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ORIGINAL ARTICLE

Identification of SARS-like coronaviruses in horseshoe bats (*Rhinolophus hipposideros*) in Slovenia

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Abstract Bats have been identified as a natural reservoir for an increasing number of emerging zoonotic viruses, such as Hendra virus, Nipah virus, Ebola virus, Marburg virus, rabies and other lyssaviruses. Recently, a large number of viruses closely related to members of the genus *Coronavirus* have been associated with severe acute respiratory syndrome (SARS) and detected in bat species. In this study, samples were collected from 106 live bats of seven different bat species from 27 different locations in Slovenia. Coronaviruses were detected by RT-PCR in 14 out of 36 horseshoe bat (*Rhinolophus hipposideros*) fecal samples, with 38.8% virus prevalence. Sequence analysis of a 405-nucleotide region of the highly conserved RNA polymerase gene (pol) showed that all coronaviruses detected in this study are genetically closely related, with 99.5–100% nucleotide identity, and belong to group 2 of the coronaviruses. The most closely related virus sequence in GenBank was SARS bat isolate Rp3/2004 (DQ071615) within the SARS-like CoV cluster, sharing 85% nucleotide identity and 95.6% amino acid identity. The potential risk of a new group of bat coronaviruses as a reservoir for human infections is highly suspected, and further molecular epidemiologic studies of these bat coronaviruses are needed.

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Introduction

The majority of coronaviruses are disease-causing agents [34]. Human coronaviruses are associated with respiratory and gastrointestinal diseases, but coronaviruses detected in animals cause severe respiratory, enteric, neurological or hepatic diseases in their hosts. Coronaviruses were responsible for severe acute respiratory syndrome (SARS), which represents the twenty-first century's first previously unknown pandemic transmissible disease. SARS coronaviruses isolated from this pandemic are genetically distinct from previously known coronaviruses of animals and humans [39]. SARS coronaviruses have also been isolated from small mammals such as civets (*Paguma larvata*) and raccoon dogs (*Nyctereutes procynoides*) from live-animal markets in the southern part of the People's Republic of China, suggesting that these animals could be the direct source of the SARS epidemic in 2003 [19]. The identification of SARS-like coronaviruses in bats and other wild animals at live-animal markets suggest that a novel human pathogen emerged as a result of interspecies transmission [19]. These findings highlight the potential human-health risk posed by coronaviruses in wild animals. To predict the risk of coronavirus host transition and disease outbreaks, we need to have a deeper understanding of coronavirus reservoirs, such as bats.

Coronaviruses belong to the genus *Coronavirus* within the family *Coronaviridae*, order *Nidovirales*. Coronaviruses are enveloped viruses with positive-stranded RNA genomes of 26–32 kb, the largest continuous RNA genomes in nature [5, 16]. Coronaviruses have a unique mechanism of viral replication that results in a high frequency of recombination and high mutation rates that allow them to adapt to new hosts and niches [26]. On the basis of genetic analysis, coronaviruses are subdivided into three

distinct groups: groups 1 and 2 include viruses that are pathogenic for mammals, and group 3 includes viruses that are pathogenic for poultry [16, 17]. Group 1 consists of human coronaviruses, porcine transmissible gastroenteritis virus (TGEV), porcine epidemic diarrhea virus (PEDV), feline infectious peritonitis virus (FIPV) and some bat coronaviruses. Group 2 includes human coronavirus, bovine coronavirus, murine hepatitis virus, porcine hemagglutinating encephalomyelitis virus and bat coronaviruses. Group 3 consists of infectious bronchitis virus and turkey coronavirus.

Recent studies from the People's Republic of China have identified bats as the most likely source of all coronaviruses [27, 30, 36, 45, 48]. Bats represent approximately 20% of all living mammal species and are distributed worldwide [22]. Bats have wide dietary diversity, including small vertebrates, nectar, pollen, fruits, blood, and insects. The majority of bat species live in social groups ranging from a few up to 20 million, the largest contiguous colony of mammals on earth [11]. All bat species have nocturnal activity and characteristic roosting behavior during the day. In Slovenia, we can find up to 30 different bat species that are characteristic of northern countries as well as species that are characteristic of southern countries, such as *Rhinolophus euryale* [37]. Insects are the main food of bat species living in Slovenia. Bats occupy diverse ecologic niches in a large range of habitats. Roost places include cavities in tree trunks, caves, cervices in rock and tree bark, bridges, cellars, wells and houses. During this time, they can have accidental contacts with humans, possibly enabling virus transmission.

