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SHORT COMMUNICATION

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Detection of coronavirus in vampire bats (*Desmodus rotundus*) in southern Brazil

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Abstract

The vampire bat (*Desmodus rotundus*) is a haematophagous animal that feeds exclusively on the blood of domestic mammals. Vampire bat feeding habits enable their contact with mammalian hosts and may enhance zoonotic spillover. Moreover, they may carry several pathogenic organisms, including coronaviruses (CoVs), for which they are important hosts. The human pathogens that cause severe acute respiratory syndrome (SARS-CoV), Middle East respiratory syndrome (MERS-CoV) and possibly coronavirus disease 2019 (SARS-CoV-2) all originated in bats but required bridge hosts to spread into human populations. To monitor the presence of potential zoonotic viruses in bats, the present work evaluated the presence of CoVs in vampire bats from southern Brazil. A total of 101 vampire bats were captured and euthanized between 2017 and 2019 in Rio Grande do Sul state, southern Brazil. The brain, heart, liver, lungs, kidneys and intestines were collected and macerated individually. The samples were pooled and submitted to high-throughput sequencing (HTS) using the Illumina MiSeq platform and subsequently individually screened using a pancoronavirus RT-PCR protocol. We detected CoV-related sequences in HTS, but only two (2/101; 1.98%) animals had CoV detected in the intestines by RT-PCR. Partial sequences of RdRp and spike genes were obtained in the same sample and the RdRp region in the other sample. The sequences were classified as belonging to *Alphacoronavirus*. The sequences were closely related to alphacoronaviruses detected in vampire bats from Peru. The continuous monitoring of bat CoVs may help to map and predict putative future zoonotic agents with great impacts on human health.

KEYWORDS

Bat, Coronavirus, *Desmodus rotundus*, PCR, sequencing

1 | BACKGROUND

Bats are important reservoirs of several pathogens, such as rabies virus (Rocha & Dias, 2020), henipaviruses (Nipah and Hendra virus) (Murray et al., 1995; Paton et al., 1999) and coronaviruses (CoVs) (Banerjee et al., 2019; Hu et al., 2015; Wong et al., 2019).

Coronaviruses can be classified into four genera: *Alphacoronavirus*, *Betacoronavirus*, *Deltacoronavirus* and *Gammacoronavirus* (Brownlie, 2017). Delta and gammacoronaviruses infect mainly birds, while alpha

and betacoronavirus genera contain important animal and human pathogens. Almost 60% of alpha and betacoronaviruses are related to bats, which are considered natural hosts of the members of these viral genera (Li et al., 2020; Wong et al., 2019). At least four different CoVs can cause symptoms of the common cold in humans: human CoV (HCoV)-229E and HCoV-NL63 (alphacoronaviruses) and HCoV-HKU1 and HCoV-OC43 (betacoronaviruses) (Corman et al., 2015; Künkel & Herrler, 1993; Sloots et al., 2006; Tao et al., 2017). In the last two decades, some betacoronaviruses that initially emerged in bats have caused severe disease outbreaks in humans: Middle East respiratory syndrome coronavirus (MERS-CoV) (De Benedictis et al., 2014) and severe acute respiratory syndrome coronavirus (SARS-CoV) (Li et al. 2005). Although the recent pandemic coronavirus disease 2019 (COVID-19) caused by SARS-CoV-2 (Zhou et al., 2020) has not been identified in any bat species, several closely related SARS-related coronaviruses have been found in *Rhinolophus affinis* bats, with 96% of genome identity (Boni et al., 2020).

The vampire bat (*Desmodus rotundus*) is a well-known haematophagous bat that feeds exclusively on the blood of domestic mammals. The feeding habit of vampire bats enhances their ability to transmit viruses and other pathogens via zoonotic spillover. *D. rotundus*, despite being well known for carrying the rabies virus (Rocha & Dias, 2020), also hosts a vast number of other viral species such as CoV. The first CoV identified in a vampire bat in Brazil was a betacoronavirus detected in 2008 in enteric content (Brandão et al., 2008). After, the presence of alphacoronavirus was reported in *D. rotundus*, through the amplification of the viral replicase gene in faecal samples (Asano et al., 2016). Another alphacoronavirus was detected in *D. rotundus* through rectal swabs in Peru (Bergner et al., 2020). Recently, a betacoronavirus closely related to MERS-CoV were found in the serum proteome of 17 vampire bats in Belize (Neely et al., 2020). The continuous monitoring of the presence of potential zoonotic viruses in bats is necessary, and the present work aimed to evaluate the presence of coronaviruses in vampire bats from southern Brazil.

