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MERS-related CoVs in hedgehogs from Hubei Province, China

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ABSTRACT

The emerging coronavirus diseases such as COVID-19, MERS, and SARS indicated that animal coronaviruses (CoVs) spillover to humans are a huge threat to public health. Therefore, we needed to understand the CoVs carried by various animals. Wild hedgehogs were collected from rural areas in Wuhan and Xianning cities in Hubei Province for analysis of CoVs. PCR results showed that 5 out of 51 (9.8%) hedgehogs (*Erinaceus amurensis*) were positive to CoVs in Hubei Province with 3 samples from Wuhan City and 2 samples from Xianning City. Phylogenetic analysis based on the partial sequence of RNA-dependent RNA polymerase showed that the CoVs from hedgehogs are classified into *Merbecovirus* of the genus *Betacoronavirus*; the hedgehog CoVs formed a phylogenetic sister cluster with human MERS-CoVs and bat MERS-related CoVs. Among the 12 most critical residues of receptor binding domain in MERS-CoV for binding human Dipeptidyl peptidase 4, 3 residuals were conserved between the hedgehog MERS-related CoV obtained in this study and the human MERS-CoV. We concluded that hedgehogs from Hubei Province carried MERS-related CoVs, indicating that hedgehogs might be important in the evolution and transmission of MERS-CoVs, and continuous surveillance of CoVs in hedgehogs was important.

1. Introduction

In recent years coronaviruses (CoVs) including severe acute respiratory syndrome coronavirus (SARS-CoV), Middle-East Respiratory syndrome coronavirus (MERS-CoV), and SARS-CoV-2 had emerged as severe threats to public health worldwide. As of 14 September 2021, WHO has reported 225,024,781 confirmed cases of COVID-19 with 4,636,153 deaths globally [1]. CoVs are RNA viruses that cause diseases in mammals and birds. The *Coronaviridae* subfamily *Coronavirinae* contains four genera including *Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus*, and *Deltacoronavirus* [2]. *Betacoronavirus* was further divided into five subgenus, namely *Embecovirus*, *Sarbecovirus*, *Merbecovirus, Nobecovirus*, and *Hibecovirus* [3–6]. Seven CoVs are known to infect humans including the 2 alphacoronaviruses HCoV-229E and HCoV-NL63, and 5 betacoronaviruses including MERS-CoV belonging to *Merbecovirus*, SARS-CoV-2 and SARS-CoV belonging to *Sarbecovirus*, and HCoV-OC43 and HCoV-HKU1 belonging to *Embecovirus* [7–13].

Because of the close genetic similarity between bat CoVs and human CoVs, bat CoVs had been proposed as the origin of human CoVs, which evolved into human CoVs through unknown intermediate animal hosts [14,15]. Hedgehogs belong to the order *Eulipotyphla*, which is phylogenetically related to the order *Chiroptera*, and has a similar lifestyle to insectivorous bats [16]. Some *Betacoronavirus* had also been detected in European hedgehogs from Germany and France [17,18]. A novel CoV (*Ea*- HedCoV HKU31) was discovered in *Erinaceus amurensis* hedgehog from Guangdong, China in 2019 [19].

These data suggested that hedgehogs may contribute to the evolution of CoVs. In order to explore the host diversity and potential animal origin of human CoVs, we analyzed the CoVs of hedgehogs collected from the rural areas of Wuhan and Xianning cities, Hubei Province, China.

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2. Materials & methods

2.1. Sample collection

Hedgehogs were trapped using mist nets in the forest sites near cesspools from Wuhan City (31°22′N, 113°41′E) in April 2019 and Xianning City (29°53′N 114°13′E) in May and October 2018 in Hubei Province in central China. The hedgehogs were identified morphologically and euthanized with chloral hydrate [20]. The intestine samples of hedgehogs were collected and stored at −80 °C. The study was approved by the Ethics Committee of Wuhan University (2018010). Hedgehogs were handled in accordance with good animal practices required by the Animal Ethics Procedures and Guidelines of the People's Republic of China. ArcGIS 10.3 was used for mapping hedgehog collection sites.

