Investigation of Animal Reservoir(s) of SARS-CoV

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Introduction

Severe acute respiratory syndrome (SARS) is a novel infectious disease in the new millennium. It has been ascertained that a new coronavirus, SARS-CoV, is the etiological agent of SARS. While the extraordinarily rapid isolation and full genome sequencing of SARS-CoV constituted a remarkable scientific achievement, identification of the actual animal reservoir(s) of SARS-CoV is more difficult. Initial evidences indicated that the masked palm civet (*Paguma larvata)* was the primary suspect of the animal origin of SARS (Guan et al., 2003; Song et al., 2005). Recent studies suggested that horseshoe bat is one of the real reservoirs (Lau et al., 2005; Li et al., 2005) and masked palm civet may have only served as an intermediate amplification host for SARS-CoV and fulfilled efficient interspecies transmission (Lau et al., 2005). This chapter will summarize the studies on the animal reservoir(s) of SARS-CoV.

Investigation of Animals and Animal Traders in Markets in 2003

Search for the animal host of SARS-CoV started early during the SARS outbreak. In May 2003, a breakthrough occurred with the identification of a SARS-CoV-like virus in animals in a live-animal market in Shenzhen, Guangdong Province, China. Guan et al. (2003) investigated 25 animals from eight different species in the market. SARS-CoV-like viruses were isolated from two species, four out of six masked palm civets and one raccoon dog (*Nyctereutes procyonoides*). Serological data suggested that three species were positive, three out of the four masked palm civets, the raccoon dog, as well as one of the two Chinese ferret-badgers (*Melogale moschata*). Five species including three hog-badgers (*Arctonyx collaris*), three beavers (*Castor fiber*), four domestic cats (*Felis catus*), three Chinese hares (*Lepus sinensis*), and two Chinese muntjac (*Muntiacus reevesi*) were shown to be negative.

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Two of the viruses from the nasal swabs of masked palm civets, SZ3 and SZ16, were completely sequenced. SZ3 and SZ16 had 18 nucleotide (nt) differences between them over the 29,709 nucleotide genome (99.94% identity), suggesting that they were closely related. The genome homology of SZ3 and SZ16 to the epidemic strain of human SARS-CoV, isolate Tor 2 (Marra et al., 2003) was 99.8%, suggesting that the animal viruses were very similar to the human epidemic strain. A 29 nt insertion was found in the ORF10 (also called open reading frame 8 or ORF8) region in these animal sequences, which was only found in some early human isolates (Guan et al., 2003; Chinese SARS Molecular Epidemiology Consortium, 2004). This 29 nt insertion was suggested as a marker for animal origin. The spike sequence of raccoon dog isolate (SZ13) was also sequenced and was found to be almost identical to the civet isolate SZ16. This led the authors to suggest that transmission or contamination from one host to the other within the market cannot be excluded (Guan et al., 2003).

The prevalence of neutralization antibody to SZ16 in humans in the same market was also evaluated. Eight out of 20 (40%) of the wild animal traders and three of 15 (20%) of those who slaughtered these animals had evidence of antibody, yet only one of 20 (5%) vegetable traders in the market was seropositive and none of the control group from individuals from outside the market (Guan et al., 2003).

Guangzhou Municipal Center for Disease Control conducted serological studies of traders from three animal markets in Guangzhou, Guangdong Province, China in May 2003 (Centers for Disease Control and Prevention (CDC), 2003). Among 508 animal traders, 66 (13%) tested positive for IgG antibody to SARS associated coronavirus by ELISA, while the control groups including hospital workers, Guangdong CDC workers, and healthy adults at clinic had an antibody prevalence of 1–3%. Among animal traders, the highest prevalence of antibody was found among those who traded primarily masked palm civets (72.7%), wild boars (57.1%), muntjac deer (56.3%), hares (46.2%), and pheasant (33.3%). Those for cat, other fowl, and snake were 18.6%, 12%, and 9.2%, respectively. None of the antibody positive traders demonstrated SARS-like disease symptoms. The prevalence of traders with IgG antibody to SARS-CoV of the three tested markets varied (6%, 11%, and 20%, respectively; $p < 0.001$). The results also provide indirect support for the hypothesis of an animal origin for SARS (Centers for Disease Control and Prevention, 2003).

A similar report investigated 635 animal traders in three animal markets (A, B, and C) in Guangzhou, Guangdong Province, China from May to June, 2003 (Xu et al., 2004a). The prevalence of IgG antibody to SARS-CoV was about 16.69% (106/635) in animal traders, significantly higher than that of the control group of vegetable traders (0.72%, 1/139). The prevalence of the traders who engaged only in masked palm civets was 58.54% (24/41), significantly higher than the 9.46% (14/148) of the traders engaged only in snakes. The prevalence of the animals traded in the three markets varied. Market A engaged mainly in the trade of wild animals, while market B engaged in domestic fowl, and market C in snakes. Market A ranked the highest prevalence of IgG positive with 25.61% (84/328), significantly higher than the 7.5% (12/160) of market B, and the 6.80% (10/147) of market C. In market A, the prevalence of IgG positive individuals occurred in traders engaged

