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Alphacoronavirus in urban *Molossidae* and *Phyllostomidae* bats, Brazil

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Abstract

Background: Bats have been implicated as the main reservoir of coronavirus (CoV). Thus the role of these hosts on the evolution and spread of CoVs currently deserve the attention of emerging diseases surveillance programs. On the view of the interest on and importance of CoVs in bats the occurrence and molecular characterization of CoV were conducted in bats from Brazil.

Findings: Three hundred five enteric contents of 29 bat species were tested using a panCoV nested RT-PCR. Nine specimens were positive and eight was suitable for *RdRp* gene sequencing. *RdRp* gene phylogeny showed that all CoVs strains from this study cluster in *Alphacoronavirus* genus, with one *Molossidae* and one *Phlyllostomidae*-CoV specific groups. Phylogenetic analyses of two S gene sequences showed a large diversity within the *Alphacoronavirus* genus.

Conclusions: This study indicated a CoV-to-host specificity and draws attention for CoV detection in *Cynomops sp*, a potential new reservoir. The phylogenetic analyses indicate that diversity of CoV in bats is higher than previously known.

Keywords: Bat, Coronavirus, *Molossidae*, *Phyllostomidae*

Background

Bats have been recognized as the natural reservoirs of a large variety of emerging and re-emerging viruses and have been implicated as the main reservoir of coronavirus (CoV). Thus the role of these hosts on the evolution and spread of CoVs currently deserve the attention of emerging diseases surveillance programs as illustrated by the finding of bats as reservoirs for SARS (Severe Acute Respiratory Syndrome) and MERS (Middle East Syndrome) coronavirus [1, 2].

CoV (*Nidovirales:Coronaviridae:Coronavirinae*) are classified into four genus: *Alpha* and *Betacoronavirus* are often found in mammals, while *Gammacoronavirus* were detected in wild birds, poultry, and marine mammals and *Deltacoronavirus* were detected in wild birds, pigs, and wild feline [3–5].

The first report of bat CoV was described in 2005 [6] in bats of *Miniopterus pusillus* species. Since then, several studies have identified the presence of CoV in bat population from various regions of the world, and to date have been detected both *Alphacoronavirus* and *Betacoronavirus* [6–13].

Although there is a great diversity of bats in Brazil, there are few studies related to bat CoV. *Betacoronavirus* has been reported in *a Desmodus rotundus* vampire bat and *Alphacoronaviruses* have been detected in *Molosuss rufus, M. currentium, M. molossus, Carollia perspicillata, C. brevicauda* and *Tadarida brasilensis* bats (14–18), but a range of bat species that might act as reservoirs for known or unknown CoVs still remains to be surveyed.