2.2. RNA extraction, PCR amplification and sequencing

RNA was extracted from the hedgehog intestine tissues with QIAamp RNA Mini Kit (Qiagen, Hilden, Germany). RNA was used as a template for PCR amplification of CoV genes using the Access RT-PCR System (Promega, Madison, WI, USA). For the initial PCR screening, CoVs were detected with degenerated primers targeting the RNA-dependent RNA polymerase (*RdRp*) gene (Table 1) by using a one-step RT-PCR and nested-PCR amplification as described previously [21]. For phylogenetic analyses, we attempted to extend the sequences of the initial PCR amplified region on both sides with degenerated primers designed by using known hedgehog CoVs sequences from GenBank and partial sequences obtained from this study (Table 1). The RT-PCR cycle included an initial denaturation at 95 °C for 5 min, followed by 30 cycles of 95 °C 30s, 50 °C 30 s, and 72 °C 90 s and a final extension of 10 min at 72 °C. The nested PCRs were performed with 1 cycle of 95 °C 5 min, followed by 35 cycles of 95 °C 30s, 55 °C 30s, 72 °C 1 min and a final extension at 72 °C for 10 min. Nuclease-free water was used as a negative control.

To amplify the receptor-binding domain (RBD) region of the spike (S) protein gene, 5 pairs of primers were designed based on 4 hedgehog CoVs (GenBank accession numbers: MK907287, NC_039207, MK679660, MG021452) (Table 1). The amplification was performed by a touchdown PCR as follows [22]:1 denaturing cycle of 95 °C 3 min followed by 4 cycles of denaturation at 95 °C for 30s, annealing with a temperature decreases 1 °C every cycle from 54 °C to a touchdown 51 °C for 40s and 35 cycles of 95 °C 30s, 50 °C 40s, 72 °C 40s, and a final extension at 72 °C for 10 min.

PCR products were analyzed with 1% agarose gel electrophoresis and detected with ethidium bromide under UV light. PCR products with expected size were excised from gels and extracted with a gel extraction kit (TSINGKE, Beijing, China) and were cloned into the pMD19-T vector (TaKaRa, Kusatsu, Shiga, Japan). Recombinant plasmids were sequenced bidirectionally.

2.3. Phylogenetic analysis

Sequence chromatograms were examined with Chromas 2.5.1 (Technelysium, Tewantin, QLD, Australia) and sequences were analyzed with the BLAST. Routine sequence management was conducted by using DNAStar software. After alignment and editing by ClustalW, a phylogenetic tree based on nucleotide sequence was constructed using the Maximum likelihood method with bootstrap values calculated by 1000 replicates with MEGA 7.0.

2.4. Receptor binding domain (RBD) sequence analysis

The RBD region at the C-terminal of S1 domain of MERS was responsible for receptor binding with human Dipeptidyl peptidase 4 (hDPP4) for viral entry to host cells. The RBD nucleotide (nt) sequence and predicted amino acid (aa) sequence of hedgehog CoVs were analyzed and compared with that of human pathogenic CoVs (MERS-CoV, SARS-CoV-2, and SARS-CoV), and bat CoVs which could use the hDDP4 and Bat CoV HKU4, respectively. Phylogenetic trees based on the whole RBD amino acid sequence were conducted using MEGA 7.0 (Fig. 1). The 12 most critical residues of RBD for binding hDDP4 in MERS-CoVs were compared in hedgehog CoVs. The alignment of RDB amino acid sequences was edited by GeneDoc 2.7.0.

3. Results

A total of 51 hedgehogs were collected from Hubei Province in May 2018 and April 2019, including 10 in Wuhan City and 41 in Xianning City adjacent to Wuhan City. All hedgehogs were identified through morphological observation as *E. amurensis* (Fig. 1).

