Isolation and Characterization of Viruses Related to the SARS Coronavirus from Animals in Southern China

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A novel coronavirus (SCoV) is the etiological agent of severe acute respiratory syndrome (SARS). SARS-like viruses were isolated from Himalayan palm civets found in a live-animal market in Guangdong, China. Evidence of virus infection was also detected in other animals (including a raccoon dog, Nyctereutes procyonoides) and in humans working at the same market. All the animal isolates retain a 29-nucleotide sequence that is not found in most human isolates. The detection of SCoV-like viruses in small, live wild mammals in a retail market indicates a route of interspecies transmission, although the natural reservoir is not known.

Severe acute respiratory syndrome (SARS) recently emerged as a human disease associated with pneumonia (1). This disease was first recognized in Guangdong Province, China, in November 2002. Subsequent to its introduction to Hong Kong in mid-February 2003, the virus spread to more than 30 countries and caused disease in more than 7900 patients across five continents (2). A novel coronavirus (SARS-CoV) was identified as the etiological agent of SARS (3, 4), and the virus causes a similar disease in cynomolgous macaques (5). Human SARS-CoV appears to be an animal virus that crossed to humans relatively recently. Thus, identifying animals carrying the virus is of major scientific interest and public health importance. This prompted us to examine a range of domestic and wild animals in Guangdong Province.

Because the early cases of SARS in Guangdong reportedly occurred in restaurant workers handling wild mammals as exotic food (6), our attention focused on wild animals recently captured and marketed for culinary purposes. We investigated a live-animal retail market in Shenzhen. Animals were held, one per cage, in small wire cages. The animals sampled included seven wild and one domestic, animal species (Table 1). They originated from different regions of southern China and had been kept in separate storehouses before arrival to the market. The animals remained in the markets for a variable period of time, and each stall holder had only a few animals of a given species. Animals from different stalls within the market were sampled. Nasal and fecal samples were collected with swabs and stored in medium 199 with bovine serum albumin and antibiotics. Where possible, blood samples were collected for serology. Before sampling, all animals were examined by a veterinarian and confirmed to be free of overt disease. Serum samples were also obtained, after informed consent, from traders in animals (n = 35) and vegetables (n = 20) within the market. Sera (n = 60) submitted for routine laboratory tests from patients hospitalized for nonrespiratory disease in Guangdong were made anonymous and used for comparison.

Nasal and fecal swabs from 25 animals were tested for SARS-CoV viral nucleic acid by using reverse transcription–polymerase chain reaction (RT-PCR) for the N gene of the human SARS-CoV. Swabs from four of six Himalayan palm civets were positive in the RT-PCR assay (Table 1). All specimens were inoculated into FRhK-4 cells as previously described for virus isolation (5). A cytopathic effect was observed in cells inoculated with specimens from four Himalayan palm civets (Paguma larvata), two of which also positive for coronavirus in the original specimen by RT-PCR. A virus was also detected by virus isolation and direct RT-PCR from the fecal swab of a raccoon dog (Nyctereutes procyonoides). No virus was detectable in six other species sampled. Electron microscopy of one infected cell supernatant (SZ16) showed viral particles with a morphology compatible with coronavirus (fig. S1). Sera from five animals had neutralizing antibody to the animal coronavirus; these were from three palm civets, a raccoon dog, and a Chinese ferret badger, respectively (Table 1).

To further validate the results from the neutralization test, a Western blot assay was used to detect SCoV-specific antibodies from these animal serum samples (Fig. 1). Indications of positive antibodies were observed from samples SZ2, SZ3, SZ11, and SZ17 (which were also positive in the neutralization assay) and from the positive control human serum. No positive signal was observed from those serum samples that were negative in the neutralization test. There was insufficient serum left over from the raccoon dog (SZ13) to be analyzed by this assay.