In this study, we investigated whether bats in Slovenia also harbor coronaviruses. To our knowledge, this is the first report of coronaviruses in bats in Slovenia. One hundred six fecal samples from bats in Slovenia were analyzed using primers for the highly conserved RNA polymerase gene. Sequence comparisons were done with available sequences in GenBank database.

Materials and methods

Sample collection

During the period from May to October 2008, a total 106 bats of 7 different species were captured at 27 different locations in Slovenia (Fig. 1). Bats were captured manually by using nets and hand nets. Live bats were captured in 16 different churches, 1 house, 1 castle, and 1 cave and by net trap at 8 different streams. All of the captured animals were placed separately into cotton bags for several minutes to calm them down before further investigations and sampling

Fig. 1 Locations of sampling shown on a map of Slovenia. Each number (1–27) represents one location where bat samples were collected. Coronavirus RT-PCR-positive samples were found at the following locations: Spodnji Log in commune Kočevje (14), Livold in commune Kočevje (15), Tinje in commune Slovenska Bistrica (17), Knežja Njiva in commune Loška dolina (22) and Mali Otok in commune Postojna (23). Other sampling locations are Vnanje Gorice (1), Verd (2), Goče (3), Krašnja (4), Volovščica (5), Trebnje (6), Dolnji Suhor (7), Kobiljača (8), Završe (9), Kobilje (10), Mali Obrh (11), Močnik (12), Račna (13), Škocjan (16), Puščava (18), Dobrova (19), Selca (20), Snežnik (21), Velike Lašče (24), Iška vas (25), Cerkno (26) and Rižana (27)

started. While being kept in the bag, the bats produced fecal pellets that were collected and transported from the field to the laboratory and stored at −70°C until use. Bats were classified according to species, sex, age, gravidity, and lactation status and typed by morphologic criteria by bat biologists. All manipulations of live animals were done in a manner consistent with our national guidelines for the capture, handling and care of bats.

Processing and analysis of samples

Fecal samples were suspended in minimum medium (RPMI 1640, Gibco) and homogenized by vortexing. Centrifugation was done at 3000 rpm for 10 min to obtain supernatants. From these supernatants, 50 µl of each sample was transferred to 500 µl of buffer AVL from the QIAamp Viral RNA Mini Kit (Qiagen) and processed further according to the instructions of the manufacturer. Total RNA was eluted in 50 µl of AVE buffer and was used as the template for reverse transcription and polymerase chain reaction (RT-PCR).

Electron microscopy

A limited number of samples ($n = 16$) that were in sufficient quantity was also examined for viruses using negatively stained direct electron microscopy (EM) of prepared stool suspension. The EM grid was incubated with 50 µl of stool suspension without a prior concentration step, washed with sterile water and incubated with 2% phosphotungstic acid (pH 4.5) for negative staining. Samples were examined using a JEM-1200 EX II transmission electron microscope (JEOL, Japan) for 20 min per grid at various sites prior to final result.

RT-PCR and sequencing

RT-PCR was performed by amplifying a 440-bp-long fragment of the highly conserved RNA-dependent RNA polymerase (*pol*) gene using the primers 5′ GGT TGG GAC TAT CCT AAG TGT GA 3′ and 5′ CCA TCA TCA GAT AGA ATC ATC ATA 3′, which were designed to amplify all known coronaviruses [43]. The RT-PCR was performed by using One Step RT-PCR Kit (Qiagen). The amplified products were analyzed by 1.8% agarose in gel electrophoresis and visualized under UV light after ethidium bromide staining. RT-PCR products from 14 positive samples were gel-purified and subjected to nucleotide sequence analysis. Two PCR products were sequenced for each strain. Sequencing reactions were performed using an ABI 310 Genetic Analyzer (Applied Biosystems) with ABI PRISM dye terminator sequencing chemistry, according to the manufacturer's instructions.