2 | MATERIALS AND METHODS

Vampire bats (*Desmodus rotundus*) (n = 101) were captured in Rio Grande do Sul (RS) state in 49 different roosts spread in 35 different municipalities, during November 2017 and April 2019 (Figure 1) as part of the official continuous rabies surveillance programme

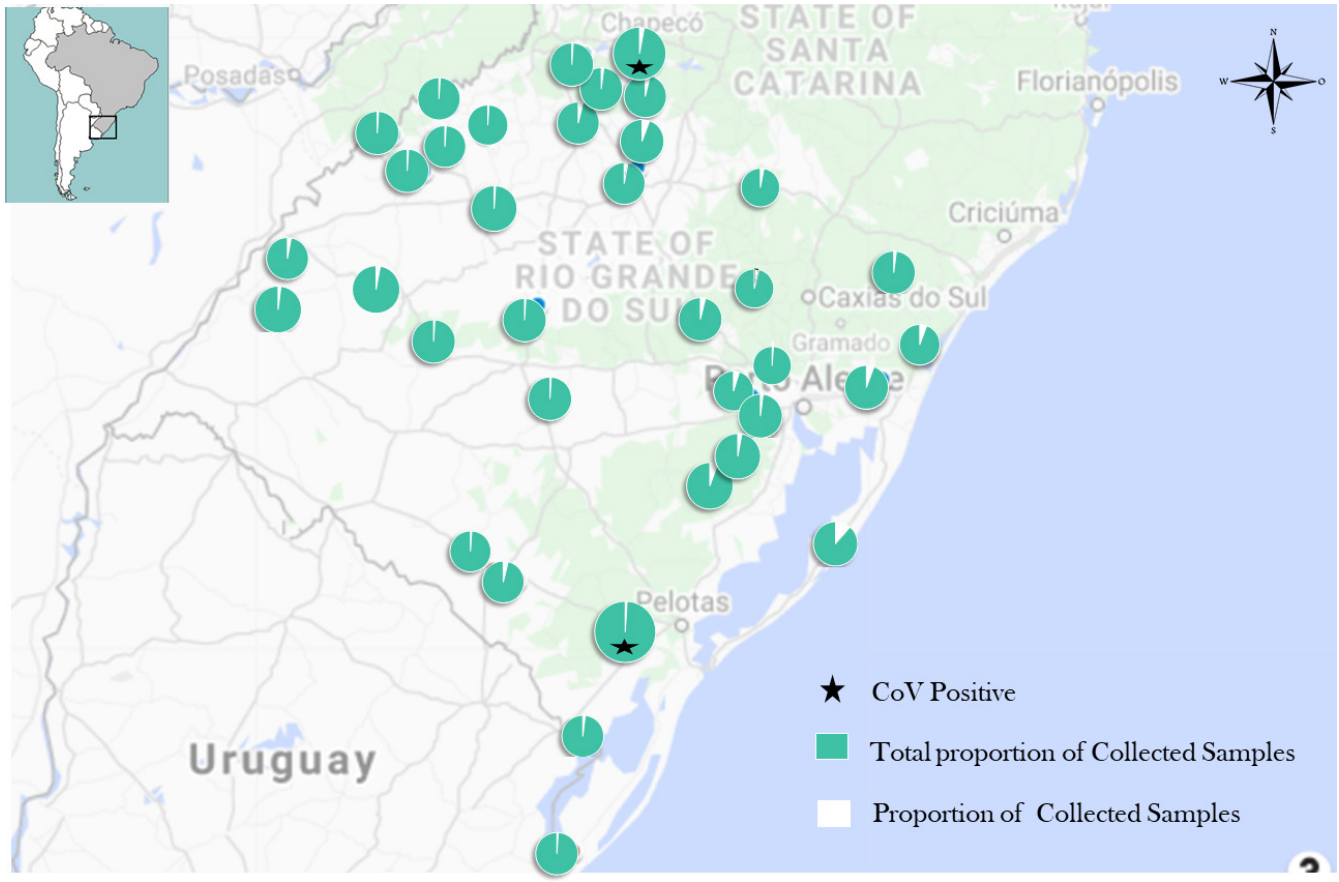

FIGURE 1 Geographical distribution of sampled vampire bats (*Desmodus rotundus*) investigated for the presence of coronaviruses (CoVs) in Rio Grande do Sul state, southern Brazil. The pie chart refers to the proportion of animals collected in the study, where the colour green represents the total proportion of collected bats and white the proportion of bats collected on this site. A black star represents location displaying positive bats for CoV detected in the present study