On the view of the interest on and importance of bat CoV surveillance, the aim of this study was survey the occurrence of bat CoV in Brazil and to perform molecular characterization of CoVs detected.
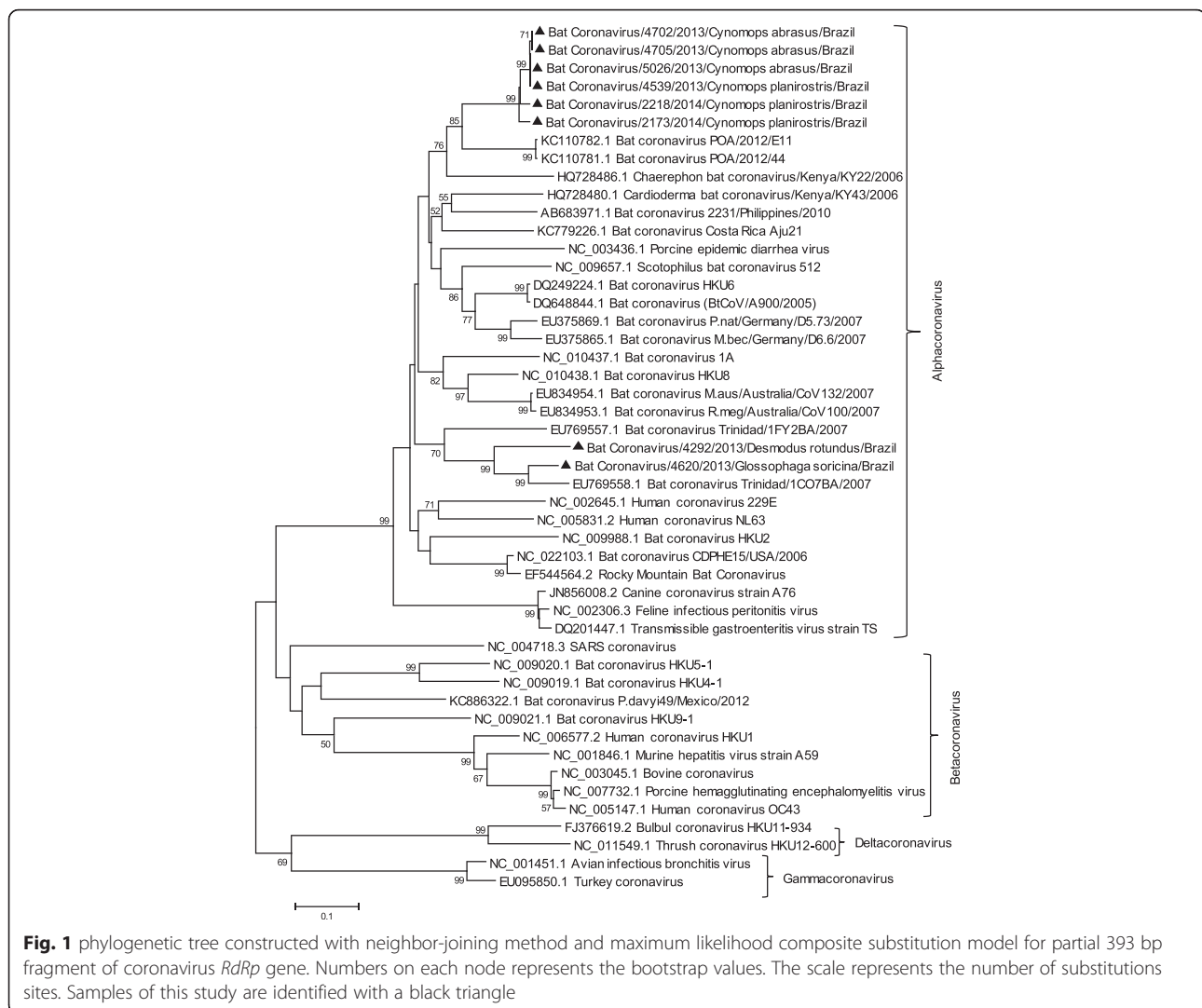
Methods

This work was conducted with samples from 73 municipalities of São Paulo State, Southern Brazil, using 305 samples of enteric content of 29 bat species of three families (*Molossidae, Phyllostomidae* and *Vespertilionidae*). These animals were submitted to *Instituto Pasteur*

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Fig. 1 phylogenetic tree constructed with neighbor-joining method and maximum likelihood composite substitution model for partial 393 bp fragment of coronavirus *RdRp* gene. Numbers on each node represents the bootstrap values. The scale represents the number of substitutions sites. Samples of this study are identified with a black triangle

(São Paulo, Brazil) from March/2013 to July/2014, as a part of rabies surveillance program, and were stored at −20 °C. Each animal was necropsied, the entire intestine was removed from abdominal cavity and all intestinal content was extracted and stored at −20 °C.

