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BRIEF REPORT

Identification of coronaviruses in bats and rodents in northern and central Argentina

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Abstract

Due to the present pandemic situation and the many animal species that are epidemiologically involved, there has been a surge of renewed interest in investigating the coronavirus (CoV) population circulating in wildlife, especially bats and rodents, which are potential reservoirs of new human pathogens. In Argentina, information about the viruses present in these mammals is very limited. To investigate the presence of coronaviruses in this country, we obtained 457 samples from hematophagous, insectivorous, and frugivorous bats and rodents from two regions of Argentina. We report here the detection of alphacoronavirus sequences in three groups of bats as well as in rodents. Phylogenetic analysis showed the closest relationships to alphacoronaviruses from Brazil.

Keywords Alphacoronavirus · Bats · Rodents

Wildlife is known to be a major reservoir of viruses causing old and new infectious human diseases. The emergence of new pathogens with zoonotic potential represents a constant threat to global public health, as has been seen with the current pandemic caused by SARS-CoV-2. While this is the most notorious incident of a zoonotic event extending worldwide, events of this nature have happened before, for example, with HCoV-NL63, HCoV-229E, SARS-CoV-1, and MERS-CoV [1]. Despite these early warnings, research on the viral population circulating in wildlife is very limited, especially in bats. Bats are the only mammals with the ability to fly, and they are also one of the most diverse groups within this class (Mammalia) of animals. Bats are known reservoirs of viruses with zoonotic potential, such as paramyxovirus, filoviruses, and lyssaviruses, but new viruses are being identified continually through next-generation sequencing (NGS) [2, 3]. Coronaviruses infecting bats have been implicated as predecessors of those causing human infections. Bats may also become new reservoirs of viruses infecting other species, which, in turn, may infect humans in a process of reverse zoonosis, as suggested for SARS-CoV-2 [4]. Thus, surveillance of viral populations, in particular, coronaviruses, in these mammals has become a priority in order to learn more about the events leading to interspecies and intraspecies transmission. The subfamily *Orthocoronavirinae* is divided into four genera, *Alphacoronavirus, Betacoronavirus, Gammacoronavirus*, and *Deltacoronavirus*. All bat coronaviruses belong to the genera *Alpha-* and *Betacoronavirus*. In addition, 15 subgenera have been established within the genus *Alphacoronavirus* (International Committee on Taxonomy

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of Viruses, [ICTV] 2021), and alphacoronaviruses from American bats have been classified into seven clades (A to G) [5].

The study described in this report was based on bat and rodent sample collection carried out in two regions of Argentina, one of which was the northwestern region of Argentina, which is in an area where some of the most important phytogeographic/ecological units of South America converge, with desert/semi-arid regions alongside subtropical rainforests. This has given rise to one of the areas of Argentina with the greatest animal diversity. Unlike the north, the central part of Argentina is characterized by extensive plains, where some of the most important agricultural and livestock activity in the country is concentrated. Due to the high level of agricultural production and the large number of food storage facilities, mainly for grains and cereals, the region provides an environment conducive to the presence and development of populations of wild animals that inevitably cohabit with domestic animals, production animals, and humans.

Several surveys over the years have contributed to our knowledge of the different bat species that coexist within these areas. In Argentina, field studies have identified 67 species of bats representing five families (Emballonuridae, Noctilionidae, Phyllostomidae, Molossidae, and Vespertilionidae) and 29 genera [6]. Despite the increasing interest worldwide in studying the populations of viruses that circulate in wildlife, there have been few such studies in bats in Argentina.

Bat and rodent samples were collected in 2020 and 2021 in northern and central Argentina, the Yungas region in the province of Jujuy, and the northeastern region of the province of La Pampa (Supplementary Fig. S2). The sampling sites were selected based on their favourable characteristics for the nesting of these animals and their contact with humans and other domestic or wild animals. Thus, ruined buildings, abandoned iron mines, and cereal storage sheds in rural or periurban areas in which nests of different bat species were already known to be present were chosen for sampling.