PCR amplification of the intestine samples of hedgehogs showed that 5 of 51 (9.8%) hedgehogs were positive to CoVs with 3 samples collected from Wuhan City and 2 from Xianning City (Fig. 1). Initially, a sequence of 441 bp was obtained in 5 hedgehogs by nested PCR using group 1 primers (Table 1) targeting a conserved region of the RdRp gene. We tried to further extend the RdRp gene sequences in 5 positive hedgehogs by nested PCR with 3 groups of degenerated primers (Table 1). The

Table 1
PCR primers for amplification of hedgehog CoVs.

Primers		Primer sequences (5′-3′)	Target genes	Product length (bp)	References
Group 1	RdRp-1F1	GGITGGGAYTAYCCIAARTGYGA	RdRp	441	[21][a]
	RdRp-1R1	CCRTCATCWGAIARWATCATCAT			
Group 2	RdRp-2F1	GYGGYTATCACTAYAARG	RdRp	961	This study[a]
	RdRp-2R1	GCCAGGTTTAACATAATAACCACC			
	RdRp-2F2	GAYGGYGGTTGYCTTAATGC		440	
	RdRp-2R2	GCACATATTAGGCATAGCTC			
Group 3	RdRp-3F1	GCTCTTATGAGTGCTAATGGC	RdRp	943	This study[a]
	RdRp-3R1	GYYCTACCWGTGCACATA			
	RdRp-3F2	GATGATTCTCTCTGATGATGG		589	
	RdRp-3R2	TAACTGTCWAGCATGTGKCC			
Group 4	RBD-F1	CACAATTDCAATGCTCTTATGG	RBD	778	This study[a]
	RBD-R1	TCTATRCACTGHCCTAACAAGC			
Group 5	RBD-F2	GAATGTGMHTTAGATGTDTTGTT	RBD	371	This study[a]
	RBD-R2	CTRGAAATATAMGCATARTTA			
Group 6	RBD-F3	ACAGTAGACGTGTGTTCACCAA	RBD	395	This study
	RBD-R3	CACTCCTGAGCGACATGCTAT			
Group 7	RBD-F4	GCTCTTATGGKCAATTTGATATG	RBD	784	This study
	RBD-R4	ACTCCRTKAATGTCATAATC			
Group 8	RBD-F5	CCAATCCTACGTGYAGAATTC	RBD	295	This study
	RBD-R5	GTTATTACATAMACCATTTG			

[a] Primers which yield aimed PCR products from hedgehogs. The left primers did not work.

Fig. 1. Geographical distribution of hedgehog collection sites. The area within the blue line is Hubei Province and the red areas are Wuhan City and Xianning City, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

RdRp sequences were extended only in 2 hedgehogs; the gene in one hedgehog was extended to 1260 bp with degenerated primer group 2 and in another hedgehog was extended to 864 bp with degenerated primer group 3. The 441 bp sequences of RdRp gene from 5 hedgehogs were 95.5% (ranging from 92.8 to 99.3%) homologous. BLAST search indicated that these 5 sequences were most closely related to *Ea*-HedCoV HKU31 (MK907287) in hedgehogs from Guangdong Province, China with 93.7%–95.8% nt identity, and also shared 83.0%–83.3% nt identity to human MERS-CoV (KU710265) and bat MERS-related CoV (MF593268). The RdRp-grouping unit (RGU) based on the 816-nt was recommended for the prediction of CoVs species classification [23]. The amino acid of the RGU of RdRp sequences obtained in the hedgehog CoV from this study was 1.2% different from the *Ea*-HedCoV HKU3, and at least 6.3% different from known *Betacoronaviruses* species, which was the recommended criterion for the classification of CoVs species [17,23,], therefore, hedgehog CoVs from this study were the same species as the *Ea*-HedCoV HKU31 strain.