from RS state. The project was authorized by ICMBio (Chico Mendes Institute for Biodiversity Conservation) under licence number #61537-1. Animal collection was supported by the official Animal Sanitary Defense Service of the Secretary of Agriculture of Rio Grande do Sul state (Secretaria da Agricultura, Pecuária e Desenvolvimento Rural, SEAPDR-RS), southern Brazil. Bats were euthanized by an overdose of anaesthetic ketamine injected in the ventral region of the animal, following the recommendations of Resolutions CFMV No. 1000/2012 and Resolution CFBio No. 301/2012. Their organs (brain, lungs, liver, heart, kidney and intestines) were harvested separately (Table S1) and stored at −80°C until virological analysis. All organs were macerated individually and diluted to 20% (w/v) in phosphate-buffered saline (PBS) (pH 7.2). The samples were first screened by high-throughput sequencing (HTS) and then by a pancoronavirus RT-PCR protocol (Woo et al., 2006).

For HTS, the samples were pooled as follows: one pool containing 100 μl of brain, lungs, liver, heart and kidney of all the bats and one pool containing 100 μl of the intestines of all bats. PBS was added to the pools to a final volume of 50 ml, which was then centrifuged at low speed at 2,000 × *g* for 30 min at 10°C. The supernatant was filtered through a 0.22-μm filter to remove debris. RNA enrichment and HTS using the Illumina MiSeq System and an Illumina v2 reagent kit (2 × 150 paired-end reads) were performed as previously reported by our group (Weber et al., 2020).

The samples (brain, liver, lungs, kidney, heart and intestines) were also individually checked for CoV presence using a pancoronavirus RT-PCR protocol (Woo et al., 2006). RNA isolation was performed using TRI Reagent® (Sigma-Aldrich, St. Louis, MO, USA) in all organs of all individual samples, followed by cDNA synthesis using GoScript™ Reverse Transcriptase (Promega, Madison, WI, USA) with random primers (Invitrogen, Carlsbad, CA, USA). Pancoronavirus RT-PCR targeting a 440 bp fragment of the RNA-dependent RNA polymerase (RdRp) gene was carried out as described previously (Woo et al., 2006) to identify the organs with CoV in each animal.

An additional pair of primers was designed in the present study targeting a fragment of 332 bp of the spike (S) gene (forward 5′- AGTCTGGGGTTCATCCATT- 3′ and reverse 5′-TTGTGCATACAGGATCGGGC - 3′) according to the sequence found using the Illumina MiSeq platform. RT-PCR targeting the S gene was performed only on the two samples that were RdRp-positive. cDNA amplification by PCR was conducted in a total volume of 25 μl containing 1× PCR buffer, 1 mM $MgCl_2$, 0.5 mM dNTP mix, 0.2 mM each primer and 1 unit GoTaq® DNA Polymerase (Promega). Reactions were performed under the following conditions: 3 min at 95°C, followed by 35 cycles of 1 min at 95°C, 1 min at 50°C and 1 min at 72°C, with a final extension at 72°C for 7 min. Specificity was confirmed by amplification of the ribosomal RNA (rRNA) gene as described (Smith et al., 2000). All the resulting amplicons were purified using the PureLink™ Quick PCR Purification Kit (Invitrogen), and both DNA strands were sequenced with an ABI PRISM 3100 Genetic Analyzer using the 238 Big Dye Terminator v.3.1 cycle Sequencing Kit (Applied Biosystems, USA). Consensus sequences were assembled using Geneious software (version 9.0.5). The nucleotide sequences obtained were compared to the GenBank database with BLASTN and BLASTP programs (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify the sequences that were most closely related to those found herein. The sequences with the best hits were retrieved, as well as reference sequences of alpha-, beta-, delta- and gamma-CoV genera, and aligned using ClustalW with MEGA6 software (Tamura et al., 2013). Pairwise genetic distances were calculated by p-distance. Amino acid phylogenetic trees were constructed using maximum likelihood (ML) inference with the JTT method (RdRp; S gene) in 1,000 bootstrap replicates.

