

Bats, Civets and the Emergence of SARS

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Abstract Severe acute respiratory syndrome (SARS) was the first pandemic transmissible disease of previously unknown aetiology in the twenty-first century. Early epidemiologic investigations suggested an animal origin for SARS-CoV. Virological and serological studies indicated that masked palm civets (*Paguma larvata*), together with two other wildlife animals, sampled from a live animal market were infected with SARS-CoV or a closely related virus. Recently, horseshoe bats in the genus *Rhinolophus* have been identified as natural reservoir of SARS-like coronaviruses. Here, we review studies by different groups demonstrating that SARS-CoV succeeded in spillover from a wildlife reservoir (probably bats) to human population via an intermediate host(s) and that rapid virus

evolution played a key role in the adaptation of SARS-CoVs in at least two nonreservoir species within a short period.

1 Introduction

Severe acute respiratory syndrome (SARS) first appeared in mid-November 2002 in Guangdong province in southern China, and continued to spread to more than 30 countries on five continents with 8,098 reported cases and 774 deaths by the end of July 2003, placing it with HIV/AIDS as one of the severe and readily transmissible new diseases to emerge in the twenty-first century (WHO 2004). The high case fatality rate and global spread led to an urgent response by an international network co-ordinated by the World Health Organization (WHO) of the United Nations, which resulted in the rapid identification of the aetiological agent (Drosten et al. 2003; Fouchier et al. 2003; Ksiazek et al. 2003; Kuiken et al. 2003; Peiris et al. 2003). The outbreak was caused by a newly emerged and previously unrecognised coronavirus, now known as the SARS coronavirus (SARS-CoV). The complete genome sequence of SARS-CoV has been determined and the virus is classified within the order *Nidovirales*, family *Coronaviridae*, genus *Coronavirus* (Marra et al. 2003; Rota et al. 2003). From December 16, 2003 to January 8, 2004, four SARS cases were detected in the city of Guangzhou, the capital city of Guangdong province of China (Liang et al. 2004; Wang et al. 2006). None of these cases was fatal or resulted in documented secondary transmission, suggesting the possibility that these sporadic outbreaks were caused by less virulent strains of SARS-CoV.

Coronaviruses are known to infect a variety of avian and mammalian species (Holmes and Lai 2001; see the chapter by Holmes and Drummond, this volume). Before the discovery of SARS-CoV, two human coronaviruses (229E and OC43) were known to cause upper respiratory tract infections that varied in frequency and severity in different disease outbreaks, but were usually mild and self-limited (Holmes and Lai 2001). Since the discovery of SARS-CoV, two new coronaviruses, NL63 (van der Hoek et al. 2004) and HKU1 (Woo et al. 2005), have been isolated from human patients with nonfatal infections. To date, SARS-CoV is the only known coronavirus capable of causing lethal infection in humans. Recently, two groups independently demonstrated that bats in the genus *Rhinolophus* are natural reservoirs of SARS-like viruses (Lau et al. 2005; Li et al. 2005), providing strong evidence that SARS-CoV is indeed a new zoonotic virus with a wildlife origin.

2 Epidemiologic Clues to an Animal Origin

Epidemiological studies of index SARS cases in Guangdong Province provided initial evidence that the agent responsible for the outbreak was zoonotic in origin. Between November 2002 and February 2003, the first cases or clusters of SARS appeared in several independent geographic locations in the Pearl River Delta region in southern Guangdong, and suggested multiple introductions of a virus or similar viruses from a common source. Several of the early cases were reportedly associated with occupations that involved contact with wildlife, including handling, killing and selling wild animals as well as preparing and serving wildlife animal meat in restaurants (Xu et al. 2004). Moreover, a study of early SARS cases (i.e. those with disease onset prior to January 2003) compared to those identified later in the outbreak found that 39% of early-onset cases were food handlers, whereas only 2%–10% of cases between February and April 2003 were associated with this occupation. Also, early-onset cases were more likely to live within walking distance of animal markets than late-onset cases (Xu et al. 2004).

To confirm the initial epidemiologic association of early-onset patients with animal handling, several groups conducted retrospective serologic surveillance in different human populations in Guangdong Province during the outbreak period. In one study by Xu et al. (2004), a total of 1,454 clinically confirmed human cases were analysed covering the period from November 2002 to April 30, 2003. Several observations supported the hypothesis of a wild animal origin for SARS. It was observed that early cases of SARS occurred independently in at least five different well-separated municipalities in Guangdong Province. The study also found that early patients were more likely than later patients to report living near a produce market, but not near a farm, and nine of 23 (or 39%) early patients were food handlers with probable animal contact.