3.1. Phylogenetic analysis

Phylogenetic analysis based on the 441 fragment of RdRp gene indicated that all the CoVs detected from hedgehogs in this study were classified into *Merbecovirus* of the genus *Betacoronavirus*. The phylogenetic tree showed that sequences from hedgehogs in this study and another hedgehog CoV from China (*Ea*-HedCoV HKU31) formed a monophyletic group, which was in the same clade with hedgehog CoVs from Germany and England (Fig. 2). Also, the hedgehog CoVs were in a phylogenetic sister relationship to human MERS-CoVs and bat MERS-related CoVs.

3.2. Sequence analysis of CoV RBD

The phylogenetic analysis indicated that the hedgehog CoVs and MERS-CoVs were closely related, which prompted us to further analyze the similarity of the RBD region between hedgehog CoVs and MERS-CoVs. The whole RBD nucleotide sequence (651 bp) of a CoV (HedCoV HBD9) was obtained from 1 hedgehog by PCR amplification with RBD-F1 and RBD-R1 primers and the partial RBD nucleotide sequences of CoVs (HedCoV HBD2, HedCoV HBD7, and HedCoV HBD9) were obtained from 3 hedgehogs by PCR amplification with RBD-F2 primers and RBD-R2 primers. Predicted amino acid sequence (217aa) based on the whole RBD nucleotide sequence of HedCoV HBD9 was 39.0% identical with human MERS-CoV. A phylogenetic tree was constructed using the RBD amino acid sequences of hedgehog CoVs and MERS CoVs. The results showed that hedgehog CoVs and MERS CoVs formed an evolutionary branch distant from SARS-CoV and SARS-CoV-2 (Figs. 3 and 4).

MERS-CoVs used hDPP4 as the receptor for infection and 12 residues (Y499, N501, K502, L506, D510, E513, E536, D537, D539, R542, W553, V555) of RBD were critical for binding the hDPP4 [24–26]. In HedCoV HBD9, 3 of 12 residues was conserved corresponding to Y499, D537, and D539 in MERS-CoV, while 6 residues were conserved in bat CoVHKU4 which is thought to have the ability to bind to hDPP4 as corresponding to Y499, K502, L506, L513, L536, and D537 in MERS-CoV (Table 2).

3.3. Nucleotide sequence accession numbers

The nucleotide sequences obtained in this study were deposited in GenBank with accession numbers: MT002834-MT002836, MW879219-MW879221 and OK117933- OK117935.

