genes and genomes among coronaviruses in animals, especially those animals in close contact with people. Only then will it be possible to monitor reservoirs of the virus, which along with a vigilant global public health surveillance program, is essential for the future control of this disease.

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