Several studies revealed a higher than normal seroprevalence of SARS-CoV antibodies among wild animal traders. Guan et al. (2003) found that eight of 20 (40%) wild animal traders sampled from a market in Shenzhen, Guangdong, in 2004 had anti-SARS-CoV antibodies in comparison to 1 from 20 (5%) vegetable traders from the same market. Yu et al. (2003) analysed serum samples taken on May 4, 2003 from animal traders in three different live animal markets in Guangzhou. Out of 508 animal traders surveyed, 13% had antibodies to SARS-CoV; 72% of traders of masked palm civets (*Paguma larvata*) were seropositive. Interestingly, none of the animal traders had SARS or atypical pneumonia diagnosed during the SARS outbreak in Guangdong, suggesting asymptomatic infection by SARS-CoV or a closely related SARS-like coronavirus. The presence

of subclinical infections was corroborated in a separate study conducted by a Hong Kong group (Zheng et al. 2004), who found that 17 of 938 (or 1.7%) adults recruited in 2001 had antibodies to SARS-CoV detected by immunofluorescence and virus neutralisation assays. These findings suggest that a small proportion of healthy individuals in Hong Kong had been exposed to SARS-CoV-related viruses at least 2 years before the SARS outbreak reached Hong Kong in mid-February 2003.

3 Detection of SARS-CoV-Like Viruses among Wildlife

In May 2003, in the middle of the SARS outbreak, a joint team from Hong Kong and Shenzhen sampled a total of 25 animals from seven wild and one domestic animal species from a live animal market in Shenzhen. It was claimed that these animals were sourced from southern China, and that they had been kept in separate storehouses before delivery to the market. The animals remained in the market 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 faecal swabs were collected for PCR and virus isolation and, where possible, blood samples were taken for serology. Among the six masked palm civets sampled, three were PCR-positive, and a SARS-CoV-like virus was isolated from four nasal swabs and one faecal swab (Guan et al. 2003). In addition, a very closely related virus was isolated from the faecal swab of the only raccoon dog (*Nyctereutes procyonoides*) sampled in the study. Two Chinese ferret badgers (*Melogale moschata*) were sampled, and although neither was PCR-positive, one displayed a neutralising antibody titre of 1:160 against SARS-CoV.

Sequencing of PCR products and virus isolates from palm civets and the raccoon dog revealed several important observations. First, the animal SARS-CoVs were almost identical in sequence to SARS-CoVs isolated from human patients, showing a 99.8% sequence identity. Second, the animal SARS-CoVs contained a 29-nt sequence, located in the C-terminal region of the genome immediately upstream from the N gene; this 29-nt sequence was absent from most of the human SARS-CoV isolates. Later it was discovered that human SARS-CoVs isolated during the early phase of the outbreaks contained the 29-nt sequence, suggesting that the deletion event occurred during adaptation of the animal-derived SARS-CoV to its new human host (The Chinese SARS Molecular Epidemiology Consortium 2004).

These data indicated that at least three different wildlife animal species in the Shenzhen market were infected by a coronavirus that is closely related to SARS-CoV. This important discovery provided the first direct evidence that

SARS-CoV existed in animals, and that the virus responsible for the SARS outbreak most likely originated from animals.

4 Multi-directional Transmission of SARS-CoV

Determining the route and direction of transmission is important for the understanding of zoonotic disease emergence and for the control of future outbreaks. For SARS-CoV, there is evidence to suggest that four possible routes of transmission, animal-to-human, animal-to-animal, human-to-human and human-to-animal, occurred during the outbreaks of SARS in 2002–2003 and 2003–2004.