Table 1. Animal species tested for coronavirus detection. Abbreviations of animal species: B, beaver (Castor fiber); CFB, Chinese ferret-badger (Melogale moschata); CH, Chinese hare (Lepus sinesis); CM, Chinese muntjac (Muntiacus reevesii); DC, domestic cat (Felis catus); HB, hog-badger (Arctonyx collaris); HPC, Himalayan palm civet (P. larvata); RD, raccoon dog (N. procyonoides) (9). N, nasal sample; F, fecal sample; titer to SZ16, neutralizing antibody titer to SZ16; * denotes positive by RT-PCR or virus isolation; ND, not done.

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Sera from humans working in the market were tested for antibody to SZ16 virus by neutralization and indirect immunofluorescence assays. Although 8 out of 20 (40%) of the wild-animal traders and 3 of 15 (20%) of those who slaughter these animals had evidence of antibody, only 1 (5%) of 20 vegetable traders was seropositive. None of these workers reported SARS-like symptoms in the past 6 months. In comparison, none of 60 control sera from patients admitted to a Guangdong hospital for nonrespiratory diseases was seropositive (Table 2).

Two of the virus isolates (SZ3 and SZ16) isolated from the nasal swabs of palm civets were completely sequenced, and the amino acid sequence was deduced. Two other viruses were partially sequenced, from the S gene to the 3’ end of the virus (GenBank accession numbers AY304486 to AY304489). Viral RNA sequences from these original swab samples from animal were confirmed in an independent laboratory (7). The full-length genome sequences had 99.8% homology to the human SCoV, which indicates that the human and animal SCoV-like viruses were closely related. Phylogenetic analysis of the S gene of both human and animal SCoV-like viruses indicated that the animal viruses are separate from the human virus cluster (Fig. 2 and fig. S2). However, the viruses SZ1, SZ3, and SZ16 from palm civets were phylogenetically distinct. The viruses SZ3 and SZ16 had 18 nucleotide differences between them over the 29,709-base pair (bp) genome, whereas the human SCoV isolated from five geographically separate sites (GZ50, CUHK-W1, Tor-2, HKU-39848, and Urbani) differed by only 14 nucleotides (nt). Nevertheless, animal virus SZ13 (rac-

Table 2. Prevalence of antibody to animal SCoV SZ16 in humans. Controls are serum specimens from patients hospitalized for nonrespiratory diseases in Guangdong made anonymous.

| Occupation       | Sample numbers | Antibody positive (%) |
|------------------|----------------|-----------------------|
| Wild-animal trader| 20             | 8 (40)                |
| Slaughterer of animals | 15         | 3 (20)                |
| Vegetable trader  | 20             | 1 (5)                 |
| Control          | 60             | 0 (0)                 |

Fig. 2. Phylogenetic analysis of the nucleotide acid sequence of the spike gene of SCoV-like viruses. Nucleotide sequences of representative SCoV S genes (S gene coding region 21477 to 25244, 3768 bp) were analyzed. The phylogenetic tree was constructed by the neighbor-joining method with bootstrap analysis (1000 replicates) using MEGA 2 (10). Number at the nodes indicates bootstrap values in percentage. The scale bar shows genetic distance estimated using Kimura’s two-parameter substitution model (11). In addition to viruses sequenced in the present study, the other sequences used in the analysis could be found in GenBank with accession number: from AY304490 to AY304495, AY278741, AY278554, AY278491, AY274119, and AY278489.
coon dog) and SZ16 (palm civet) were genetically almost identical, and transmission or contamination from one host to the other within the market cannot be excluded. When the full genome of the animal (n = 2) and human (n = 5, see above) virus groups were compared, the most striking difference was that these human viruses have a 29-nt deletion (5'--CCTACTGGT-CCTACTGGT--3', residue 27869 to 27897) that is 246 nt upstream of the start codon of the N gene (Fig. 3). Of human SCoV sequences currently available in GenBank, there was only one (GZ01) with this additional 29-nt sequence. In addition to that, there were 43 to 57 nucleotide differences observed over the rest of the genome. Most of these differences were found in the S gene coding region. The existence of the additional 29-nt sequence in the animal viruses results in demolishing the open reading frames (ORFs) 10 and 11 in the animal viruses were compared with 11 human viruses. Further extensive surveillance on the origin of the SARS outbreak.