Data analysis

Nucleotide sequences obtained from the RT-PCR products were assembled with the DNASTAR program and compared to the known sequences of the *pol* genes of coronaviruses in the GenBank database, using the Basic Local Alignment Search Tool (BLAST) program. A 405-bp region of the sequences obtained, which was present in most of the GenBank entries, was used for phylogenetic analysis. A multiple alignment of the nucleotide and protein coding sequences was constructed using Clustal W to determine genetic relationships among all of the sequences using neighbor-joining criteria. Final phylogenetic tree construction was performed with the Phylip program [12].

Results

From May to October 2008, live bats were caught and classified according to species, age, gravidity and lactation status (Table 1). A total of 106 fecal samples from 7 bat species were collected. To identify coronaviruses from these samples, an RT-PCR method using a primer pair from a conserved region was used to screen the field

Table 1 Summary of bat fecal samples tested for coronaviruses, divided according to species, with numbers of collected male and female bats, lactating and gravid status

Species	Number of positive samples by RT-PCR	Number of negative samples by RT-PCR	Male	Female		
			Young (adult)	Young (adult)	Lactating	Gravid
Horseshoe bat *(Rhinolophus hipposideros)*	14	22	6 (1)	7 (0)	6	16
Daubenton's bat *(Myotis daubentonii)*	0	26	9 (3)	10 (0)	0	4
Whiskered bat *(Myotis mystacinus)*	0	4	2 (0)	1 (0)	0	1
Mouse-eared bat *(Myotis myotis)*	0	31	16 (0)	5 (1)	3	6
Kuhl's pipistrelle *(Pipistrellus kuhlii)*	0	3	2 (0)	0 (0)	0	1
Nathusius's pipistrelle *(Pipistrellus nathusii)*	0	2	1 (0)	1 (0)	0	0
Serotine bat *(Eptesicus serotinus)*	0	4	0 (2)	1 (0)	0	1

samples. Positive PCR products were detected in 14 out of 106 fecal samples examined. Coronavirus-positive bats were found at 5 different locations in Slovenia (Fig. 1) with 100% virus prevalence in Spodnji Log, 66.6% virus prevalence in Livold and Tinje, 33.3% virus prevalence in Mali Otok and 20% virus prevalence in Knežja njiva. Of the seven bat species studied, only one species, horseshoe bat (*Rhinolophus hipposideros*), was positive for coronaviruses (Table 1). Fourteen (38.8%) out of 36 horseshoe bat fecal samples were positive by RT-PCR. Four of the positive samples were from young males and ten of them were collected from females. Six of the positive females were classified as lactating, and four of the positive females were classified as young females.

The amplicon of each positive sample was subjected to DNA sequencing. To characterize the overall diversity of coronavirus sequences, including 14 sequences from this study (Table 2), a phylogenetic tree (Fig. 2) of a 405-bp fragment of the *pol* gene was generated from available sequences from the National Center for Biotechnology Information database (NCBI). Analysis of nucleotide and amino acid sequences of this region revealed that Slovenian coronavirus strains from this study belong to the group 2 coronaviruses. The grouping of other coronaviruses into three main groups (Group 1, Group 2, and Group 3) was done according to the previously defined groups. When comparing the 14 sequences obtained, the Slovenian strains were found to have 100% amino acid identity and 99.5–100% nucleotide identity. The obtained sequences of 12 strains form group 2 (Fig. 2) and share 60.8–85% nucleotide identity to our sequences. When compared to the closest previously characterized coronavirus in GenBank,

Table 2 Data related to the 30 coronavirus strains used for phylogenetic analysis