in wild animals, market managers, traders' children, traders engaged in domestic fowl, traders engaged in snakes, and traders engaged in frozen animal food was 59.34%(54/91), 20.59(7/34), 16.00% (4/25), 15.22% (7/46), 10.40% (13/125), and 9.68% (3/31), respectively. By questionnaire, it was discovered that during the SARS epidemic, the prevalence of symptoms of acute upper respiratory infection was higher in the animal markets (33.63%, 113/336) than in the vegetable markets (15.83%, 22/139). A retrospective study also indicated that in the animal markets, the prevalence of symptoms of acute upper respiratory infection was significantly higher in individuals with IgG antibody against the SARS-CoV virus 49.28% (34/69) than those who were negative (30.35%, 78/257). In the animal market, the prevalence of IgG antibody to SARS-CoV was significantly higher in those who had symptoms of acute upper respiratory infection (30.77%, 35/114) than those who were healthy (20.085, 44/218). The data indicated that infection with SARS-CoV in traders of animal markets is possibly related to their direct exposure to wild animals, particularly to masked palm civets, and during the period of the SARS epidemic, some of the traders did become infected with the SARS-CoV virus (Xu et al., 2004a).

Xu et al. (2004b) studied the early SARS epidemic in 2003 and the indexed patients in each of the seven earliest affected municipalities in Guangdong Province, China. All the indexed patients had a date of onset before 31 January 2003, with the first patient onset on 16 November 2002. In five municipalities (Foshan, Jiangmen, Zhongshan, Guangzhou, and Shenzhen), outbreaks appear to have occurred independently, but the outbreak in Heyuan may be linked to that in Shenzhen and the outbreak in Zhaoqing to that in Guangzhou. It was discovered that two of the seven indexed patients were restaurant chefs; food handlers (i.e., people who handle, kill, or butcher animals) were overrepresented among early-onset cases with no contact history; and patients with early onset were more likely than patients with late onset to live near an agricultural produce market. However, none of the early patients was a commercial farmer nor was living near a farm associated with increased risk. The authors suggest that wild animals rather than a livestock or poultry might be the original source of the SARS outbreak (Xu et al., 2004b).

Apart from the above published data, there were a couple of news reports about the investigation of animal reservoirs in China. On 24 May 2003, an animal origin investigation group from the ministry of agriculture claimed that they have collected samples from 1,700 animals including 59 species, and they had found sequences 99.9% identical to that of SARS-CoV from bats, monkeys, palm civets, and snakes. (www.chinanews.com, 24 May 2003). This research group included scientists from Harbin Veterinary Research Institute, Chinese Academy of Agriculture, Changchun University of Agriculture and Animal Sciences, South China Agriculture University, Guangdong Provincial Veterinary Station of Epidemic Prevention and Supervision, and Guangdong CDC. Another investigation result was released from Beijing Agriculture University on 19 June 2003 (The Beijing Youth Day, 19 June 2003). They claimed that 732 samples were collected from 65 animal species including 54 wild animals and 11 domestic animals. All of the samples were negative by reverse transcription–polymerase cycle reaction (RT-PCR). Among these animals there were 76 masked palm civets, including 25 from Guangdong, 10 from Yunnan, 3 from

Guangxi, 3 from Jiangxi, 20 from Shaanxi, 4 from Shanxi, and 11 from Beijing (the Beijing Youth Day, 19 June 2003). However, no detailed results of these news reports related researches have been published so far.

Investigation of the Restaurant and Market Animals and Their Relationship to the Mild SARS Cases in the Winter of 2003–2004

Between 16 December 2003 and 8 January 2004, a total of four patients were independently hospitalized in the city of Guangzhou, Guangdong Province, China, with flu-like syndromes, which were later diagnosed as SARS cases (Liang et al., 2004). All the four cases were mild and had no secondary transmission. The epidemiological information collected by the Guangdong Center for Disease Control and Prevention and the Guangzhou Center for Disease Control and Prevention indicated that although none of these patients had a contact history with previously documented SARS cases, they all had direct or indirect contact history with wild animals in geographically restricted areas. The second patient worked in a local restaurant TDLR and the fourth patient dined in the same restaurant where palm civet and other exotic dishes were served, whereas the third patient dined in a neighboring restaurant SJR. The first patient was the only patient with no contact with TDLR or SJR; however, had contact with house rats in his apartment a few days before disease onset (Song et al., 2005).

Two teams have published their results about the SARS-CoV-like viruses in civets and their links to the mild SARS cases of winter 2003–2004 (Song et al., 2005; Kan et al., 2005).

Song et al. (2005) sequenced most of the SARS-CoV viral genome from the first two of the four human patients (GZ03-01 and GZ03-02), two palm civets from the Guangzhou food market (PC4-136, PC4-227) and one sample from the palm civet cage at the restaurant TDLR (PC4-13). They were also able to sequence seven additional spike sequences (PC4-115, PC4-127, PC4-137, PC4-145, PC4-199, PC4-205, and PC4-205) from masked palm civets from the Guangzhou food market and partial spike gene from the third patient (GZ03-03).