Feces suspensions (v/v, 10 %) were prepared with DEPC-treated water. Suspensions were clarified at 12,000 × *g* for 30 min at 4 °C and the supernatants were used in the assays. Extraction of total RNA was carried out with TRIzol Reagent™ (*Life Technologies*, Carlsbad, CA, USA) according to the manufacture's instruction followed by reverse transcription with Random Primers and M-MLV™ *Reverse Transcriptase* (*Life Technologies*, Carlsbad, CA, USA) as per manufacturer's instructions.
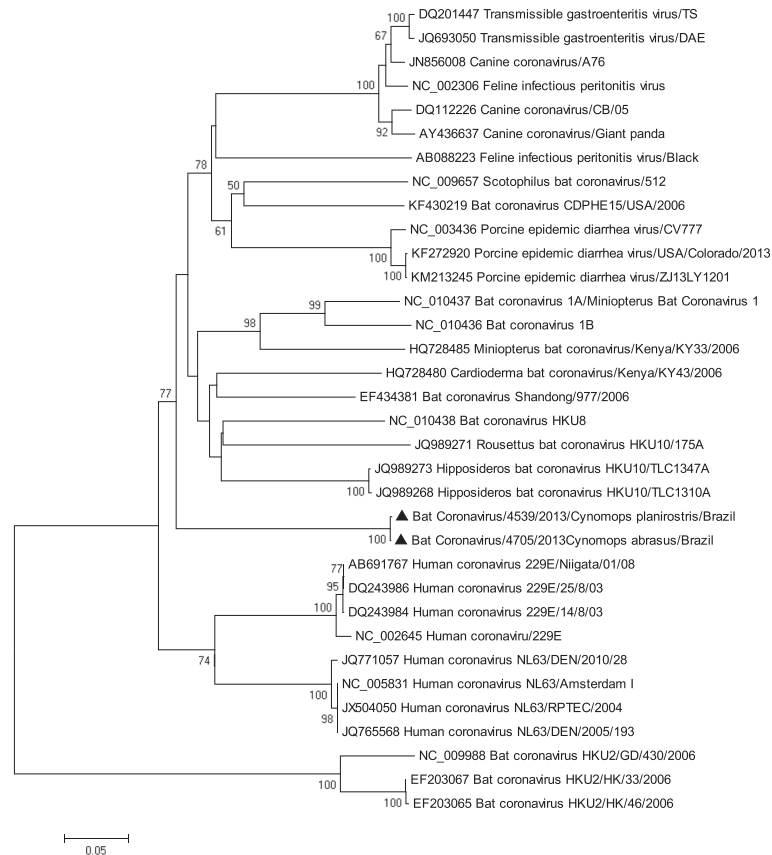
All samples were submitted to a pancoronavirus nested RT-PCR targeting the RNA-dependent RNA-polimerase (*RdRp*) [14], using *Taq Platinum™ DNA Polymerase* (*Life Technologies*, Carlsbad, CA, USA) as per manufacturer's

instructions. Positive samples were submitted to a RT-PCR targeting to S gene for phylogenetic inference [9].

Amplicons were purified using the ExoSap-IT® reagent (USB, Cleveland, OH, USA) or Illustra™ GFX™ Gel Extraction Kit (GE Healthcare, Buckinghamshire, UK) and bi-directional Sanger sequencing with the respective primers was carried out with BigDye v.3.1™ and ABI 3500 Genetic Analyzer™ (*Life Technologies*, Carlsbad, CA, USA).

Chromatograms generated were subjected to Phred online application (http://asparagin.cenargen.embrapa.br/phph/) for assessment of their quality and the final consensus sequences were obtained with CAP Contig application in Bioedit 7.2.5 program [15]. Sequences obtained were aligned with homologous sequences retrieved from GenBank using CLUSTAL/W software in Bioedit 7.2.5 program [15].

Alignments were used for phylogenetic trees construction with distance optimization criterion with neighbor-joining

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Fig. 2 phylogenetic tree constructed with the neighbor-joining method and maximum likelihood composite substitution model for partial 547 bp fragment of coronavirus *S* gene. Numbers on each node represents the bootstrap values. The scale represents the number of substitutions sites. Samples of this study are identified with a black triangle

algorithm and Composite Maximum Likelihood evolutionary model with 1000 "bootstrap" repetitions, using the MEGA 6 program [16].

Results and discussion

Nine out of the 305 samples (2,95 %) were found positive and DNA sequences were obtained for the *RdRp* for eight of them: sequences 4292/2013/*Desmodus rotundus*; 4539/2013/*Cynomops planirostris*, 4620/2013/*Glossophaga soricina*, 4702/2013/*Cynomops abrasus*, 4705/2013/*Cynomops abrasus*, 5026/2013/*Cynomops abrasus*, 2173/2014/*Cynomops planirostris*, 2218/2014/*Cynomops planirostris* (Genbank accession numbers KU552072 to KU552079).

Five species tested were positive: *Cynomops abrasus* (3/11 = 27.3 %), *Cynomops planirostris* (3/5 = 60 %), *Desmodus rotundus* (1/41 = 2.4 %), *Glossophaga soricina* (1/33 = 3 %) and *Platyrrhinus lineatus* (1/12 = 8.3 %). Only the sample of *P. lineatus* was not confirmed by DNA sequencing.