Animals were captured using mist nets in the case of bats and trap cages in the case of rodents to guarantee the safety of both the animals and the operators. The nets were placed at the exit of the nests before sunset to ensure the capture of the bats at the time of their exit. All animal procedures were performed according to a protocol approved by the Faculty of Veterinary Sciences, University of Buenos Aires (CICUAL 2020/9).

Each bat caught in the net was immediately handled by our team, taking the necessary precautions for such handling. Oropharyngeal swabs and, when possible, individual faecal samples were obtained from these animals. Due to their small size, no attempt was made to collect nasal or rectal swabs. The identification of species and, when possible, sex was done based on morphological characteristics. Each animal, before its release, had hair removed from its back to identify it as a "sampled animal" to avoid repeated captures. In addition, tissue samples were obtained from the carcasses of animals provided by local individuals who hunt them regularly. In these cases, samples of the spleen, liver, stomach, and intestine were taken. Faecal samples collected from areas inhabited by several bat colonies were pooled. For this purpose, a plastic layer was prepared in strategic areas during the night, and after two hours, several individual depositions were collected.

All samples were collected in the field in sterile tubes filled with nucleic acid preservative solutions to prevent degradation of viral RNA until processing. Swab and faecal samples were placed in DNA/RNA Shield 1X solution (Zymo Research, Irvine, CA, USA); tissue samples were transported in RNAlater™ stabilization solution (Invitrogen, Waltham, MA USA) and stored at -80º C until processing.

Faecal samples were homogenized in 250 µL of sterile 1X PBS for approximately 1 minute, followed by centrifugation at 3000 rpm for 5 minutes. Then, 20 µL of the supernatant was taken and brought to a final volume of 100 µL with 1X PBS before RNA extraction to reduce interference by PCR inhibitors. Oropharyngeal swab suspensions were vortexed for 5 minutes, and 100 µL was used for RNA extraction using a Quick-RNA Viral Kit (Zymo Research, Irvine, CA, USA) following the manufacturer's protocol. RNA extraction from tissues was performed using TRIzol Reagent (Invitrogen, Waltham, MA USA).

cDNA synthesis was performed by reverse transcription using random primers (EasyScript First-Strand cDNA Synthesis SuperMix kit, Transgene Biotech, Beijing, China), following the manufacturer's instructions. The presence of coronavirus RNA was evaluated by nested PCR (nPCR) with 5 µl of cDNA, 200 µM dNTPs, 1× buffer, 1 unit of GoTaq polymerase (Promega, Madison, WI, USA), and 25 pmol of pan-coronavirus primers [7]. In the first round, the primers RdRp For1 (5'-GGKTGGGAYTAYCCKAARTG-3') and RdRp Rev2 (5'-TGYTGTS WRCARAAYTCRTG-3') were used. For the second round, 5 µl of the first-round product was used with the primers RdRp For3 (5'-GGTTGGGACTATCCTAAGTGTGA-3') and RdRp Rev4A (5'-CCATCATCAGATAGAATCATCAT-3'). Both PCR reactions were run with the following program: 2 minutes at 94°C, 40 cycles of 94°C 1 minute, 48°C (first round) or 55°C (second round) 1 minute, 72°C 1 minute, and a final cycle at 72°C for 5 minutes.

These primers target a 440-bp conserved region of the ORF1ab gene that encodes part of the viral replication complex, specifically non-structural protein 12 (Nsp12), the central catalytic subunit of the enzyme [7]. Prior to

processing samples from bats and rodents, the specificity of the pan-corona primers was tested with different coronaviruses present in veterinary vaccines (canine, feline, bovine, and avian) or in previously confirmed SARS-CoV-2-positive human samples). Amplification was successful in all cases, and the identity of the bands was verified by Sanger sequencing.