The whole genome sequences indicated that the identities of SARS-CoV-like viruses from the civets and that of the human patients were about 99.89% homologous (Song et al., 2005). Phylogenic analysis indicated that the sequence of the masked palm civets in 2004 were closer to that of the mild human cases of winter 2003–2004 than to that of the masked palm civets found in 2003 (SZ3 and SZ16).

Kan et al. (2005) were able to get samples from Xinyuan Live Animal Market in Guangzhou, Gangdong Province, China in January 2004 just before the animals in the market were culled. They collected rectal and throat swabs from 91 civets and 15 raccoon dogs randomly selected from 18 vendors with booths located in four blocks dedicated to the sale of civets and raccoon dogs. They also collected environmental specimens from those blocks, including animal-cage swabs, cash-table swabs, and wall swabs. RT-PCR results indicated that 84 of the 91 civets were

positive with both rectal and throat swabs. The other seven palm civets tested positive with throat or rectal swabs only. Of the 15 raccoon dogs, 12 tested positive with both throat and rectal swabs, while 3 tested positive with throat swabs only. Of the 24 environmental specimens, 22 tested positive.

Two whole-genome sequences (A022G and B039G) of SARS-CoV-like viruses were directly determined from palm civet samples taken from the market, and two whole-genome sequences (Civet007G and Civet020G) from the restaurant TDLR civets were obtained by Kan et al. (2005). In addition, two spike sequences from raccoon dogs (A030G and A031G) and 13 spike sequences from masked palm civets from the market were also obtained. It was found that the spike sequence of case 1 of 2003–2004 winter patients (GD03T0013) was identical to that of one of the civets in Xinyuan Live Animal Market (B012G), suggesting that this patient might have caught the virus from palm civets from Xinyuan Live Animal Market (Kan et al., 2005). The spike sequences of the two raccoon dogs (A030G and A031G) and the masked palm civet (A022G) were identical. Phylogenetic research indicated that these three sequences (A030G, A031G, and A022G) might be the original prototype of all the sequences found in this research (Kan et al., 2005). The spike sequences of Civet007G and Civet020G from the restaurant TDLR had only five nucleotides difference from that of A030G, A031G, and A022G. In addition, the spike sequences of Civet007G and Civet020G were identical to that of other ten masked palm civets from Xinyuan Live Animal Market, including A001G, A013G, B033G, B039G, B040G, C013G, C014G, C017G, C019G, and C028G.

The 29-nt sequence that was recognized as the marker of animal origin (Guan et al., 2003; Chinese SARS Molecular Epidemiology Consortium, 2004) was detected in all of the completed sequenced viruses from masked palm civets (Guan et al., 2003; Song et al., 2005; Kan et al., 2005), with only a difference in PC4-227 that the insertion was comprised of 27 nt instead of 29 nt.

When it was found that the sequence of the first SARS case in 2004 was almost identical to that of one of the animals in the market, the local Guangdong government took aggressive action in culling all the masked palm civets in the farms and food markets (Normile, 2004; Watts, 2004). It was estimated that in January 2004, about 10,000 masked palm civets were culled in Guangdong Province, China.

If the finding in 2003 had revealed that masked palm civets are the possible origin of the SARS outbreak, the discovery of the winter 2003–2004 further confirmed that masked palm civets were the source of human infection with SARS-CoV. However, whether the masked palm civet is the primary animal reservoir or an intermediate vector of SARS-CoV remains unclear.

Investigation of Farmed Masked Palm Civets and Comparison with Market Animals

The ideal way to find out whether masked palm civets are animal reservoir of SARS-CoV is to conduct surveillance of the animals in their native habitats. However, as masked palm civets are normally solitary in nature, it is not an easy task to capture

them for epidemiology study. In contrast, it was estimated that there were about 40,000 masked palm civets being raised in about 600 farms all over China in 2003 (China Daily, 6 January 2004). Also, it was reported that there were 41 civet farms in Guangdong Province at the time of the slaughter campaign in January 2004 (Tu et al., 2004). Therefore, several investigations to determine the prevalence of SARS-CoV-like virus in farmed masked palm civets were conducted.

Tu and co-workers (2004) investigated serum samples from masked palm civets in four farms and in one market in Guangdong province of China during the slaughter campaign in January 2004. Intestinal tissues and serum samples were taken from 56 animals: 38 civets from four farms in different regions of Guangdong Province (10 from Zhuhai, 10 from Shanwei, 9 from Shaoguan, and 9 from Qingyuan) and 18 civets from the Xinyuan Live Animal Market in Guangzhou. They found anti-SARS-CoV antibodies in 78% of the market animals (14 out of 18), while the overall prevalence in farm animals was ~10% (4 of 38), with the highest prevalence of 40% (4 of 10) in Farm Shanwei. SARS-CoV antibody levels in the four animals at the farm in Shanwei were lower than those from the market, and two samples found positive by a virus neutralization test failed to react on immunofluoresence antibody assay or Western blot. Intestinal tissues collected from the 56 civets including both market and farmed animals were tested by RT-PCR using nucleocapsid (N), membrane (M), or spike (S) gene specific primers, and none of the samples was positive (Tu et al., 2004).