4.1 Animal-to-Human Transmission

When SARS-CoV was identified as the causative agent of the SARS outbreaks, the first question asked was whether this new virus arose from a pre-existing human virus by an evolutionary process which enhanced its virulence or whether it was an animal virus newly introduced into the human population. Retrospective serologic studies indicated that there were no antibodies to SARS-CoV in the human population prior to the SARS outbreak, suggesting that SARS-CoV was not an existing human coronavirus (Ksiazek et al. 2003). Epidemiologic studies, as discussed above, revealed that animal handlers and people working in the food industry had a higher representation than other groups among early SARS patients. Molecular epidemiologic studies confirmed that the earliest genotypes of human SARS-CoV from the 2002–2003 outbreaks were most closely related to those of animal SARS-CoV isolates (Guan et al. 2003; The Chinese SARS Molecular Epidemiology Consortium 2004). During the sporadic outbreaks of 2003–2004, a total of four patients were independently infected with the SARS-CoV (Liang et al. 2004). There was no direct link between any of the four cases and none of the patients had direct or indirect contact history with previously documented SARS cases; all of them had a history of contact with animals. Furthermore, genome sequences of SARS-CoVs from human patients in 2003–2004 were almost identical to those isolated from civets in the market at the same time period, but more divergent from the human SARS-CoVs obtained during the 2002–2003 outbreaks. Taken together, these results demonstrated that animal-to-human transmission was responsible for the introduction of SARS-CoV into the human population.

4.2

Animal-to-Animal Transmission

In the market study conducted by Guan et al. (2003), it was shown, by virus isolation, RT-PCR or serum neutralisation assay, that all of the six masked palm civets were exposed to SARS-CoV. Considering that these animals were sampled at the same time in the same market, but originated from different regions of southern China, it is most likely that some, if not all, of them got infected in the market through animal-to-animal transmission. In the same study, it was shown that the raccoon dog isolate (SZ13) had an S-gene sequence which was identical to that of one of the civet isolates (SZ16) but differed from the other two civet isolates (SZ1 and SZ3) which displayed S-gene sequence variation. This observation strongly indicated the occurrence of inter-species transmission among the animals in the market.

Animal-to-animal transmission has also been demonstrated in experimental situations. Martina et al. (2003) showed that ferrets (*Mustela furo*) and domestic cats (*Felis domesticus*) are susceptible to infection by SARS-CoV and that they can efficiently transmit the virus to previously uninfected animals that are housed with them.

4.3

Human-to-Human Transmission

Numerous epidemiologic studies documented the rapid human-to-human transmission of SARS-CoV, which spread the virus to more than 30 countries in less than 5 months (WHO 2004). One important example was the spread of SARS-CoV from mainland China to Hong Kong by a Chinese doctor attending a conference there. Through the individuals he infected at a Hong Kong hotel, this single human source was mainly responsible for the subsequent spread of SARS to the rest of the world (Tsang et al. 2003; Zhong et al. 2003).

The major routes of SARS CoV transmission are believed to be droplets, aerosols and fomites (Peiris et al. 2004). In general, the average number of secondary cases of infection generated by one infected individual (R_0) was low (see the chapter by Real and Biek, this volume), approximately 2.2–3.7 (Anderson et al. 2004), a figure much lower than the R_0 of influenza, which ranges from 5 to 25. However, for countries with a moderate to large number of cases, super-spreading events (SSEs) played a pivotal role in large-scale transmission of the virus. In such circumstances, a few infected individuals caused a much higher number of secondary infections. In addition to the SSE in the Hong Kong hotel, other SSEs occurred in a hospital setting in Hong Kong, an air flight from Hong Kong to Beijing and in healthcare settings in Beijing, Singapore and Toronto

(Anderson et al. 2004). In the SSE in a Beijing hospital, one patient infected 33 out of 74 persons that had close contact with the patient. These secondary cases resulted in a further 43 cases before this chain of transmission subsided (Shen et al. 2004).

4.4

Human-to-Animal Transmission

The exact cause for the rapid transmission of SARS-CoV among the more than 100 residents at the Amoy Gardens apartment block in Hong Kong remains a mystery. Although there have been reports suggesting environmental spread through U-traps contaminated with SARS-CoV in bathrooms, other studies also indicated a possible role played by domestic animals such as rats and cats (Lu and Qu 2004; Martina et al. 2003; Ng 2003). Domestic cats living in the apartment complex were found to be infected with SARS-CoV (Martina et al. 2003), suggesting possible human-to-animal transmission. This notion was supported by the subsequent experimental infection of domestic cats with a human SARS-CoV isolated from a Hong Kong patient (Martina et al. 2003). Experimentally infected cats were asymptomatic, but were able to infect other co-housed cats.