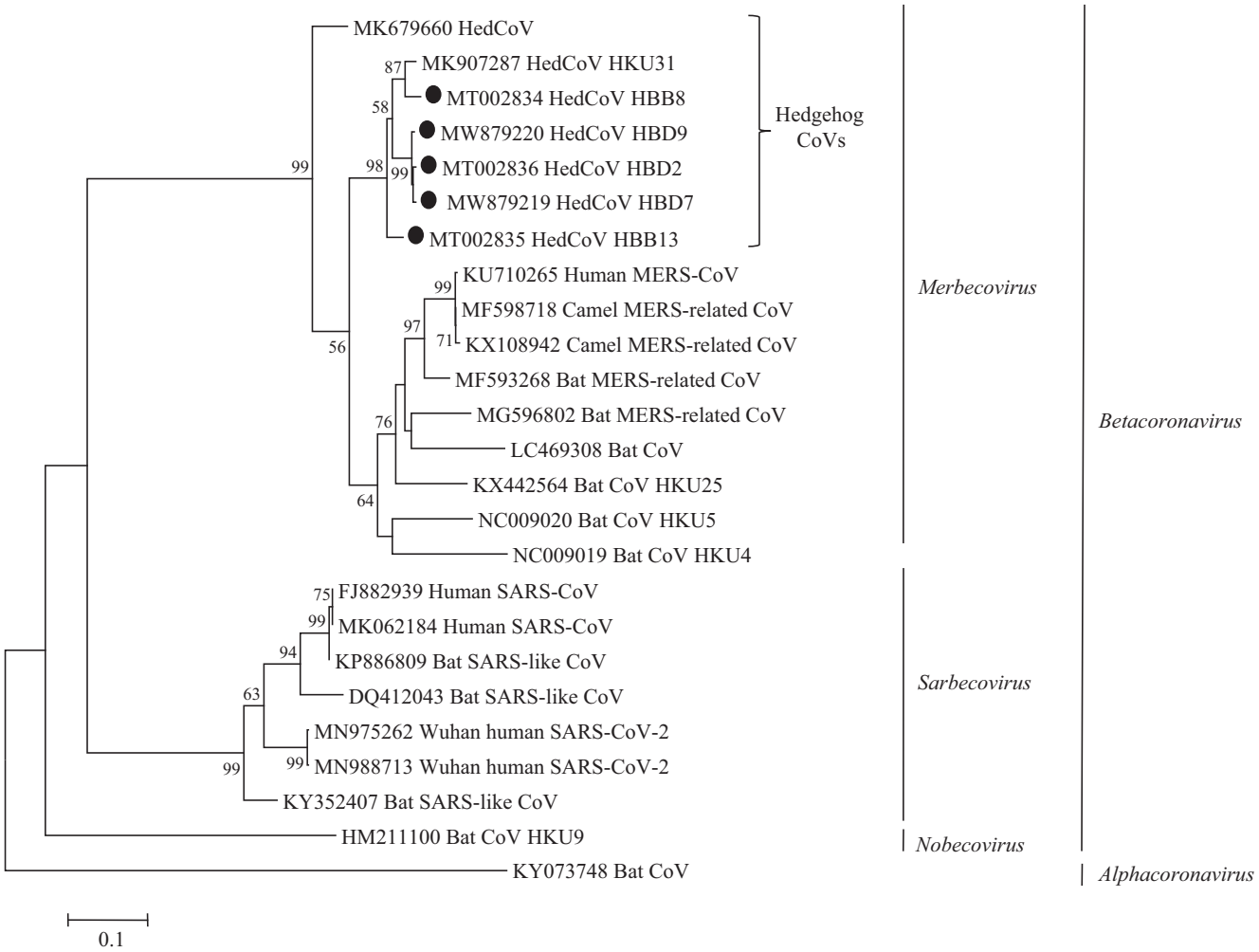

Fig. 2. Phylogenetic tree of CoVs based on sequences of the RdRp gene fragment (441 bp). The phylogenetic tree was constructed with a Maximum-likelihood method using MEGA 7.0, and bootstrap values >50% from 1000 replicates were shown on the nodes. The strains detected in this study were indicated with solid cycles.

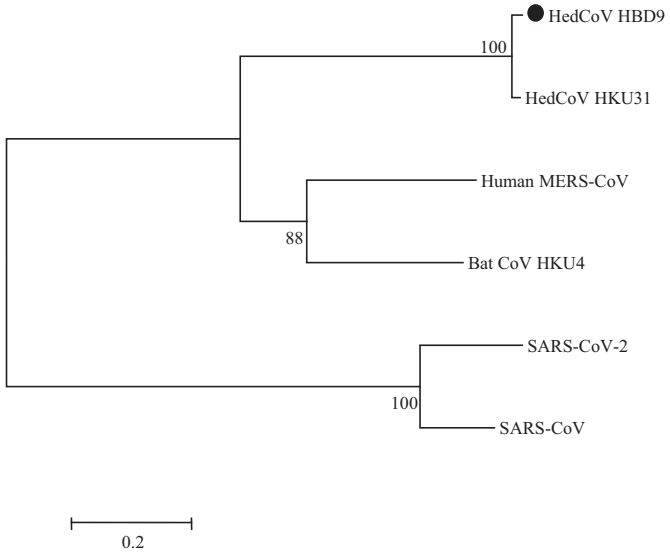

Fig. 3. Phylogenetic tree with the RBD amino acid sequences using MEGA 7.0. The parameters were the same as Fig. 2.