In the same paper, Tu et al. (2004) also investigated 47 civet serum samples that had been previously collected in early June 2003 from two civet farms in Luoning City of Henan Province and Changsha City of Hunan Province. All the samples were negative by virus neutralization test and immunofluoresence antibody assay. The authors suggested that the high prevalence of SARS-CoV in market civets might be associated with trading activities, which resulted in overcrowding and the mixing of different animal species (Tu et al., 2004).

Kan et al. (2005) investigated 1,107 palm civets from 25 farms in 12 provinces in China during January and September 2004. These provinces included Anhui, Beijing, Fujian, Guangxi, Henan, Hebei, Hubei, Hunan, Jiangsu, Jiangxi, Shanxi, and Shaanxi. The criteria for the selection of farms for sampling included their sale of animals from a booth at the Xinyuan Live Animal Market and their claims to trade ~80% of their animals to Guangdong province. In contrast to the market masked palm civets and raccoon dogs, which were all positive for SARS-CoV-like viruses, all of the 1,107 civets sampled from farms tested negative for SARS-CoV-like virus by RT-PCR (Kan et al., 2005). The authors were able to trace one farmer who sold masked palm civets to Xinyuan Live Animal Market. All the seventeen masked palm civets in Xinyuan Live Animal Market from this farm tested positive for SARS-CoV like virus by RT-PCR. However, all the masked palm civets (n = 169) at his farm in Henan Province detected negative for SARS-CoV-like virus by the same RT-PCR method (Kan et al., 2005). The authors suggested that the palm civets were infected at the market by other palm civets or by other animals harboring the virus rather than at the farm.

If the animals were infected at the market, then new arrivals should possess a relatively low or no viral load, and the viral load should increase after arriving at

the market. Kan et al. (2005) tested viral loads in a few masked palm civets whose arrival dates were traceable. Quantitative measurement of SARS-CoV-like virus in rectal swabs taken from the masked palm civets was determined by fluorescent real-time RT-PCR based on the N gene. It was found that animals started shedding virus ($10^{3.68}$ viral copies per ml) as early as 2 days after arrival (there were no data for day 1 and that only one animal was observed at the 2-day time point). An average of $10^{4.43}$ viral copies per ml of specimen was observed for six masked palm civets which had been at the market for 4 days. The peak virus load (an average of $10^{6.91}$ viral copies per ml) was observed in animals which had been at the market for 7 days; this declined by day 15 ($10^{4.17}$ viral copies per ml). This pattern of viral load change was found similar to the results in experimentally infected masked palm civets. When palm civets were experimentally infected, the viral genome was detected by RT-PCR in throat and anal swabs from 3 to 18 days post inoculation (Wu et al., 2005). However, the viral load in rectal swabs remained relatively high in the animals that arrived at the market for 17 days ($10^{6.4}$ viral copies per ml), 52 days ($10^{6.56}$ viral copies per ml), and 180 days ($10^{5.49}$ viral copies per ml) (Kan et al., 2005), unlike the inoculated experimental masked palm civets that virus were not detectable by RT-PCR for throat or anal swabs after 18 days post inoculation (Wu et al., 2005). However, viral genomic DNA could be detected in spleen and lymph nodes up to 34 and 35 days post inoculation in experimentally infected masked palm civets (Wu et al., 2005). It is suggested that there might be a persistent viral infection or reinfection of masked palm civets in the market (Kan et al., 2005).

Hu et al. (2005) developed a multitarget real-time PCR assay for detecting SARS-CoV in clinical samples and also SARS-CoV-like viruses in masked palm civets. They used probes and primers based on sequences of the N gene, open reading frame (ORF) 3, and ORF 8. It was found that the detection of N gene was much more sensitive than that of ORF3 and ORF8. They tested seven randomly selected throat swabs of masked palm civets from a farm located in Hubei Province and found that one was positive by using three primer and probe sets, with N gene of $10^{7.99}$ copies/ml, ORF3 of $10^{2.7}$ copies/ml, and ORF8 of $10^{3.36}$ copies/ml. Two were positive by using two primer and probe sets and the other four were positive by using one primer and probe set (Hu et al., 2005).

At the moment, the data about the farmed masked palm civets are still confusing. It is quite possible that prevalence of infection in different farms varies. It could not be ruled out that in the farms, viral load is relatively lower than in the markets, and when less sensitive assays were used, the prevalence of viral infection in the farms was underestimated.

Investigation of Wild Masked Palm Civets

So far, the data of SARS-CoV prevalence in wild masked palm civets are very limited. Poon et al. (2005) trapped 21 masked palm civets from Hong Kong Special Administrative Region, China. They collected respiratory and fecal swab samples and detected the existence of coronaviruses by RT-PCR using consensus primers

targeted to the conserved region of coronavirus RNA polymerase. Blood samples were taken for neutralization assay for SARS-CoV. None of the masked palm civets was positive for SARS-CoV by both serological and molecular tests (Poon et al., 2005). The authors suggested that although the result did not exclude the possibility that masked palm civet is the natural host of SARS-CoV, it at least indicated that SARS-CoV is not broadly circulating in wild masked palm civets.