Strain code	Host species	Group	Reference	Accession no.
HKU2	Bat	1	Woo et al. [53]	DQ249235
HKU7	Bat	1	Woo et al. [53]	DQ249226
HKU8	Bat	1	Woo et al. [53]	DQ249228
1B	Bat	1	Chu et al. [9]	NC_010436
1A	Bat	1	Chu et al. [9]	NC_010437
TGEV	Pig	1	Almazan et al. [1]	NC_002306
FIPV	Cat	1	Unpublished	AY994055
HCoV-NL63	Human	1	van der Hoek et al. [47]	NC_005831
HCoV-229E	Human	1	Thiel et al. [46]	NC_002645
PEDV	Pig	1	Kocherhans et al. [24]	NC_003436
512/2005	Bat	1	Tang et al. [45]	NC_009657
HKU6	Bat	1	Woo et al. [53]	DQ249224
HKU9-1	Bat	2	Woo et al. [54]	NC_009021
CoV-HKU1	Human	2	Woo et al. [52]	NC_006577
MHV	Mammalian	2	Unpublished	NC_006852
BCoV	Cattle	2	Chouljenko et al. [8]	NC_003045
PHEV	Pig	2	Vijgen et al. [50]	NC_007732
HCoV-OC43	Human	2	Vijgen et al. [49]	NC_005147
Rp3/2004	Bat	2	Li et al. [30]	DQ071615
SZ16	Bat	2	Guan et al. [19]	AY304488
SARS-CoV	Human	2	Marra et al. [31]	NC_004718
Rf1/2004	Bat	2	Li et al. [30]	NC_009695
HKU3	Bat	2	Li et al. [29]	DQ022305
Rm1/2004	Bat	2	Li et al. [30]	NC_009696
SLO1A0066	Bat	2	This study	GQ404795
SLO1A0050	Bat	2	This study	GQ404796
SLO1A0082	Bat	2	This study	GQ404797
TCoV	Turkey	3	Stephensen et al. [43]	AF124991
Peafowl/GD/KQ6/2003	Peafowl	3	Sun et al. [44]	AY641576
IBV	Chicken	3	Brierley et al. [3]	NC_001451

GenBank accession numbers represent available sequences of the *pol* gene that have been included in the phylogenetic tree. Only unique Slovenian bat coronavirus sequences (SLO1A0050, SLO1A0066, SLO1A0082) were presented with accession numbers