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Materials and Methods
Figs. S1 and S2
References and Notes
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Table 3. Nucleotide sequence variation of the S gene of animal and human SCoV. The nucleotide residues are based on AU278554 numbering. Nonsilent mutations are highlighted in red. Dash indicates a nucleotide deletion.

| Virus  | CATTACATATTTACGGCGAGTGTTCCTCTGCGTGCGCGCTGT | CATTACATATTTACGGCGAGTGTTCCTCTGCGTGCGCGCTGT |
|-------|---------------------------------------------|---------------------------------------------|
| GZ01  | CATTACATTTACGGCGAGTGTTCCTCTGCGTGCGCGCTGT  | CATTACATTTACGGCGAGTGTTCCTCTGCGTGCGCGCTGT  |
| GZ43  | CATTACATTTACGGCGAGTGTTCCTCTGCGTGCGCGCTGT  | CATTACATTTACGGCGAGTGTTCCTCTGCGTGCGCGCTGT  |
| GZ60  | CATTACATTTACGGCGAGTGTTCCTCTGCGTGCGCGCTGT  | CATTACATTTACGGCGAGTGTTCCTCTGCGTGCGCGCTGT  |
| GZ50  | CATTACATTTACGGCGAGTGTTCCTCTGCGTGCGCGCTGT  | CATTACATTTACGGCGAGTGTTCCTCTGCGTGCGCGCTGT  |
| CUIHK-W1 | CATTACATTTACGGCGAGTGTTCCTCTGCGTGCGCGCTGT | CATTACATTTACGGCGAGTGTTCCTCTGCGTGCGCGCTGT |
| HKU-36871 | CATTACATTTACGGCGAGTGTTCCTCTGCGTGCGCGCTGT | CATTACATTTACGGCGAGTGTTCCTCTGCGTGCGCGCTGT |
| HKU-39848 | CATTACATTTACGGCGAGTGTTCCTCTGCGTGCGCGCTGT | CATTACATTTACGGCGAGTGTTCCTCTGCGTGCGCGCTGT |
| HKU-60078 | CATTACATTTACGGCGAGTGTTCCTCTGCGTGCGCGCTGT | CATTACATTTACGGCGAGTGTTCCTCTGCGTGCGCGCTGT |
| HKU-68906 | CATTACATTTACGGCGAGTGTTCCTCTGCGTGCGCGCTGT | CATTACATTTACGGCGAGTGTTCCTCTGCGTGCGCGCTGT |
| Urbani | CATTACATTTACGGCGAGTGTTCCTCTGCGTGCGCGCTGT | CATTACATTTACGGCGAGTGTTCCTCTGCGTGCGCGCTGT |
| Tor2  | CATTACATTTACGGCGAGTGTTCCTCTGCGTGCGCGCTGT | CATTACATTTACGGCGAGTGTTCCTCTGCGTGCGCGCTGT |

**Nucleotide Residue**

|   | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
|   | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 |
|   | 6 | 6 | 6 | 6 | 7 | 7 | 1 | 1 | 1 | 2 | 2 | 2 | 4 | 5 | 5 | 5 | 5 |
|   | 2 | 9 | 9 | 9 | 0 | 0 | 3 | 5 | 9 | 0 | 0 | 5 | 0 | 0 | 5 | 4 | 1 |
|   | 8 | 2 | 7 | 2 | 5 | 7 | 8 | 7 | 2 | 7 | 5 | 6 | 3 | 6 | 8 | 3 | 5 |
|   | 2 | 0 | 1 | 2 | 0 | 6 | 0 | 7 | 2 | 5 | 7 | 8 | 7 | 2 | 7 | 5 | 6 |

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