3 | RESULTS AND DISCUSSION

In the present study, 101 vampire bats were initially screened for CoVs using HTS and subsequently validated by RT-PCR followed by DNA sequencing. In HTS analysis, 201,366 sequences were obtained and assembled into 805 contigs in the pool containing brain, liver, heart, lungs and kidneys. In the pool containing the intestines, 235,584 sequences were obtained and assembled into 344 contigs, of which three were identified as CoV-related. These three sequences were 317, 324 and 340 nucleotides in length and presented higher nucleotide identity (88.2%–95.1%) with bat alphacoronavirus isolate AMA_L_F (GenBank accession number MT663548) detected in *D. rotundus* in Peru (Bergner et al., 2020). Two sequences were homologous to the RdRp gene, and the other sequence was homologous to the S glycoprotein.

In the RdRp RT-PCR (Woo et al., 2006) analyses of individual organ samples, CoV was not detected in the brain, liver, heart, lung or kidney. However, two out of the 101 intestine samples (1.98%) were positive for CoV by RT-PCR targeting of the RdRp gene (Table 1). This result agrees with previous works indicating that CoVs are mainly shed by respiratory and/or digestive routes (Corman et al., 2015; Künkel & Herrler, 1993; Sloots et al., 2006; Tao et al., 2017). Some important alphacoronaviruses are frequently reported in animals, such as feline coronavirus (FCoV) and canine coronavirus (CCoV),which are important enteric pathogens (Brownlie, 2017; Le Poder, 2011; Vogel et al., 2010) These data suggest a possible faecal route. The data reinforce the faecal route

TABLE 1 Results obtained in RT-PCR targeting a 440 bp fragment of the RNA-dependent RNA polymerase (RdRp) gene of coronaviruses in different organs of *Desmodus rotundus* individuals

Tissue	Number of positive individuals
Brain	000/101 (%)
Heart	000/101 (%)
Intestine	002/101 (%)
Kidney	000/101 (%)
Liver	000/101 (%)
Lung	000/101 (%)