In another potential example of human-to-animal transmission, SARS-CoV was isolated from a pig during a surveillance study in farming villages outside of Tianjin, where a SARS outbreak occurred in the spring of 2003 (Chen et al., 2005). The genome sequence of the pig isolate (designated TJF) revealed it to be closely related to the human isolate BJ01 obtained from a patient in Beijing, 120 km from Tianjin, but only distantly related to SARS-CoVs isolated from animals. More importantly, the TJF genome contained the 29-nt deletion, the genetic feature characterising SARS-CoV which circulated among human patients during the later phases of the 2002–2003 outbreaks, but never observed in any of the animal SARS-CoV isolates. The authors concluded that direct human-to-pig transmission was the most likely explanation for these results.

5

Susceptibility of Different Animal Species to Infection by SARS-CoV

During investigations of new zoonotic diseases, it is important to differentiate the roles that different animals may play in distinct stages of disease emergence (see the chapters by Childs et al. and Childs, this volume). It is especially important to distinguish between the reservoir host, which may or may not be responsible for direct pathogen transmission to humans, and the intermediate or amplifying host which

introduced the pathogen into the human population. Due to the sudden emergence of SARS, it was extremely difficult to obtain reliable epidemiologic data to pinpoint the source of the outbreak. The vast number of live animals being traded in animal markets in southern China further complicated the investigation process. Experimental animal infection studies therefore became an important component of the SARS-CoV investigation. They provided the proof that SARS-CoV was the causative agent of SARS, helped define the range of animals susceptible to this new virus, elucidated the mechanisms of virus transmission, and established useful animal model(s) for pathogenesis studies and the testing of vaccines and antivirals.

Since the first experimental infection of cynomolgus macaques by Fouchier et al. (2003), rhesus macaques, African green monkeys, cats, ferrets, mice, pigs, hamsters, guinea pigs and civets have also been shown to be susceptible to experimental infection by SARS-CoV (Liang et al. 2005; Martina et al. 2003; Roberts et al. 2005; Subbarao et al. 2004; Weingartl et al. 2004; Wentworth et al. 2004; Wu et al. 2005). Together with the three naturally infected animal species identified in the market study (Guan et al. 2003), more than ten different mammalian species have so far been shown to be susceptible to SARS-CoV. It can be expected that many more susceptible species will be identified in the future.

Rats have also been identified as another potentially susceptible host and may have played a role in the transmission and spread of SARS-CoV in the well-publicised outbreaks of SARS in the Amoy Gardens apartment block in Hong Kong (Ng 2003). Also, the first confirmed SARS case in 2004 in Guangdong was reported not to have had any contact with animals with the exception of rats (Liang et al. 2004). In our inoculation studies, we have obtained serologic evidence to indicate that SARS-CoV was able to replicate asymptotically in rats (B.T. Eaton, L.-F. Wang et al., unpublished results). It is clear that further studies are required to clarify the role played by rats in the transmission of SARS-CoV.

In contrast, two independent studies conducted in Canada (Weingartl et al. 2004) and the USA (Swayne et al. 2004) indicated that none of the avian species tested, which included chicken, turkey, goose, duck and quail, was susceptible to SARS-CoV infection under laboratory conditions. These findings suggest that domestic poultry were unlikely to be the reservoir or associated with dissemination of SARS-CoV in the animal markets of southern China.

6 The Role of Palm Civets in SARS Outbreak: Natural Reservoir or an Amplifying Host?

In the study by Guan et al. (2003), SARS-CoV-like viruses were isolated from palm civets and a raccoon dog in a live animal market in southern China and serologic evidence indicated that a third species, the Chinese ferret-badger,

was also infected by a similar virus. In spite of the diversity of animals susceptible to SARS-CoV-like viruses, subsequent attention focussed on palm civets, probably because of the larger number of these animals being traded in the market. However, despite the initial speculation that palm civets might be the source of SARS-CoV, several studies demonstrated that there was no widespread infection in wild or farmed civets and that infection in this and other species in animal markets was more likely a reflection of an "artificial" market cycle in naïve species than an indication of the natural reservoir of SARS-CoV.

The first clue came from serological surveillance conducted by Tu et al. (2004). In this study, a total of 103 civet serum samples were taken from a number of civet farms and a market in different regions of China. No SARS-CoV antibody was detected in any of the 47 sera taken in June 2003 from two different farms in Hunan and Henan Provinces. The same was true for 28 serum samples obtained in January 2004 from three different farms in Guangdong Province. In contrast, out of the 18 serum samples taken from an animal market in Guangdong during the same period in January 2004, 14 (or 79%) had significant level of neutralising antibodies to SARS-CoV, indicating widespread infection by a virus that is closely related to SARS-CoV.