4. Discussion

We found *Merbecovirus* of genus *betacoronaviruses* from 5 hedgehogs (*E. amurensis*) collected from Wuhan and Xianning cities in Hubei Province, China. The prevalence of CoVs in hedgehogs was very high (9.8%) in Hubei Province, which was higher than the positive rate of hedgehogs in Germany (1.6%) and Guangdong Province (7.4%), but lower than the positive rate of hedgehogs in France (50%) [17–19]. Previous studies on hedgehog CoVs were scarce, especially in China. Our study indicated that hedgehogs are an important animal host of CoVs and may play an important role in the evolution and transmission of CoVs.

CoVs not only cause respiratory and gastrointestinal diseases in humans, but also cause diseases in animals. Bats were recognized to be natural hosts for a variety of zoonotic pathogens, and the potential origin of SARS-CoV, MERS-CoV, and SARS-CoV-2 [15,27–31]. Horseshoe bats were found to carry SARS-like CoVs in Asia, Europe, Africa, and other regions and were believed to be natural reservoirs of the 2003 SARS pandemic [28,32,34,35]. In contrast, the host range of MERS-like CoVs is more extensive, including diverse bat species in the genera *Tylonycteris, Pipistrellus, Hypsugo*, and *Verspetiliom* of the order *Chiroptera*, and hedgehogs including *E. amurensis* and *E. europaeus* [26,34–38,23,39–46]. The animal order *Eulipotyphla* including hedgehogs, shrews, moles, and solenodons and the order *Chiroptera* are phylogenetically related [16], which suggests that there might be a