The nested PCR products obtained by electrophoresis were purified using an EasyPure Quick Gel Extraction Kit (TransGene Biotech®, Beijing, China). DNA concentration was estimated using a NanoDrop Lite instrument (Thermo Scientific, Waltham, MA, USA). Sequencing was performed by the Sanger method, and similarity to known coronaviruses was assessed by BLAST analysis against the nucleotide dataset in the GenBank database. All sequences were submitted to GenBank, with accession numbers ON228222-23, ON237741-43, ON246265-73, and ON256703-05.

A dataset was compiled to include (i) sequences determined in this work (n = 17), (ii) the most similar sequences from GenBank to those from this work, obtained from a BLAST analysis (the five hits with the highest score, identity > 85% and coverage > 70%, excluding duplicates, n = 22), (iii) reference sequences for all subgenera of the genus *Alphacoronavirus* as defined by the ICTV (n = 15), (iv) representative sequences of previously identified alphacoronaviruses belonging to clades A to G [5] (n = 48), (v) other alphacoronavirus sequences from Argentina, available in GenBank on October 25, 2022 (n = 20), and (vi) sequences from members of the genus *Betacoronavirus* to be used as an outgroup (n = 4).

A multiple sequence alignment was performed with MAFFT v.7.4, using default parameters [8], and manually edited to trim the ends, using Bioedit v.7.2 [9] to obtain a final alignment of 126 sequences 360 nucleotides in length. A phylogenetic tree (majority-rule consensus) was obtained using Bayesian inference in MrBayes v.3.2.7 software [10], using the GTR + I + G nucleotide substitution model, selected by ModelFinder [11] under the Bayesian information criterion (BIC). The analysis was run to convergence, which was assessed by effective sample size values higher than 200 using Tracer v. 1.7.1 [12], with the first 10% of generations discarded as burn-in. In addition, a maximum-likelihood (ML) phylogenetic tree was obtained using IQ-TREE v2.1 [13], using the same substitution model as for the Bayesian analysis. To evaluate the reliability of the groups and branches obtained in trees, the Shimodaira-Hasegawa-like approximate likelihood ratio test (SH-aLRT) (1,000 replicates) [14] and ultrafast bootstrap approximation (UFB) (10,000 replicates) [15] methods were used. The phylogenetic trees were visualized using FigTree v.1.4.4. Divergence among sequences was estimated for nucleotide and amino acid sequences using MEGA 11 [16], with uncertainty evaluated as the standard error of the mean (SEM), using the bootstrap method (1,000 replicates).

Three hundred sixty-one samples obtained from hematophagous, insectivorous, and frugivorous bats from the areas selected for sampling were analysed: 178 corresponding to oropharyngeal swabs, 117 to individual faecal samples, and 34 to colony faecal pools (Table 1). Likewise, 93 samples from wild rodents, 29 individual faeces, 13 oropharyngeal swabs, and three pools of faeces were collected and analysed. A total of 80 tissue samples (spleen, liver, stomach, and intestine) from eight bats and 12 rats were also analysed (Table 1).

All samples were tested by RT-nPCR, and 17 were positive: fifteen from bat samples (nine oropharyngeal swabs, four individual faecal samples and faecal pools from two bat colonies), and two from individual rodent faeces. All tissue samples and oral swabs from rodents were negative. Most of the positive samples were from the Yungas region in the province of Jujuy, and only one was from the northeast region of La Pampa province (Table 1). It should be noted that the integrity of the RNA was not checked by amplification of a housekeeping gene, so a negative result could have been due to sample degradation rather than a real absence of viral sequences.

Analysis of partial RdRp sequences showed a high degree of nucleotide sequence similarity to alphacoronaviruses previously reported in bats in South America (particularly in Brazil) and the United States. Phylogenetic analysis of partial RdRp sequences confirmed that all of the viral sequences identified in bats and rodents belonged to members of the genus *Alphacoronavirus* (Fig. 1 and Supplementary Fig. S1). The trees obtained using different methods showed congruent topologies for all of the relevant groups. The sequences obtained in this work clustered into the previously named clades A (n = 2), B (n = 10), G1 (n = 2), and G2 (n = 3) (Fig. 1 and Supplementary Fig. S1).

