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Wild animal surveillance for coronavirus HKU1 and potential variants of other coronaviruses

Key Messages

1. Although CoV-HKU1 was not identified in any of the studied animals, a coronavirus closely related to SARS-CoV (bat-SARS-CoV) was identified in 23 (19%) of 118 wild Chinese horseshoe bats by reverse transcriptase polymerase chain reaction (RT-PCR).
2. Complete genome sequencing and phylogenetic analysis showed that bat-SARS-CoV formed a distinct cluster with SARS-CoV as group 2b coronaviruses, distantly related to known group 2 coronaviruses.
3. Most differences between the bat-SARS-CoV and SARS-CoV genomes were observed in the spike gene. The presence of a 29-bp insertion in ORF 8 of bat-SARS-CoV genome, not in most human SARS-CoV genomes, suggests that it has a common ancestor with civet SARS-CoV.
4. Antibody against recombinant bat-SARS-CoV nucleocapsid protein was detected in 84% of Chinese horseshoe bats using an enzyme immunoassay. Neutralising antibody to human SARS-CoV was also detected in those with lower viral loads.
5. This study also revealed a previously unknown diversity of coronaviruses in bats, which are important natural reservoir for coronaviruses including SARS-CoV-like viruses.

Introduction

Coronaviruses are found in a wide variety of animals in which they cause respiratory, enteric, hepatic, and neurological diseases of varying severity. Owing to their unique mechanism of viral replication, coronaviruses have a high frequency of recombination. This tendency for frequent recombination and high mutation rates enable them to adapt to new hosts and ecological niches. Although the isolation of SARS-CoV from caged animals (Himalayan palm civets and a raccoon dog) from wild animal live markets in mainland China suggests that they are the reservoir from which the SARS epidemic originated,¹ several lines of evidence suggest that the civet may have served as an amplification host only for SARS-CoV.²⁻⁵ In January 2005, we reported the discovery of a novel coronavirus, coronavirus HKU1 (CoV-HKU1), from a patient with pneumonia in Hong Kong.⁶ The patient was a 71-year-old Chinese man who had just returned from Shenzhen, China three days before admission. The patient's recent travel history raised a suspicion that he had acquired the virus directly or indirectly through contact with wild animals. We therefore conducted a one-year wild animal surveillance study in Hong Kong to find possible animal reservoirs for CoV-HKU1 or other coronaviruses. Upon identification of coronaviruses, complete genome sequencing and serological studies were performed.

Methods

This study was conducted from 16 December 2005 to 15 December 2007 and entailed a one-year surveillance of non-caged mammals in the wilds of Hong Kong. The study was performed in collaboration with the Agriculture, Fisheries and Conservation Department, and the Food and Environmental Hygiene Department. It was approved by the Committee on the Use of Live Animals in Teaching and Research, The University of Hong Kong.

A total of 489 wild animals, including 309 bats, 160 rodents and 20 monkeys were captured from various locations in Hong Kong. As initial surveillance results in bats were more promising, more bats were captured than originally planned to replace the reduced number of captured monkeys. All animals were released back to the wild after sample collection.

Swabs were taken and kept in viral transport medium at 4°C before processing.⁷ Where possible, blood was collected from the bats for serological studies by a veterinary surgeon. All nasopharyngeal and anal swabs were tested for coronavirus RNA by RT-PCR, using conserved primers targeted to a 440-bp fragment of the *pol* gene.⁸ The sequences of the PCR products were compared with known sequences of the *pol* genes of coronaviruses in the GenBank. Based on the initial results, attempts to isolate bat-SARS-CoV were made by inoculating RT-PCR positive specimens to FRhK-4, HRT-18G, Huh-7, Vero E6, C6/36, and Caco-2 cells, as well as chicken embryonated eggs.