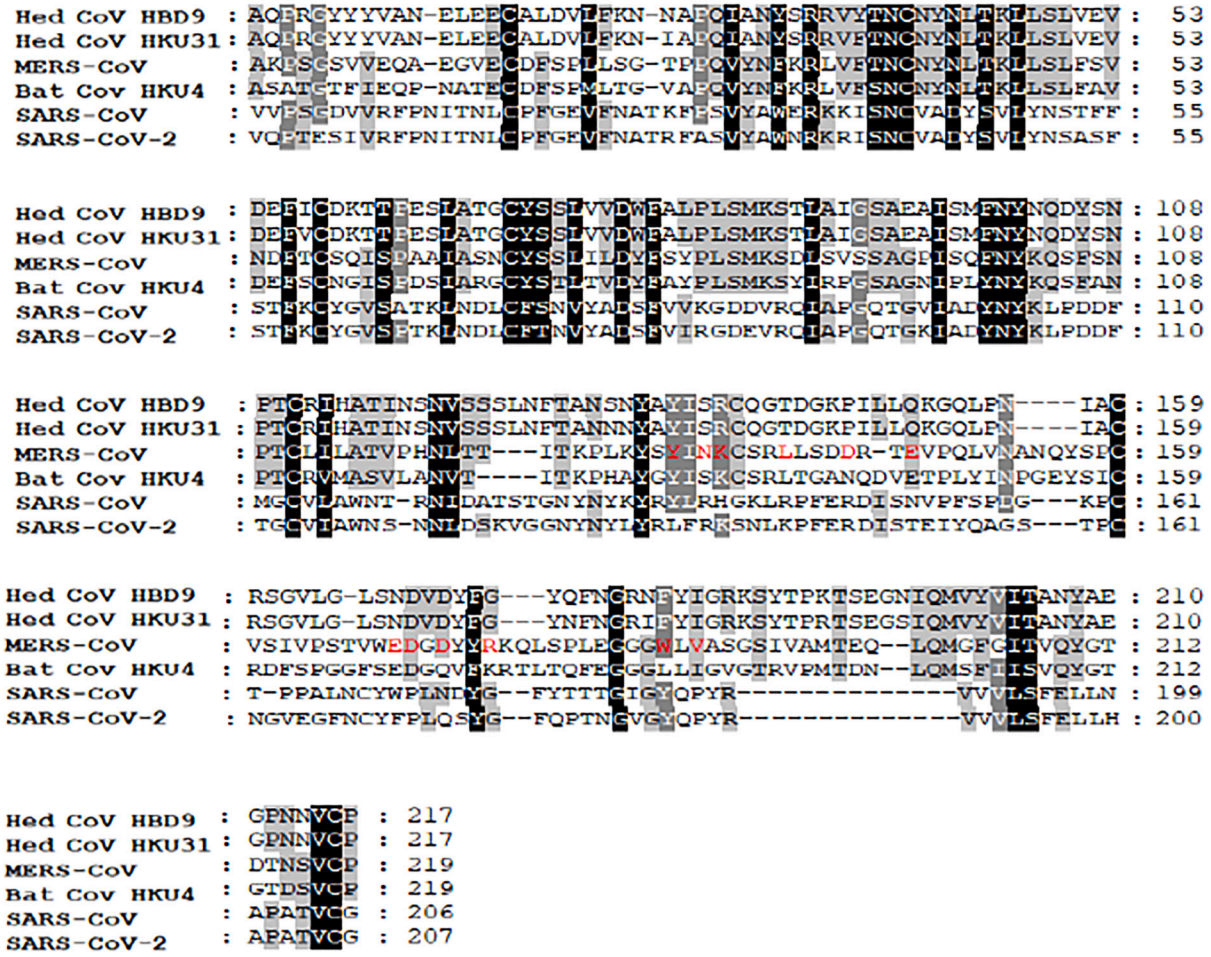

Fig. 4. RDB aa sequences alignment by MEGA 7.0 and editing with GeneDoc2.7.0. The conserved residues were highlighted in black and grey shadings and red residues presented the critical residues for the binding of RBD and hDPP4 in MERS-CoVs. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Variation of 12 critical residues of RBD in HedCoV HBD9.

MERS-CoV	Y 499	N 501	K 502	L 506	D 510	E 513	E 536	D 537	D 539	R 542	W 553	V 555
HedCoV HBD9	[a]Y	S	R	T	P	Q	N	[a]D	[a]D	G	F	I
Bat CoV HKU4	[a]Y	S	[a]K	[a]L	N	[a]E	[a]E	[a]D	Q	K	L	I

[a] Indicated the conserved residues.

correlation between pathogens carried by hedgehogs and bats, especially insectivorous bats. Therefore, continuous surveillance and investigation of insectivorous animals such as hedgehogs and bats are of great significance in exploring the origin and evolution of various CoVs, and as an early warning of newly emerging CoVs in their natural animal reservoirs.

Hedgehog CoVs from Hubei Province in our study was phylogenetically closely related to, but different from hedgehog CoV strains (*Ea*-HedCoV HKU31) from Guangdong Province, and CoVs strains from Europe [19]. The variation of CoV strains among hedgehogs from different places was probably due to the instability of CoVs transmission between different hosts and regions. Usually, different CoVs were restricted to their hosts, and there was a tight barrier against CoV host switching, preventing humans from acquiring novel CoVs from animals [47]. However, some CoVs could spill over from their animal hosts to humans such as SARS-CoV, MERS-CoV, and SARS-CoV-2 by cross-species transmission. Based on the results of this study, hedgehog CoV strains were phylogenetically closely related to human MERS-CoVs and bat MERS-related CoVs.

The RBD region of MERS-CoV was the key domain responsible for binding with the hDPP4 receptor to mediate viral entry to host cells. Bat CoV HKU5 and bat CoV HKU25 were suggested to be able to bind to the human hDPP4 [26,43]. Bat NeoCoV and bat MERS-like CoV PREDICT/PDF-20180 shared the most amino acid (aa) identity (86–87%) to the human MERS-CoV but lacked the ability to bind hDPP4 [41,42]. The RBD region of hedgehog CoV in our study was highly homologous to the RBD of the human MERS-CoV and the bat MERS-like CoVs. Previous studies showed that 12 critical aa residues of the RBD were critical for binding the hDPP4, the RBD receptor [24–26]. In this study, the predicted RBD of HedCoV HBD9 possessed 3 out of 12 aa critical residues for binding to hDPP4, which was less than that of batCoVHKU4 (6 conserved residues) and batCoV HKU25 (5 conserved residues), but more than that of batCoV HKU5 (1 conserved residue) [17,43].