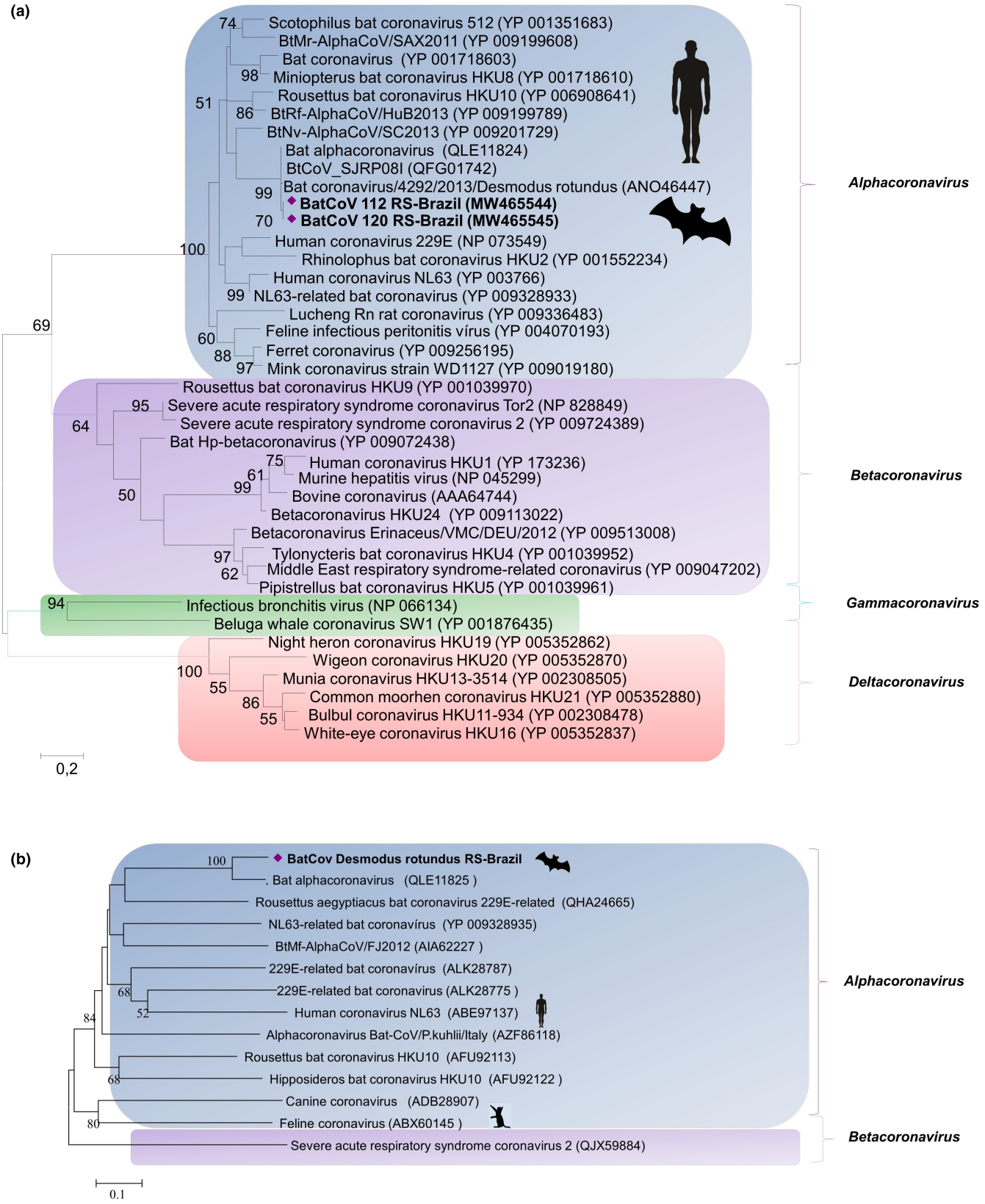

FIGURE 2 Amino acid phylogenetic trees constructed using sequences of RNA-dependent RNA polymerase (RdRp) (a) and spike genes (b). The phylogenetic trees were constructed using MEGA 6 software using the maximum likelihood algorithm method based on the JTT model in 1,000 replicates. The sequences obtained in the present study were deposited in the GenBank database under accession numbers MW465544, MW465545 and MW473477

of a putative spread of the alphacoronavirus detected in *D. rotundus* in the present study.

The partial RdRp sequences obtained by RT-PCR were purified and submitted for DNA sequencing. RdRp sequences from the two different bats showed 100% nucleotide identity. However, the two bats were collected approximately 700 km apart (Figure 1) and both were adult males and sexually active. Since this bat species does not migrate over long distances, it is unlikely that there was transmission of the same strain between these individuals, suggesting that this virus may circulate in *D. rotundus* over large geographical scales. The sequences present 100% nucleotide identity with the same alphacoronavirus also detected in *D. rotundus* in Peru (Bergner et al., 2020). In the partial RdRp phylogenetic tree (Figure 2a), BatCoV 112 RS Brazil and BatCoV 120 RS Brazil, the sequences recovered here clustered with other members of the genus *Alphacoronavirus*, supported by a 100% bootstrap value. Both sequences grouped in the same terminal node with a CoV detected in *D. rotundus* in Peru (GenPept accession number QLE11824), with *D. rotundus* from Southeast Brazil (ANO46447) and with a CoV detected in *Phyllostomus discolor* also in Southeast Brazil (QFG01742) supported by a 99% bootstrap value.

These two samples were also tested by RT-PCR primers specific for the S gene designed in the present study, and one of them was positive. The sample was purified and submitted for DNA sequencing. Nucleotide BLAST search indicated that it displayed 89.4% identity with bat alphacoronavirus isolate AMA_L_F (GenBank accession number MT663548) detected in *D. rotundus* in Peru. A partial S gene amino acid phylogenetic analysis was also performed (Figure 2b), showing that the sequence grouped with the same *D. rotundus* CoV sequence detected in Peru (GenPept accession number QLE11825).

The unquestionable emergence of the human pathogens SARS-CoV, MERS-CoV and SARS-CoV-2 from bat hosts during the last two decades has encouraged several studies for surveillance and monitoring other potential emerging viruses (Bergner et al., 2020; Corman et al., 2013; Li et al., 2020). Moreover, the alphacoronaviruses HCoV-NL63 and HCoV-229E, associated with the common cold, apparently also originated in bats (Banerjee et al., 2019).

In the present study, different organs of 101 vampire bats were screened for CoVs using HTS and a pancoronavirus RT-PCR protocol. Alphacoronaviruses similar to strains reported in Peru and Southeast Brazil were detected, suggesting a putative widespread presence of these related viruses in South American *Desmodus rotundus*. Apparently, the CoV detected in the present study was detected in faeces, suggesting an enteric route of transmission that would be consistent with other CoVs. The continuous monitoring of CoVs in bats may help to understand the epidemiology of these viruses and putative future human outbreaks caused by CoVs.

CONFLICTS OF INTEREST

The authors declare no competing interest.

ETHICAL APPROVAL

The project was authorized by ICMBio (Chico Mendes Institute for Biodiversity Conservation) under licence number #61537-1.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available in this paper and in Supplementary Information.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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