Previous studies from Argentina have revealed the presence of sequences from clades A and B [17]. However, in this work, members of clades G1 and G2 were also found to be present (Fig. 1 and Supplementary Fig. S1).

Two sequences obtained from *Myotis* sp. clustered in clade A, whose members were previously associated with other species (*Tadarida* sp. and *Molossus* sp.) [5]. These sequences from Jujuy province (northern Argentina) showed a closer relationship to sequences from Brazil (*Molossus*) than to other sequences from Argentina (*Molossus* and *Tadarida*) (Fig. 1 and Supplementary Fig. S1).

In clade B, two faecal samples from wild rodents were found to contain alphacoronavirus sequences related to those found previously in insectivorous bats (*Myotis*, *Histiotus*, *Tadarida*), forming a highly supported monophyletic group

Table 1 Results of detection of CoV RNA in faecal or oral samples of bats and rodents in the northern and central Argentina

Bat/rodent species		Number of samples				Positive by sequencing		Capture region
Genus	Species	Oropharyngeal swabs	Feces (individual)	Fecal pools	Tissue	No. positive	Positive sample	
Desmodus	*rotundus*	0/24	2/6	0/4	--	2/34	Feces Feces	Northern Argentina
Myotis	sp.	3/30	3/22	0/10	--	6/62	Feces Feces Feces Oroph. swabs Oroph. swabs Oroph. swabs	Northern Argentina
Molossus	*molossus*	0/25	0/21	0/5	--	0/51	--	Northern Argentina
Histiotus	*laephotis*	2/23	0/18	1/2	--	3/43	Fecal pool Oroph. swabs Oroph. swabs	Northern Argentina
Eptesicus	*furinalis*	0/9	0/6	--	--	0/15	--	Northern Argentina
Tadarida	*brasiliensis*	1/12	0/7	0/2	--	1/21	Oroph. swabs	Northern Argentina
Artibeus	*lituratus*	2/10	0/5	0/2	--	2/17	Oroph. swabs Oroph. swabs	Northern Argentina
Rattus	*norvegicus*	--	2/16	0/2	--	2/18	Feces Feces	Northern Argentina
Tadarida	*brsiliensis*	0/15	0/11	0/3	0/32	0/61	--	Central Argentina
Myotis	*Sp.*	0/30	0/21	1/6	--	1/57	Fecal pool	Central Argentina
Rattus	*norvegicus*	0/13	0/13	0/1	0/48	0/75	--	Central Argentina
Total samples		8/191	7/146	2/37	0/80	17/454		

within this clade (clade B; Fig. 1 and Supplementary Fig. S1). Other sequences from *Molossus* and *Tadarida* reported previously in Central Argentina formed a separate cluster. It is noteworthy that two isolates from *Myotis* in central Argentina (one reported in this work) formed a basal group within clade B, supporting the suggestion that *Myotis* was the ancestral host of members of clade B [17].

Regarding clade G, two sequences from northern Argentina (*Artibeus*) were found to cluster into clade G1, closely associated with sequences from Brazil and Panama (*Artibeus*). Interestingly, we found alphacoronavirus sequences in bat species that were different from those that had already been reported in those species, such as in the case of an oropharyngeal swab from an insectivorous bat *(Myotis* sp.) in which we found sequences of alphacoronaviruses that had been reported in hematophagous bats (*Desmodus rotundus*) (clade G2 in Fig. 1 and Supplementary Fig. S1).

The authors of previous published virome studies performed in Argentina on several bat species (*Tadarida brasiliensis, Molossus molossus, Eumops bonariensis, Eumops patagonicus*, and *Eptesicus diminutus* [18, 19] reported the finding of alphacoronavirus sequences at least in *Tadarida brasiliensis*, but no GenBank accession numbers could be found for these sequences, and they were therefore not included in the phylogenetic analysis.