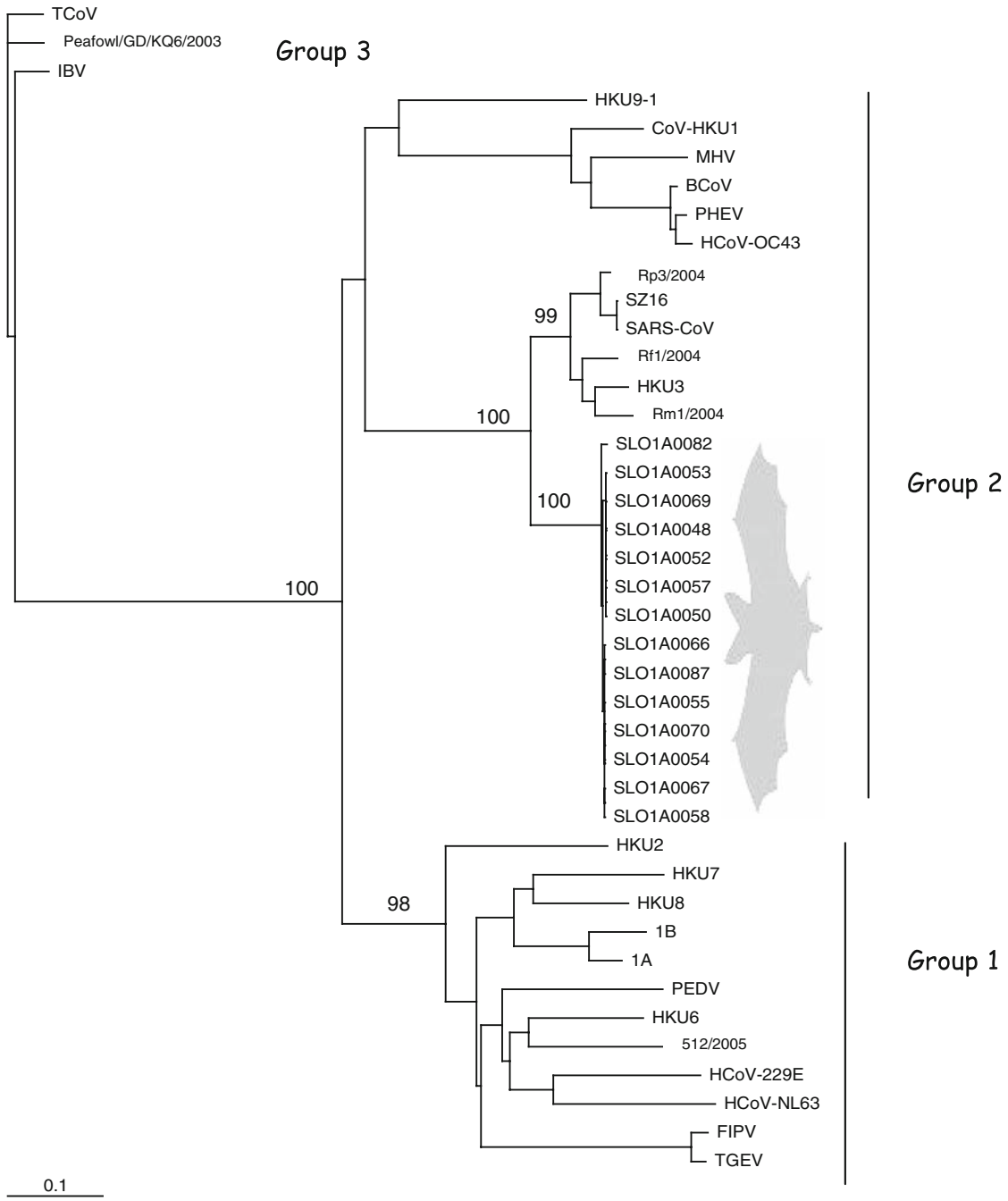

Fig. 2 Phylogenetic tree based on nucleotide sequences of a partial *pol* gene sequence with representative isolates from bats, other mammals and birds (Table 2). Analyses were conducted using the PHYLIP program with partial sequences (405 nt) of each isolate taken from GenBank (corresponding to nt 15,234–15,638 of the genome of bat strain Rp3/2004 (DQ071615), using the neighbor-joining algorithm and a bootstrap test of phylogeny. Boostrap values were obtained from 1,000 datasets, and only nodes with bootstrap values higher than 70% are presented. The new group of 14 coronaviruses from this study was found to belong to Group 2 (bootstrap value 100%)

the Slovenian strains share 85% nucleotide identity and 95.6% amino acid identity to strain Rp3/2004 (DQ071615). The genetic group of 14 coronaviruses from Slovenia forms a separate cluster with a significant bootstrap value (100%) within Group 2 (Fig. 2).

As described in "Materials and methods", 16 samples were examined by EM, of which 10 where previously characterized as RT-PCR positive for coronaviruses. There was a limited amount of stool suspension, and thus it was not possible to perform any concentration technique prior

to the EM examination. A clear coronavirus structure was detected in four of the coronavirus RT-PCR-positive samples examined (Fig. 3).

Discussion

To our knowledge, this is the first report of coronaviruses in bats in Slovenia. With more than 1,100 species, bats are among the most divergent and widely distributed mammals [41]. Bats are reservoirs for rabies virus and other lyssaviruses and were recently shown to be reservoirs for other important emerging viruses. More than 60 different RNA viruses have already been detected in bats, thus suggesting an important role in transmitting and maintaining zoonotic viruses [4, 20, 51]. It is known that Old World fruit bats are a reservoir for Hendra virus, which can cause severe respiratory illness in humans and horses [13, 23, 25, 32, 33, 35, 38, 40] and Nipah virus, which can cause lethal encephalitis and respiratory illness in humans and pigs [6, 7, 10, 18]. Bats can also be reservoirs for Ebola virus and Marburg viruses [2, 28].

The need for understanding the transmission, ecology and evolution of coronaviruses in wildlife was highlighted by a study showing that SARS coronaviruses caused large human epidemics in 2003. Those isolates were more closely related to isolates from palm civets than to the human epidemic strain of SARS coronavirus [42]. Sequence analyses of SARS coronavirus genomes after the epidemic suggested that multiple independent species-jumping events of SARS coronavirus from wild animals to humans have occurred [29, 42]. Interspecies transmissions of animal viruses to humans are a constant threat to human health. In order to obtain a better understanding of the prevalence of coronaviruses in our country, we collected 106 fecal samples from 7 different bat species.