Investigation of SARS-CoVs in Bats

The study of animal reservoirs of SARS-CoV was not limited on masked palm civets. As bats are an important reservoir for many zoonotic viruses including rabies virus, lyssavirus, Hendra and Nipha viruses, Menangle virus, St. Louis encephalitis virus (Halpin et al., 2000; Mackenzie et al., 2001; Mackenzie and Field, 2004), it was also one of the investigating targets for animal reservoir of SARS-CoV. An early study of bats by Poon et al. (2005) investigated 81 bats belonging to 12 different species by RT-PCR using conserved sequence of coronavirus. A novel bat coronavirus (Bat-CoV) was identified in three different species from the same genus, *Minoopterus magnater, M. pusillus, and M. schreibersii*. The Bat-CoV, from which the sequenced fragments shared 41–62% homology to that of SARS-CoV, is not a SARS-CoV-like virus and belongs to Group I coronavirus (Poon et al., 2005).

However, recently two independent research teams have published exciting results of finding SARS-CoV-like viruses in bats and suggested that the horseshoe bat is a reservoir of SARS-CoV-like viruses (Lau et al., 2005; Li et al., 2005). Lau et al. (2005) investigated bats located in Hong Kong Special Administrative Region, China. They detected 118 nasopharyngeal and anal swabs from 59 bats representing 8 species, 5 genera, and 3 families. By using RT-PCR, 23 of 59 anal swabs were found positive from Chinese horseshoe bats (*Rhinolophus sinicus*) (Lau et al., 2005) (also see Table 1).

Three genomes of bat SARS-CoV-like viruses were sequenced by Lau et al. (2005), that of B24, B43, and B41. The genomes of the bat SARS-CoV-like virus were very similar to that of SARS-CoV except for the regions of the Spike gene, ORF 3 and ORF 8. The three genomes had 88% nucleotide and 93% amino acid identity to human and civet SARS-CoVs. The 29-bp insertion was shown to exist in bat SARS-CoV like viruses, although this sequence demonstrated 12 nt substitutions. Phylogenetic analysis showed that bat SARS-CoV-like viruses formed a distinct cluster with SARS-CoVs (Lau et al., 2005) and a distantly related group 2 coronavirus (Siddell, 1995; Lai and Holmes, 2001).

Among the bats investigated, positive prevalence of the antibodies against recombinant bat-SARS-CoV N protein was 67% (12/18) by Western blot and 84% (31/37) by enzyme immunoassay, compared with only 42% (8/19) for human SARS-CoV neutralizing antibody titer (≥1:20). And for those bats with neutralizing antibodies, a lower viral load was found in their anal swabs (Lau et al., 2005).

Table 1 Detection of prevalence of antibodies and RNAs of SARS-CoV like viruses in bats

Sampling location	Bat species	Antibody test against N protein: positive/total (%)	RT-PCR analysis: positive/ total (%) Fecal swabs	Respiratory swabs
Guangxi	*Rousettus leschenaulti*	1/142 (1.4%)[a]	0/165	0/55
	Rhinolophus pearsonii	13/46 (28.3%)[a]	3/30 (10%)	0/11
	Rhinolophus pussilus	2/6 (33.3%)[a]	0/6	0/2
Guangdong	*Rousettus leschenaulti*	0/42[a]	0/45	ND
	Cynopterus sphinx	0/17[a]	0/27	ND
Tianjin	*Myotis ricketti*	ND	0/21	0/21
	Rhinolophus pussilus	ND	0/15	ND
	Rhinolophus ferrumequinum	0/4[a]	1/8 (12.5%)	ND
Hubei	*Rhinolophus macrotis*	5/7 (71%)[a]	1/8 (12.5%)	0/3
	Nyctalus plancyi	0/1[a]	0/1	ND
	Miniopterus schreibersi	0/1[a]	0/1	ND
	Myotis altarium	0/1[a]	0/1	ND
Hong Kong	*Hipposideros armiger*	ND	0/12	0/12
	Miniopterus magnater	ND	0/23	0/23
	Miniopterus pusillus	ND	0/24	0/24
	Myotis chinensis	ND	0/3	0/3
	Myotis ricketti	ND	0/2	0/2
	Nyctalus noctula	ND	0/2	0/2
	Rhinolophus affinus	ND	0/2	0/2
	Rhinolophus sinicus	12/18(67%)[b]	23/59(39%)	0/59

Modified according to Lau et al., 2005 and Li et al., 2005; ND, not done

[a] Sandwich ELISA based on SARS-CoV N protein (Li et al., 2005)

[b] Western blot with recombinant N protein of bat SL-CoV (Lau et al., 2005)

Li et al. (2005) detected 402 respiratory and fecal swab samples from 408 bats representing 9 species, 6 genera, and 3 families, from four provinces in China, including Guangdong, Guangxi, Hubei and Tianjin (Table 1). Three species from the genus *Rhinolophus* (horseshoe bats) in the family *Rhinolophidae* demonstrated a high SARS-CoV antibody prevalence: 13/46 (28%) in *R. pearsonii* from Guangxi; 2/6(33%) in *R. pussilllus* from Guangxi; and 5/7 (71%) in *R. macrotis* from Hubei. A total of five positive fecal samples were detected, all of them from the genus *Rhinolophus*, three in *R. pearsonii* from Guangxi, and one each in *R. macrotis* and *R. ferrumequinum*, from Hubei (Li et al., 2005). Neutralization tests using human SARS-CoV, however, were all negative. As there is no SARS-CoV-like viral isolates from bats yet, it remains unknown whether the serum from bats can neutralize the virus from bats or not.