The 3 conserved residues of the RBD of HedCoV HBD9 corresponded to Y499, D537 and D539 in human MERS-CoV, while Y499 in MERS-CoV was responsible for forming a hydrogen bond with hDPP4 and a

single-residue substitution of this residue could interrupt the binding, and D537 and D539 together form a negative surface and disrupting the negative surface lead to significant reduction of the binding and viral entry [25]. BatCoV HKU4 and HKU5 were both discovered from bats collected in Guangdong Province five years before the MERS outbreak [23,38,], however, the ability of RBD for binding hDPP4 varied. The number of conserved residues of 12 aa critical residues for binding to hDPP4 in the hedgehog CoV of this study was among that of bat CoV-HKU4 and HKU5, which indicated that only 2–3 nucleotide mutations were needed to change the RBD of the hedgehog CoV to the RBD of human MERS.

The previous SARS epidemic, persistence of MERS, and ongoing pandemic of COVID-19 served as a constant reminder of the importance of surveillance and identifying possible newly emerging CoVs in human and natural animal reservoirs. Sixteen years after the SARS epidemic in 2003, SARS-CoV-2 has caused a larger-scale pandemic than SARS-CoV with 79.6% genomic homology to SARS-CoV since 2019 [48]. MERS-CoV has been identified in dromedaries in several countries and 27 countries have reported cases with 858 known deaths since 2012 [49]. Considering the mutation of CoVs and wild rang of hosts such as bats, dromedaries, and hedgehog, whether the next mutation of MERS-CoV with more transmissible and virulence will appear is worthy of great attention and a big challenge to public health. The RdRp gene sequences of the MERS-related CoVs obtained from 5 hedgehogs in this study vary from each other, and they are also different from the MERS-related CoVs obtained from hedgehogs in Guangdong Province. This result shows that the hedgehog is the host of MERS-related CoVs, and the hedgehog is an important animal host for gene mutation and recombination of MERS-related CoVs.

4.1. Limitations

There were several limitations in our study. Firstly, small sample size was a limitation which indicated that the prevalence of the hedgehog CoVs might change when more samples are studied. Secondly, the whole RBD nucleotide sequence of CoVs was not obtained from all hedgehogs which were positive to the RdRp gene of CoVs, and the hedgehog CoVs were not successfully isolated.

5. Conclusion

Our study demonstrated that hedgehogs from Hubei Province, China carried *Merbecovirus* with a high positive rate and the hedgehog CoVs were phylogenetically closely related to human MERS-CoVs and bat MERS-related CoVs. It suggested that hedgehogs might be important animal hosts in the evolution of *Merbecovirus* and MERS-related CoVs should be surveilled in hedgehogs in more areas of the world to study the evolution of MERS-CoVs and to prevent spillover of animal CoVs to humans.

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Availability of supporting data

The datasets analyzed in the study were not public available due to the regulation but were available from the corresponding author on reasonable request.

Authors' contributions

DL contributed to conduct the experiments and draft the manuscript. XQG contributed to conduct the experiments and revise the manuscript. HJH, ZML, LNY, XLG, SHD and XX contributed to conduct the experiments. HY revised manuscript. XJY contributed to study design, revise the manuscript and supervision.

All authors have seen and approved this version of the manuscript, and the manuscript is not currently submitted for publication elsewhere.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Wuhan University (2018010). Hedgehogs were handled in accordance with good animal practices required by the Animal Ethics Procedures and Guidelines of the People's Republic of China.

Declaration of competing interest

All authors declare no conflict of interest.

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