The results presented here represent one of the few reports on coronaviruses in bats in Argentina. As expected, the sequences from this study show a high degree of similarity to those of previously reported alphacoronaviruses from the same bat species in Brazil [20, 21] (Fig. 1), showing, for example, 0.9–1.4% nucleotide sequence divergence (and 0% amino acid sequence divergence) for *Artibeus lituratus* in clade G1 and 1.1–1.4% nucleotide sequence divergence (and 0% amino acid sequence divergence) for *Myotis* sp. in clade B (Supplementary Tables S1 and S2). This observation may just be due to the greater number of alphacoronaviruses reported in this country than in other countries of South America. Thus, more representative sequences are needed to evaluate whether the amplified fragment analyzed here really allows the identification of subtle differences among alphacoronaviruses within South American species. We did not detect infection of bats by two different alphacoronaviruses, as has been described by others [22, 23], but in several cases, a particular bat species was found to harbour alphacoronavirus sequences that had been associated with another bat species (alphacoronavirus from *Molossus rufus* detected in *Myotis* sp. and *Myotis* sp.

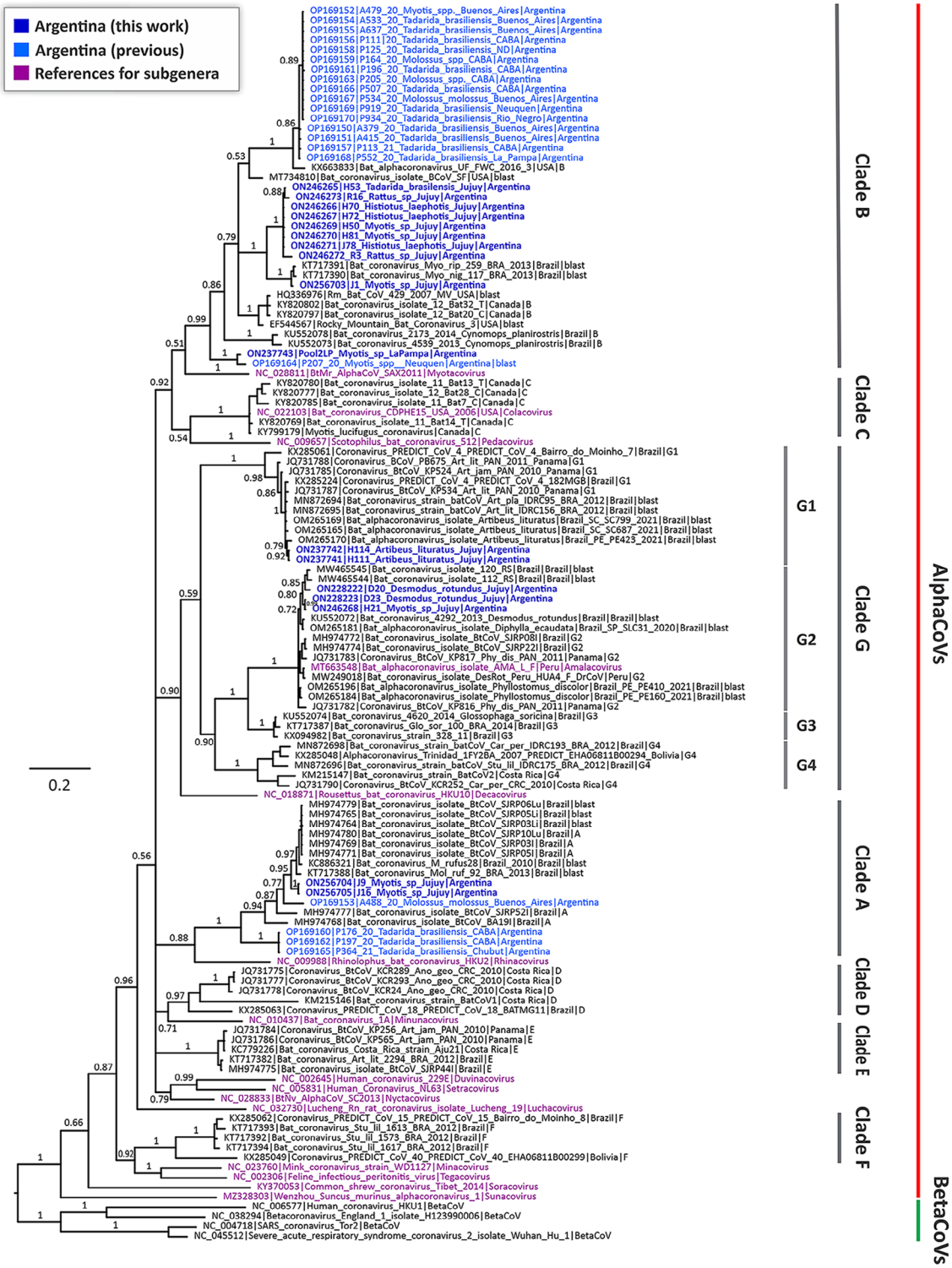

Fig. 1 Phylogenetic tree based on sequences from members of the genus *Alphacoronavirus* (indicated as "AlphaCoVs"), obtained by Bayesian methods. The analysis included sequences identified in this work (blue colour, bold), sequences obtained from the GenBank database that showed the highest score in BLAST analysis (indicated as "blast" at the end of the sequence names), sequences from other studies from Argentina (sky-blue colour), reference sequences from different *Alphacoronavirus* subgenera (purple colour), and sequences belonging to the previously defined clades A-G. Posterior probability values higher than 0.5 are shown at nodes for relevant groups. Sequences from members of the genus *Betacoronavirus* (indicated as "BetaCoVs") were used as an outgroup to root the phylogenetic tree.

alphacoronavirus sequences detected in *Histiotus laephotis* and *Tadarida brasiliensis* bats). This result is not surprising, since we sampled sites where several bat colonies were cohabitating, and similar findings have been reported previously [24, 25]. As seen for the two rodent samples from Jujuy, in which bat coronavirus sequences could be also detected, these observations may reflect the environmental source of the sample but not necessarily indicate an active infection. Nevertheless, the data also provide evidence that these viruses are circulating among the different bat species, suggesting the possibility of coinfections and the potential for viral recombination.