One virus from the fecal samples (Rp3) was completely sequenced and its genome demonstrated 92% nt identity to Tor 2 strain (Li et al., 2005). The polymerase, spike, envelope, membrane, and nucleocapsid proteins, which are present in all coronaviruses, were similarly sized in Rp3 and Tor 2, with sequence identities ranging from 96% to 100%. Partial sequences from the other four samples (Rf1, Rm1, Rp1,

and Rp2) indicated that recombination occurred within the genomes of bat SARS-CoV-like viruses.

As bats are natural reservoirs of several new and reemerging viruses, it is not a surprise that it is also a reservoir of SARS-CoV-like virus. Nipah virus, for example, spread in Malaysia in 1998 and in Bangladesh in 2004, was in high-level serological prevalence in bat genus *Pteropus* and isolated from *Pteropus hypomelanus* and *P. lylei* in Malaysia, Bangladesh, and Cambodia (Chua et al., 2001; Hsu et al., 2004; Reynes et al., 2005). Nipah virus isolated in bat also showed a greater genetic diversity than that isolated in human. The discovery of bat SARS-CoV-like viruses suggests that genetic diversity exists among zoonotic viruses in bats, increasing the possibility of variants crossing the species barrier and causing outbreaks of disease in human populations. It is therefore essential to enhance our knowledge and understanding of reservoir host distribution, animal–animal and human–animal interaction. It is also interesting to find out why bats can be reservoir of so many viruses, whether there is an association with immunology or ecology of bats.

Are There Other Animal Species that Serve as Reservoirs of SARS-CoV?

So far, the sequence data showed that the average genome homology of SARS-CoV-like virus from horseshoe bat to the SARS-CoV is about 92%, while the homology of SARS-CoV-like virus of masked palm civets to the SARS-CoV is above 99.6%. This indicated that horseshoe bat is a distantly related animal reservoir of SARS-CoV-like virus, while masked palm civet can be the direct origin of SARS. Further investigations might reveal that higher homology in bats or lower homology in masked palm civets. However, it is likely that in the transmission chain between horseshoe bats and masked palm civets there are still other species(s) missing. One way is to look at the ecological circles of both bats and masked palm civets to fish out possible links and suspects. An extensive survey of wild animal species for SARS-CoV-like viruses should provide us with information about alternative animal reservoirs.

Poon et al. (2005) conducted a survey of the prevalence of coronaviruses in wild animals in Hong Kong between the summer of 2003 and the summer of 2004. They investigated small mammalian, avian, and reptile species living in natural reservoirs or city parks in Hong Kong. A total of 162 animals from 44 species were tested by RT-PCR for conserved sequences of RNA-dependent RNA polymerase, helicase-ExoN and S-encoding sequences of SARS-CoV. Apart from three species of bats, that of *Miniopterus magnater, Miniopterus pusillus, Miniopters schreibersii*, from which a new group I coronavirus was isolated, all other species were negative. However, the animal numbers for each species they investigated were limited, with only *Cynoptus sphinx* ($n = 15$), *Miniopterus magnater* ($n = 16$), *Miniopterus pusillus*, ($n = 19$), *Hystrix hodgsoni* ($n = 10$), and *Paguma larvata* ($n = 21$) over 10 animals (Poon et al., 2005). This limited sample size of animal species might not detect viruses that are circulating at a low frequency.

Rodents are another type of popular reservoir of many viral pathogens. The first patient in winter 2003–2004 had direct contact with a rat in his apartment (Liang et al., 2004). Apart from bats, Lau et al. (2005) also captured 60 rodents and 20 monkeys from summer 2004 to spring 2005 in Hong Kong Special Administrative Region, China. The 60 rodents belong to three different species, including 12 Chestnut spiny rats (*Niviventer fulvescens*), 4 Buff-bellied rats (*Rattus rattus flavipectus*), and 44 Sikkim rats (*Rattus sikkimensis*), and all samples were tested negative by RT-PCR (Lau et al., 2005).