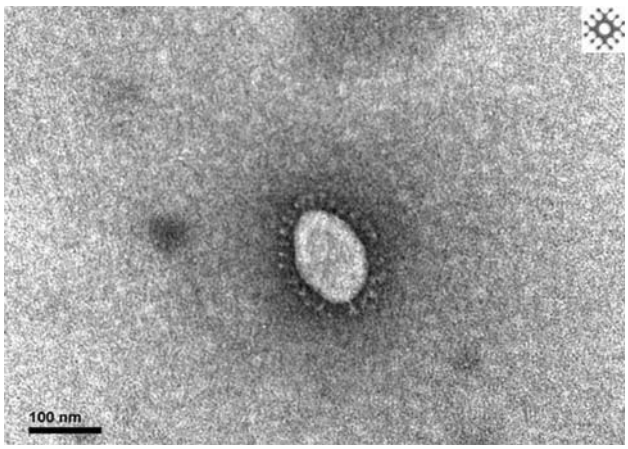

Fig. 3 EM micrograph of a coronavirus particle detected in a bat stool suspension

To understand how coronaviruses might be transmitted and maintained in bat population, factors such as species, age, sex, gravidity and lactation status were observed. Out of 14 coronavirus-positive horseshoe bats, 4 of them were young males, 6 were lactating females, and 4 were young females. These results confirm that young bats of both sexes can be virus positive, as well as lactating bats, but virus was not detected in gravid bats. The data obtained in our study are similar to those from studies conducted in Germany [15]. Virus can be transmitted between young bats and lactating mothers, and from these data, it is highly possible that young bats provide a susceptible population for amplifying the coronavirus and transmitting it to lactating females in maternity colonies. Comparable to the experience with respiratory infections in humans, virus would replicate less efficiently in adults than in young animals because of partial immune protection, which is the result of infection in earlier life. This could explain the lower prevalence of virus in adult bats.

From the results of this study, we can speculate that our data do not reveal a complete picture of the presence of coronaviruses in bats in Slovenia because the RT-PCR method relies on detection of viral sequences. Animals that have already recovered from coronavirus infection could be negative in our assay but positive for antibodies. While sample sizes of each animal species are limited, viruses that are circulating at a low copy number may be missed. However, detecting coronaviruses by direct electron microscopy without prior concentration shows that a high concentration of coronaviruses could be excreted from bats' feces. According to the estimated minimal particle concentration for EM diagnostics, it can be presumed that the coronavirus concentration in EM-positive samples is above 10^6 particles/ml [21]. The conserved primers used in our study were based on available coronavirus sequences and were used successfully for amplification of this new group of coronaviruses. However, these primers might not be able to detect coronaviruses that are genetically more divergent from previously known coronaviruses.

The new group of bat coronavirus sequences had typical features of coronaviruses. All Slovenian sequences had the highest percentage of amino acid identity with the group 2 coronaviruses. When compared with other bat isolates from group 2 coronaviruses (Rf1/2004, Rp3/2004, HKU3, Rm1/2004, HKU9-1), the Slovenian strains had from 75.6 to 96.3% amino acid identity and from 64% up to 85.8% nucleotide identity. The novel genetic group of coronaviruses presented in our study is probably the result of the first detection of coronaviruses in bats on our territory, and the high diversity of these viruses, resulting from the high mutation rate of RNA viruses due to infidelity of their polymerases and a higher chance of recombination as a result of their unique replication mechanism [55]. Before

the SARS epidemic in 2003, a total of 19 coronaviruses were known, 2 of which were isolated from humans, 13 from other mammals and 4 from birds. Since the epidemic, 2 novel human [14] and at least 10 previously unrecognized coronaviruses from bats have been described [53]. The diversity of coronaviruses in bats is astonishing, and there are probably many other unknown coronaviruses in other animal species.

A recent phylogenetic analysis of coronaviruses in bats, other mammals, humans and birds has suggested that bats could be original host from which all coronavirus lineages are derived [54]. The first detection of coronaviruses from horseshoe bats in Slovenia and genetic analysis of sequences obtained from them has confirmed that viruses from this study are related to SARS coronaviruses from group 2 isolated from human. Horseshoe bats commonly roost in churches, houses and castles, where humans can be exposed and ecologic overlap between bats and humans can occur.

In conclusion, it is possible that another epidemic caused by an emerging coronavirus originating from bats could occur in the future. Detection, isolation and molecular epidemiology studies of bats in various geographic locations and animal species could help to elucidate host range, receptor specificities and the potential of coronaviruses in new emerging infectious diseases.

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