Molecular analysis was used to investigate the distribution and evolution of SARS-CoV in palm civets and to compare the prevalence of the virus in palm civets in markets and on farms. Following the detection of SARS-CoV in market palm civets at the end of 2003, palm civets were culled in Guangdong Province in an attempt to prevent the potential reemergence of SARS. This provided a unique opportunity for Kan et al. (2005) to sample a relatively large number of animals for molecular epidemiological studies. A total of 91 palm civets and 15 raccoon dogs were sampled in the Xinyuan animal market in Guangzhou in January 2004. The animals were selected from 18 vendors with booths located in four blocks dedicated to the sale of civets and raccoon dogs. PCR analysis indicated that all of the animals sampled were positive and that most animals yielded positive rectal and throat swabs. In the same study, a total of 1,107 palm civets were sampled from 25 farms in 12 provinces from January to September 2004, but none of them was positive when analysed by the same PCR tests. These farms were selected on the basis that they used to sell animals to one of the booths at the Xingyuan animal market or that they claimed to have previously provided more than 80% of their animals to markets in Guangdong province.

In an animal surveillance study conducted in Hong Kong between the summer of 2003 and 2004, Poon et al. (2005) sampled 21 wild trapped palm civets in addition to other mammalian, avian and reptile species. Serological and PCR analyses indicated that none of the animals surveyed was positive for SARS-CoV.

Moreover, when palm civets were experimentally infected with two different strains of human SARS-CoV, one with a 29-nt deletion isolated in Beijing (Qin et al. 2003) and another containing the 29-nt sequence isolated in the early phase of the outbreak from Guangzhou (GZ01), all of the animals developed clinical symptoms including fever, lethargy and loss of aggressiveness (Wu et al. 2005). These results indicated that palm civets were equally susceptible to infection by SARS-CoV with or without the 29-nt sequence.

Taken together, the lack of widespread infection in wild or farmed palm civets and the display of overt clinical symptoms following experimental infection suggest that palm civets are unlikely to be the natural reservoir of SARS-CoV. Instead, the animal's high susceptibility to SARS-CoV and its wide distribution in markets and restaurants made it an ideal amplifying host that is believed to have played an important role in both the major 2002–2003 and sporadic 2003–2004 outbreaks.

7

Identification of Horseshoe Bats as Natural Reservoirs for SARS-Like Viruses

Bats are reservoir hosts of several zoonotic viruses (Calisher et al. 2006), including the Hendra and Nipah viruses, which have recently emerged in Australia and East Asia, respectively (Chua et al. 2000; Murray et al. 1995; Wang and Eaton 2001; see the chapter by Field et al., this volume). They are susceptible and respond asymptotically to infection with many viruses (Sulkin and Allen 1974; Calisher et al. 2006). These characteristics and the increasing presence of bats and bat products in food and traditional medicine markets in southern China and other Asian countries (Mickleburgh et al. 2002) suggest that bats could be a potential natural reservoir host of SARS-CoV. Recently, two groups have independently reported the presence of SARS-like viruses in different species of horseshoe bats within the genus *Rhinolophus*.

In one study conducted from March to December of 2004, a total of 408 bats representing nine species, six genera and three families from four locations in China (Guangdong, Guangxi, Hubei and Tianjin) were sampled by trapping in their native habitat (Li et al. 2005). Blood, faecal and throat swabs were collected for antibody and PCR analyses. Three communal cave-dwelling species from the genus *Rhinolophus* in the family Rhinolophidae had a high SARS-CoV antibody prevalence: 13 of 46 (28%) in *R. pearsoni* from Guangxi; two of six (33%) in *R. pussilus* from Guangxi; and five of seven (71%) in *R. macrotis* from Hubei. The high seroprevalence and wide distribution of seropositive bats is consistent with the pattern of serology expected from a pathogen's wildlife reservoir host (Hudson et al. 2002).