Live-animal markets (wet markets) provide an environment for cross-species transmission of virus (Webster, 2004) and therefore it is an ideal place to look for susceptible hosts and the possible origin of SARS-CoV (Guan et al., 2003; Lau et al., 2005). Wang et al. (2005) investigated an animal market in Guangzhou, Guangdong Province, China on 5 January 2004 just before the culling of masked palm civets (Normile, 2004). Thirty one animals were sampled including 20 domestic cats (*Felis catus*), 5 red foxes (*Vulpes vulpes*), and 6 Lesser rice field rats (*Rattus losea*). Real-time PCR revealed that four cats, three red foxes, and one Lesser rice field rat were positive for SARS-CoV, indicating that the market was seriously contaminated. At the time, the environment of the market was also contaminated with SARS-CoV-like viruses (Lau et al., 2005), and it is unknown whether those positive animals were susceptible hosts rather than important reservoirs of the virus. On 20 January 2004, 2 weeks after the culling of animals and the disinfection of the market, 119 animals from the same market were tested. The animals included 6 rabbits (*Oryctolagus cuniculus*), 13 cats, 46 red jungle fowl (*Gallus gallus*), 13 spotbill duck (*Anas platyhynchos*), 10 greylag goose (*Anser anser*), and 31 Chinese francolin (*Franclnus pintadeanus*). Only a rectal swab for one greylag goose tested positive for SARS-CoV-like virus by RT-PCR, indicting the disinfection of the market was successful. Later, 102 animals including 14 greylag goose, 3 cats, 5 rabbits, 9 spotbill ducks, 2 Chinese francolins, 8 common pheasants (*Phasianus colchicus*), 6 pigeons, 9 Chinese muntjacs, 19 wild boars (*Sus scrofa*), 16 Lesser rice field rats, 5 dogs, 1 mink (*Mustela vison*), 3 goats, 2 green peafowl (*Pavo muticus*) were sampled from the market between April to November 2004. Only one rectal swab from a wild boar tested positive (Wang et al., 2005). No details were reported about the isolates from the greylag goose or the wild boar.

Experimental animal inoculation of viruses may help to identify natural viral reservoirs. Such experimental tests have indicated that SARS-CoV might be able to infect a wide-range of hosts, including masked palm civets (Wu et al., 2005), monkeys (Fouchier et al., 2003; McAuliffe et al., 2004; Rowe et al, 2004; Qin et al., 2005), cats and ferrets (Martina et al., 2003), mice (Subbarao et al., 2004; Wentworth et al, 2004; Glass et al., 2004; Roberts et al., 2005b), pigs and chickens (Weingartl et al., 2004), guinea pigs (Liang et al., 2005), and Golden Syrian Hamster (Roberts et al., 2005a). However, as most experimental tests were performed with the purpose of creating animal models for vaccine evaluation, not much is known about the transmission of the virus from the inoculated animals. Pigs and chickens, for example, may support SARS-CoV replication to a very limited degree but are not likely to play a role as an amplifying host (Weingartl et al., 2004).

Some attention was also paid to domestic animals. Chen et al. (2005) conducted a survey of SARS-CoV-like viruses in six major domestic animal species that are in close contact with humans. They surveyed 242 animals, including 108 pigs, 60 cattle, 20 dogs, 11 cats, 11 chickens, and 30 ducks in Xiqing Country of Tianjin, China, where a SARS outbreak occurred in late spring 2003. Two pigs were found antibody positive, while the other 240 animals were found antibody negative. One of the two pigs was tested positive by RT-PCR and two viral isolates were obtained from its blood and fecal samples, designed TJB and TJF, respectively. The pig was followed up for 4 weeks until its blood tested negative with RT-PCR. The genome of TJF was completely sequenced and was very close to the human epidemic strain (differing by only 18 nt from the BJ01 whole genome sequence). Because the genome of TJF does not contain the insertion marker of 29 nt, the authors suggested that TJF was transmitted from humans to the pig. As swineherds in rural areas often obtain leftovers from restaurants in the cities for use as hogwash without thoroughly fermenting, the authors suggested that the pig was most likely infected from virus-contaminated animal feed (Chen et al., 2005).

Although there are several susceptible hosts for SARS-CoV, all the above researches did not give an obvious hint to possible animal reservoirs further than horseshoe bats and masked palm civets. Also, the linkage between the horseshoe bats and masked palm civets is still missing.

Possible Factors that Contributed to the Increasing Risks of Disease Outbreaks

It remains unknown why the first SARS outbreaks appeared in the late 2002 in Guangdong, China, and whether this was the first time that SARS-CoV infected and caused disease in humans. Using immunofluorescence and neutralization assays, Zheng et al. (2004) detected antibodies to human SARS-CoV and/or an animal SARS-CoV-like virus in 17 of 938 (1.8%) healthy adults from Hong Kong in 2001, suggesting that a small proportion of healthy people in Hong Kong had been exposed to SARS-related viruses at least 2 years before the first SARS outbreak. However, Yu et al. (2005) had a different result. They used different assays to analyze 1,621 serum specimens collected from military recruits from the People's Republic of China in 2002 for SARS-CoV antibodies. Eleven samples were found positive by ELISA and six of them confirmed by IFA, but only three were confirmed by protein microarray analysis and antigen-capturing ELISA. None of the eleven samples was positive in neutralization test. The authors suggested that the people from mainland China either had only rarely been exposed to SARS-CoV before the 2003 SARS outbreak or had not been exposed to SARS-CoV at all (Yu et al., 2005).