The occurrence of CoV per specie demonstrates a high frequency in *Cynomops* genus: 60 % for *C. abrasus* and

27.3 % for *C. planirostris*, indicating these species must have an important role in CoV maintenance in bat population in this region. In South America, CoV detection on bats have shown a low occurrence of CoV per species [12, 17, 18], however, none of these studies included the *Cynomops* genus. Though CoVs have been described in Brazilian *Molossidae* bats [19, 20], *Cynomops sp* were previously unknown hosts for CoVs.

The *Alphacoronaviruses* found in *Cynomops sp* (*Molossidae*) were closely related with *Alphacoronaviruses* already detected in South Brazil (*RdRp* nt identities 81.4–82.9 %).

D. rotundus is one of the three hematophagous bat species and can be found only in the Americas, from northern Mexico to northern Argentina [21]. Although the presence of *Betacoronavirus* has already been described [22], this is the first report of an *Alphacoronavirus* in common vampire bat, showing that this species can carry both *Alpha* and *Betacoronavirus*.

G. soricina is a nectarivorous neotropical bat that might be found inside houses and has already been described as a host for *Alphacoronaviruses* [23]. Although

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separated by a large geographic area of more than 4000 km, *G. soricina* CoV found in São Paulo showed high nucleotide and amino acids identities when compared with *G. soricina* CoV of Trinidad and Tobago (90.3 and 98.4 %, respectively). *G. soricina* species does not migrate over long distances [24], therefore it is unlikely that transmission has occurred among these specimens, suggesting a virus-host adaptation.

RdRp phylogeny (Fig. 1) showed that all CoVs strains from this study clustered in the *Alphacoronavirus* genus, with one *Molossidae* and one *Phyllostomidae*-CoV specific groups. The *Molossidae* CoV of this study clustered with *Molossidae* bat CoV from South Brazil with high bootstrap value (85 %). Although phylogenetic analysis indicates a geographic relation, the *Alphacoronaviruses* of this study was separated by a geographic area of 1000 km approximately from *Molossidae* batCoV previously detected in Brazil [19]. Besides, there are few studies regarding batCoV in Brazil, which difficult a phylogeographic relatedness hypothesis confirmation. *Phyllostomidae* CoV of this study clustered with sequences of *Carollia perspicillata* and *G. soricina*, two *Phyllostomid* bats from Trinidad and Tobago, with high bootstrap value (70 %) (Fig. 1). Taking together these results supports the theory that host specificity is more important than geographic pattern as previously noticed [13, 17, 25, 26].

Two 547 nt sequences of S gene (GenBank accession numbers KU552080 and KU552081) were obtained for *C. planirostris* and *C. abrasus* CoV. The nucleotide identity between these two sequences was high (99.8 %), suggesting the transmission of CoV among different bats species. However, the nucleotide identity with sequences retrieved from GenBank was low, varying from 39,1 and 65,8 %, showing a large genetic diversity with *Alphacoronavirus* from others countries.

The phylogenetic tree performed for partial S gene (Fig. 2) shows that the samples of this study formed a separate group from others *Alphacoronavirus* sequences retrieved from GenBank, with bootstrap value of 100 %. Although the fragment analyzed was small, the tree indicates that samples of this study are unique, forming a completely separate group.

Environmental changes caused by man have promoted a major impact on ecology, affecting the movement of several wild animals species from their natural habitat to urban or rural areas [27], increasing the chances of contact between humans and domestic animals with wild animals. Furthermore approximately 75 % of emerging infectious diseases have zoonotic origin and wildlife as source of infection [28, 29]. Therefore, it is essential to survey and identify possible sources of infection, especially in relation to bats that are considered important reservoirs of viral agents [30, 31].

Conclusion
The significance of CoVs detected during this survey on Public Health remains to be investigated, but the finding of CoV in new viral reservoirs justifies the need for CoV surveillance. This study indicates a CoV-to-host specificity and draws attention for CoV detection in *Cynomops sp*, suggesting the importance of this species for CoV maintenance in the region studied. The phylogenetic analyses indicate a great diversity of CoV in bats, particularly for S gene.

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