The serological findings were corroborated by PCR analyses using primer pairs derived from the nucleocapsid (N) and polymerase (P) genes of SARS-CoV. A total of five positive faecal samples were detected, three in *R. pearsoni* from Guangxi and one each in *R. macrotis* and *R. ferrumequinum*, respectively, from Hubei. Genome sequence analysis indicated that SARS-like coronaviruses (SL-CoVs) present in bats have an almost identical genome organisation to those of SARS-CoVs isolated from humans or civets, sharing an overall sequence identity of 92%. The most variable regions were located in the 5' end of the S gene, which codes for the surface spike protein involved in receptor binding, and in the ORF10-coding region immediately upstream from the N gene, which contains the coding region for putative nonstructural proteins of unknown function (Marra et al. 2003; Rota et al. 2003) and is known to be prone to mutation and deletions of various sizes (Guan et al. 2003; Song et al. 2005; The Chinese SARS Molecular Epidemiology Consortium 2004). When these regions were excluded from the comparison, the sequence identity increased to 94% between SL-CoVs and SARS-CoVs (Li et al. 2005). It was interesting to note that the ORF10-coding region of bat SL-CoVs contained the 29-nt sequence present in civet SARS-CoV isolates and human SARS-CoV isolates from the early phase of the outbreak, but absent from human isolates obtained in the later phases of the outbreak (The Chinese SARS Molecular Epidemiology Consortium 2004). This finding suggests that SL-CoVs and SARS-CoVs may have a common ancestor.

In another study reported by Lau et al. (2005), it was found that 23 (39%) of 59 anal swabs of wild Chinese horseshoe bats (*Rhinolopus sinicus*) contained genetic material closely related to SARS-CoV when analysed by PCR. It was also found that up to 84% of the horseshoe bats examined contained antibodies to a recombinant N protein of SARS-CoV. This study was conducted using wild animals from unpopulated areas of the Hong Kong Special Administration Region of China. Analysis of three full-length genome sequences derived from PCR products revealed similar findings to those reported by Li et al. (2005) in that the bat viruses shared an overall 88% nucleotide and 93% sequence identity to ten human and civet SARS-CoVs isolated from different locations and at different times during the SARS outbreaks, and the major differences were located in the S gene and ORF10-coding region. The bat viruses from Hong Kong also contained the 29-nt sequence in the ORF10 region.

The genetic diversity observed among bat-derived SL-CoVs together with the high prevalence and wide distribution of seropositive bats, as revealed by two independent groups, are consistent with bats being the wildlife reservoir host of SL-CoVs. As shown by Li et al. (2005), comparison of partial sequences from SL-CoVs isolated from three different horseshoe bat species revealed a much higher genetic diversity than those observed among all the reported

sequences of civet and human SARS-CoVs. Furthermore, sequence analysis also indicated that human and civet SARS-CoV nestle phylogenetically within the spectrum of SL-CoVs, suggesting that the viruses responsible for the SARS outbreaks were members of this diverse coronavirus group, tentatively named the group 2b coronaviruses or G2b-CoVs (Wang et al. 2006). This notion was strengthened by the comparison of genetic relatedness among the different bat viruses detected in Hong Kong and mainland China. As mentioned above, the overall genome sequence identity between the human or civet SARS-CoV and the bat viruses Rp3 (isolated from *R. pearsoni*) and B24 (isolated from *R. sinicus*) was 92% and 88%, respectively. The sequence identity between Rp3 and B24 is 89%, suggesting that Rp3 has a closer evolutionary relationship to the civet/human isolates than to the B24 isolate of a different bat species.

Further surveillance studies in the region are required to investigate the distribution and diversity of the G2b-CoVs in different bat species, and to find the location and reservoir species of the SARS-CoVs responsible for the SARS outbreaks in 2002–2003 and 2003–2004.

8

Factors Contributing to the Emergence of SARS

Emergence of zoonotic viruses maintained by wildlife reservoir hosts is a complex and poorly understood sequence of events. Childs (2004) and Childs et al., this volume, recognised four transitions in the process by which zoonotic viruses are transmitted and infect other species. Two of these transitions, inter-species contact and cross-species virus transmission (i.e. spillover) are essential and sufficient to cause epidemic emergence. Two other transitions, sustained transmission and virus adaptation within the spillover species, are not required for emergence, but will determine the magnitude and scope of subsequent disease outbreaks. These transition events are discussed below in relation to the potential mechanism of SARS emergence.