The complete genome of bat-SARS-CoV was sequenced using RNA extracted from anal swabs of three bats as a template. The complete S genes of bat-SARS-CoV from 14 positive samples, with adequate amounts of RNA available, were sequenced using primers targeted to S. Quantitative RT-PCR

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was used to determine the viral loads in positive samples. Serological studies were performed using western blot and enzyme immunoassay (EIA) based on (His)₆-tagged recombinant N protein of bat-SARS-CoV. Neutralisation assays to SARS-CoV were also performed.

Results

A total of 309 bats of 13 species, 160 rodents of 5 species, and 20 monkeys (*Macaca mulatta*) were sampled. RT-PCR for a 440-fragment of *pol* gene of coronaviruses was positive in anal swabs from 39 bats. None of the specimens from rodents and monkeys was positive. Sequencing results suggested the presence of at least three different coronaviruses, including one closely related to SARS-CoV. This virus possessed 88% nucleotide identities to SARS-CoV and was found in 23 Chinese horseshoe bats.⁹ Attempts to stably passage these viruses in cell lines were unsuccessful.

Phylogenetic analysis showed that the bat-SARS-CoV, together with SARS-CoV from humans and civets, belongs to a group distantly related to known group 2 coronaviruses. The three genomes possessed 88% nucleotide and 93% amino acid identities to 10 human and civet SARS-CoV with genome sequences available. Most differences between the bat-SARS-CoV genomes and human and civet SARS-CoV genomes were observed in the S gene, ORF 3, and ORF 8. Compared to SARS-CoV from humans and civets, there were 11 insertions and 15 deletions in the bat-SARS-CoV genome. The 29-bp region, deleted in most human SARS-CoV, is present, as in civet SARS-CoV.

Among tested sera from Chinese horseshoe bats, 12 (67%) of 18 were positive for bat-SARS-CoV antibody by western blot and 31 (84%) of 37 by EIA with titre $\geq 1:400$; but only 8 (42%) of 19 for human SARS-CoV neutralising antibody with titre $\geq 1:20$. Interestingly, those with neutralising antibody had lower viral loads in their anal swabs ($P=0.016$, Student's *t* test).

Discussion

Although none of the animal samples possessed coronavirus closely related to CoV-HKU1, the bat-SARS-CoV was identified, which is closely related to SARS-CoV from humans and civets, suggesting that bats are likely the animal reservoir of SARS-CoV-like viruses. Moreover, a diversity of coronaviruses in bats was also discovered and warrants further investigations. Bat-SARS-CoV was identified from 23 anal swabs from the species *R. sinicus*; 67% and 84% of tested sera from Chinese horseshoe bats were positive for antibodies against recombinant bat-SARS-CoV N protein by western blot and EIA, respectively. Further studies are required to find out if there are yet unidentified intermediate hosts between bats and civets.

Hong Kong has extensive natural areas with 52 terrestrial

mammals, including 22 bat species. The Chinese horseshoe bat (*R. sinicus*), belonging to the family *Rhinolophidae* of the order *Chiroptera* under *Microphiroptera* (microbat), is an insectivorous species widely distributed in forested areas throughout Hong Kong and China (<http://www.hkbiodiversity.net>). The species *R. sinicus* was previously called the *R. rouxii* subspecies *sinicus* but recent karyotyping study has elevated its status to a separate species. Although no data on its migration patterns are available, members of *Rhinolophus* may migrate up to 30 km for hibernation in winter. Interestingly, the nearest wild life market in Shenzhen found to have animals with SARS-CoV is only 17 km away from the locations with bats harbouring bat-SARS-CoV in Hong Kong. The phylogenetic distance from SARS-CoV and the presence of the 29-bp insertion in ORF 8 of bat-SARS-CoV genomes suggest that bat-SARS-CoV is unlikely to result from transmission of SARS-CoV from humans to bats. Instead, bat-SARS-CoV and civet SARS-CoV are likely to have a common ancestor. Nevertheless, the direction of inter-species transmission of SARS-CoV-like viruses or their ancestral relationships cannot be directly inferred. Continuous surveillance for coronaviruses in these flying mammals with roosting behaviour is necessary to assess their potential threats to human health.

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