Still, some results require further investigation, as we found alphacoronavirus sequences from hematophagous bats in insectivorous bats (*Myotis* sp.) (Fig. 1 and Supplementary Fig. S1) where no proximity between these two species was seen. Another interesting finding has been the presence of alphacoronaviruses in *Myotis* sp. from central Argentina, forming a basal group within clade B, which includes sequences from South and North America that were obtained from several bat species, which emphasizes the importance of continuous work on the identification of new viral lineages in this geographical region.

No betacoronaviruses were identified in the samples collected in this study. Betacoronaviruses have been described in South American bat species, some of them represented in this study, such as *Artibeus* sp. and *Desmodus rotundus* [21, 26]. Due to the small number of samples collected and locations surveyed, the presence of betacoronaviruses cannot be excluded, but there seems to be enough evidence to conclude that alphacoronaviruses are the most common coronaviruses found in bats in Argentina, in agreement with reports from other South American countries [27–29].

Considering the crucial role bat coronaviruses play in zoonotic events like the one involving SARS-CoV-2, any information about their circulation in these mammalian species is extremely valuable for monitoring and understanding their evolution. Thus, the alphacoronavirus sequences reported here contribute to the global surveillance efforts to monitor these viruses and their potential as human pathogens.

Supplementary information The online version contains supplementary material available at https://doi.org/10.1007/s00705-023-05703-y.

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Author contributions The final paper was approved by all authors. AB, SLM, MM, and GC wrote the research project and received the grant. FLA, MM, and CB oversaw the collection of samples. FLA and AB performed the laboratory work. FLA and AB prepared the draft manuscript, data analysis, and genome annotation. CT performed the phylogenetic analysis.

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Data availability Partial sequences of the viral RdRp gene were deposited in the NCBI GenBank database and were assigned the accession numbers reported in this article.

Declarations

Conflict of interest The authors declare that there is no conflict of interest.

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