8.1

Inter-species Contact and Spillover

There are a number of possibilities for contact between horseshoe bats, the putative reservoir host (H_R), and one or more secondary hosts (H_C). This could happen in the bat's natural habitat and in a variety of other situations. Horseshoe bats are cave-dwelling animals which feed mainly on moths and beetles and may have the opportunity to come into close proximity with other animals which live in or explore caves. It is interesting to note that in the study by Li et al. (2005),

serological findings indicated that *Rousettus leschenauti*, a much larger cave-dwelling fruit bat, may also be infected by a closely related G2b-CoV, although at much lower frequency. Contact between bats and other animal species may also arise because bats are used as a source of medicinal components and live bats are among a large number of different animal species that are traded in animal markets. From the studies by Li et al. (2005) and Lau et al. (2005), we know that the main route of excretion of G2b-CoV from naturally infected bats is via faecal shedding. The opportunity of virus transmission between H_R and H_S is therefore further enhanced since direct contact of bats and other animals may not be absolutely required for the virus to pass to a H_S . Live animal trading in China and Asia thus provides the most likely circumstances for inter-species contact.

As revealed in an epidemiology study conducted during the peak of the SARS outbreaks in China, most animal traders handle more than one animal species, thus providing numerous opportunities for animal-to-animal contact. This could happen during transportation, where animal cages are often piled on top of each other, or in the market where more than 100 different animal species can be housed under a single roof simultaneously. Wholesale animal markets or warehouses also offer the possibility of sustained opportunity for inter-species contact because animals may be kept together for an extended time before being sold individually. The notion of inter-species transmission in wholesale or retail markets is supported by the finding in two different studies that G2b-CoVs were detected in civets and raccoon dogs in the market, but not in the farms which claimed to have supplied the animals to the particular markets surveyed (Tu et al. 2004; Kan et al. 2005).

The second transition (i.e. cross-species transmission or spillover) requires not only inter-species contact, but also the susceptibility of H_S animals to the virus. For SARS-CoV, this does not seem to be a major constraint. As discussed above, a large number of mammalian species have been demonstrated to be susceptible to SARS-CoV infection, either under experimental conditions or by natural infection in markets. Spillover is defined as introduction, replication and release of virus from the H_S (Childs 2004). For SARS-CoV, there was ample evidence to suggest that this has happened in more than one H_S species, including civets, raccoon dogs, ferret badgers and humans.

8.2

Sustained Transmission and Virus Adaptation

Three separate surveillance studies indicate that, at least for the civet populations in markets, intra- H_S transmission of SARS-CoV occurred readily (Guan et al. 2003; Tu et al. 2004; Kan et al. 2005). SARS-CoV reactive antibody was found in 79% of civets in January 2004 in one study and 100% of civets tested in

another study contained SARS-CoV genomic RNA. Civet trading was banned in May 2003 after the first SARS outbreaks, but was resumed in August 2003. Considering that there was no evidence of widespread infection of SARS-CoV among civet populations on farms and in the wild (Tu et al. 2004; Kan et al. 2005), it can be concluded that re-appearance of the virus in the civet population in markets in late 2003 to early 2004 was a result of separate spillover event(s) which occurred after the resumption of civet trading in August 2003. This would suggest sustainable intra- H_s transmission among civets after spillover events. Similarly, intra- H_s transmission among different human populations was documented in many different cities, especially in Guangzhou, Hong Kong, Beijing, Singapore and Toronto (Anderson et al. 2004). It is conceivable that such intra- H_s transmission would have been sustained for a much longer period if draconian quarantine measures had not been implemented.

Virus adaptation is the fourth transition considered to be important in determining the scope and magnitude of a disease outbreak after a spillover event (Childs 2004). Several studies demonstrated rapid evolution of the SARS-CoV sequence, especially in the receptor-binding domain (RBD) of the spike protein gene, a location believed to be important for virus adaptation to the different H_s species, i.e. civet and human.

In the first detailed molecular epidemiology study (The Chinese SARS Molecular Epidemiology Consortium 2004), 61 SARS-CoVs derived from early, middle and late phases of the SARS outbreaks in 2003 were analysed by genomic sequencing. It was discovered that genotypes characteristic of each phase could be identified, and that the earliest genotypes were the most similar to those of SARS-CoVs isolated from animals. Moreover, it was shown that while the neutral mutation rate of the viral genome was constant during the different phases of the outbreak, the amino acid substitution rate of the coding region slowed during the course of the outbreak, indicating rapid adaptation to the human host. As expected, the spike protein-coding gene showed the strongest initial responses to host selection pressures.