The peak of demand of masked palm civets as a delicacy in Guangzhou, China occurred in 2000–2002; whether this provided an environment and a period for the viruses to accumulate mutations and evolve the capacity to infect humans needs further investigation.

One of the factors that might contribute to the human infection may reside in the genotypes of the virus isolated from SARS patients. Recent studies have shown that two amino acids (aa 479 and 487) might be responsible for the transmission of SARS-CoV-like virus from the masked palm civet to humans (Yang et al., 2005; Qu et al., 2005). It was demonstrated that with the mutation of either R/K479 (of masked palm civets) into N479 (of human) or S487 (of palm civets) into T487 (of human), the pseudotype viruses carrying mutated spike of civet SARS-CoV-like viruses could infect the cells expressing human receptor ACE2. On the other hand, if both N497 and T487 were mutated into R/K497 and S487, the pseudotype virus with spike of human SARS-CoV lost its infectivity to human cells (Qu et al., 2005). The mutation of N479 had been found in some of the sequenced masked palm civets from the market as well as in some of the mild human cases of winter 2003–2004 (Song et al., 2005; Kan et al., 2005). It was suggested that this mutation can cause animal to human transmission, and by further mutation such as S487T, those viruses might cause human to human transmission and a subsequent epidemic (Song et al, 2005; Qu et al., 2005). In fact, so far we have not found the original strain that caused the 2003 epidemic of SARS. The animal viruses sequenced from the Shengzhen animal market (Guan et al., 2003) had too many mutations compared to that of the human epidemic strains, suggesting that these isolates were not directly responsible for the 2003 epidemic.

The distribution of the virus in the environment and its access to humans could be another factor that contributes to the risk. Although we are not sure whether masked palm civets and raccoon dogs are natural hosts for SARS-CoV or not, their high prevalence of SARS-CoV in the wet markets certainly played an important role in the previous SARS outbreaks. Surveillance of Xinyuan Live Animal Market on 5 January 2004 revealed that 100% samples from the masked palm civets and raccoon dogs were positive for SARS-CoV by RT-PCR and 22/24 (92%) environment specimens were also positive (Kan et al., 2005). A similar surveillance indicated that 3/5 (60%) of the red foxes, 4/20 (20%) of the domestic cats, 1/6 (17%) of lesser rice-field rats were positive for SARS-CoV by RT-PCR, in the market (Wang et al., 2005). The fact that so many species and even environmental samples from animal cages, cash tables, or walls were detectable for viral RNA serves as dangerous signals for the existing of emerging SARS-CoV-like viruses in the market at that time. The coincidence of the four mild cases could be regarded as a reflection of the high risk in the winter 2003–2004. And it is believed that the culling of masked palm civets and the close of the wet markets in Guangdong in January 2004 did contribute to the absence of subsequent outbreaks.

Identification of Future Risks and Actions

After the SARS epidemic in 2003, apart from a few mild cases in the winter of 2003–2004 and laboratory contraindications, it seems that there is no evidence of the reemergence of SARS. Certain changes have been made in China.

For example, masked palm civet is hardly seen in the restaurants or markets in Guangdong China, and Xinyuan Live Animal Market has now changed into a market for frozen food. However, as we have only very limited knowledge of the ecology of the SARS-CoV virus in nature, we are unable to make accurate predictions without further scientific surveillance and research.

A wider investigation of SARS-CoV-like viruses in different animal species is still needed to fill the gaps in the transmission chain of SARS-CoVs and to understand the viral evolution. The identification of different viral genotypes in natural hosts would provide information as to the important factors in the species barrier and human infection with SARS-CoV. The culling of the masked palm civets in Guangdong in January 2004 was an urgent need at that time. However, further studies of the distribution of the SAR-CoV in the environment will provide information on its natural animal reservoirs and methods to contain its epizootic transmission.

Wet markets are an important source of emerging viruses (Webster, 2004) and therefore their surveillance can be used as an early-warning system. After the culling of masked palm civets and disinfection, only two animals were tested positive out of 221 animals from the market during the period of January to November, 2004 (Wang et al., 2005). In November and December 2004, 12 and 10 masked palm civets were sampled from Guangzhou and Shenzhen, respectively, including five of which had been at the live market for 2 days, none of them tested positive. Therefore, it was suggested that the reemergence of human infection from animal origins was low for the winter of 2004–2005 (Wang et al., 2005) and this has been proved to be the case.

For surveillance, it is important to develop a sensitive tool to detect antibody prevalence in different animals. It has been shown that the spike genes from some masked palm civets were difficult to generate neutralizing antibodies (Yang et al., 2005) and that some antibody positive sera from bats could not neutralize SARS-CoV infection (Li et al., 2005). Although recombinant N protein could be used as an antigen for detecting antibodies against SARS-CoV-like viruses in bats (Lau et al., 2005, Li et al., 2005), it may not be the case for other animals or for different viral genotypes. The relationship of different genotypes of SARS-CoV-like viruses with their serological cross-reaction needs to be further identified.

As SARS infections of humans have been controlled, much important SARS related research has been stopped due to limited resources. However, at least for the research on animal reservoir of SARS-CoV, more investigations should be made to further understand the future risks of the reemergence of SARS.

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