In a separate study focusing on SARS-CoVs isolated from humans and civets during the 2003–2004 outbreaks, Song et al. (2005) discovered that the ratio of nonsynonymous/synonymous nucleotide substitution in viruses isolated from civets collected 1 year apart and from different geographic locations, was very high. This suggested a rapid process of virus evolution in civets, much like the adaptation process revealed for human SARS-CoV isolates in the first study (The Chinese SARS Molecular Epidemiology Consortium 2004). These results also indicated that civets were not likely to be an H_R , and highlighted their potential role as an H_s involved in transmitting the virus from bats to humans. The authors concluded that major genetic variations in critical genes, particularly the S gene, are essential for the progression from animal-to-human transmission to sustained human-to-human transmission, which eventually led to the first SARS outbreaks in 2002–2003.

The rapid evolution of SARS-CoVs in palm civets in markets in Guangdong was also confirmed by Kan et al. (2005) who analysed a total of 17 animal-derived sequences isolated in January 2004 and compared them to those from animals and humans isolated in 2003. Their study revealed that viruses in palm civets in the live animal markets had undergone a process of evolution that generated viruses with the potential to infect humans. Within the 17 animal-derived sequences, there were three which did not contain any of the novel signature variation residues (SNV) that characterised previously isolated pathogenic viruses. The authors postulated that such viruses were the evolutionary starting point of a process which introduced seven SNVs and caused the substitution of six amino acid residues in the spike protein. The resulting virus jumped to humans and was the cause of the low pathogenic infection of humans in 2003–2004. A further 14 SNVs caused 11 amino acid residue changes and resulted in the high-pathogenicity viruses which were responsible for human infection during early phase of the 2002–2003 outbreaks. Finally, six SNVs with four amino acid changes produced the group of viruses that were responsible for the global epidemic in the middle to late phases of the SARS outbreaks.

The metalloproteinase, angiotensin-converting enzyme 2 (ACE2), has been identified as the functional receptor for SARS-CoV infection (Li et al. 2003). In a comparative study of binding affinity of different S proteins to human and civet ACE2, it was shown that S proteins of SARS-CoVs isolated from civet and the mild human cases in 2004 bind to human ACE2 much less efficiently than the S proteins of SARS-CoV isolated from human patients during 2002–2003 outbreaks (Li et al. 2003). Similar findings were obtained in a separate study by Yang et al. (2005). It was found that the S protein from viruses isolated from a patient in late 2003 and from two civets depended less on the human ACE2 receptor and were markedly resistant to antibody inhibition.

These data demonstrated that SARS-CoVs were successful in both maintaining intra- H_s transmission among at least two different H_s species and in adapting to the new hosts via rapid virus evolution. These attributes made possible the rapid global spread of SARS-CoV to cause the most severe infectious disease outbreak of the twenty-first century.

9 Conclusions

Less than 3 years after the first emergence of SARS, rapid progress has been made in the identification and genetic analysis of the aetiological agent and its molecular epidemiology, the identification of the host receptor and molecular characterisation of the virus–host interaction, and the rapid development of

diagnostic assays and vaccine and therapeutic candidates. The recent identification of horseshoe bats as the natural reservoir of this new group of G2b-CoVs will undoubtedly play an important role in facilitating our understanding of SARS emergence and in the prevention of future outbreaks. Bats have been identified or implicated as the natural reservoir host for an increasing number of new and often deadly zoonotic viruses. In addition to the emergence of G2b-CoVs from insectivorous *Rhinolophus* species, Hendra virus, Nipah virus and, most recently, Ebola virus have been shown to have fruit bat reservoir hosts (Chua et al. 2002; Halpin et al. 2000; Leroy et al. 2005; see the chapters by Field et al., and Gonzalez et al., this volume). Bats typically respond asymptotically to virus infection and display a capacity to permit persistent virus infections (Sulkin and Allen 1974). Their wide distribution and abundant status (one mammalian species in five is a bat) makes them prime candidates for reservoirs of viruses which may, like G2b-CoVs, jump the species barrier and infect humans and other animals. Information on the ecology of bats and the nature of their response to virus infection may not only be scientifically interesting, but may also provide fundamental information on how best to cope with further outbreaks of disease caused by bat-borne viruses (Calisher et al. 2006).

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