No molecular evidence for influenza A virus and coronavirus in bats belonging to the families *Phyllostomidae, Vespertilionidae, and Molossidae* in the state of São Paulo, Brazil

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Abstract

This study aimed to evaluate, by molecular methods, the presence of influenza A virus (IAV) and coronavirus in non-hematophagous bats collected in the state of São Paulo, Brazil. Samples of lung tissue and small intestine from 105 bats belonging to three families (*Phyllostomidae, Vespertilionidae, and Molossidae*) were collected in 22 municipalities in the state of São Paulo. Genetic identification of bats species was performed by amplification and sequencing of a fragment of 710 bp of the mitochondrial COI gene. In the detection of IAV, genomes were performed by RT-PCR, aiming at the amplification of a 245-bp fragment of the IAV matrix (M) protein gene. For coronaviruses, two fragments of 602 and 440 bp corresponding to segments along the gene encoding the RNA-dependent RNA polymerase (RdRp) were targeted. The detection limit for each of the PCRs was also determined. All samples analyzed here were negative for both viruses, and the lower limit of detection of the PCRs for the amplification of influenza virus A and coronavirus was estimated at $3.5 \times 10^3$ and 4.59 genomic copies per microliter, respectively. Although bats have been shown to harbor a large number of pathogens, the results of the present study support the theory that virus circulation in bats in the wild often occurs at low viral loads and that our understanding of the complex infectious dynamics of these viruses in wild conditions is still limited.

Keywords Alphainfluenzavirus · RT-PCR · Chiropterans · *Coronaviridae* · RdRp · Spillover

Introduction

Bats represent 20% of all mammals and are widely distributed in all continents, except Antarctica [1, 2]. The wide diversity, extensive distribution, longevity, adaptation to altered ecosystems, great trophic and habitat diversity, ability to fly long distances, and high interaction between species of both the same order and between other species of mammals, including humans, are all features that may contribute to their role as reservoirs of viruses of public health importance [3].

An important and growing number of viruses, some of them deadly, have been related to different species of bats [2, 4]. Alphainfluenzavirus (AIV) is responsible for causing four major influenza pandemics in humans in the last 100 years [5, 6]. Influenza A viruses H17N10 and H18N11 were recently discovered in bats in South America, and these viruses show a great genetic diversity when compared to previously described AIVs [7–9]. The phylogenetic analyses suggest that these bat influenza viruses are ancestors of the IAVs known so far and infect different vertebrates [9]. This discovery led to new theories about the evolution of influenza viruses, as current influenza viruses may have originated in bats before spreading to birds [9, 10].
Another group of viruses highly reported in bats is the coronaviruses. It is estimated that at least 3200 coronaviruses are circulating in bats; in consequence, bats play an important role in the evolution of α-CoVs and β-CoVs [11]. In the last 20 years, coronaviruses have started to circulate among humans and cause outbreaks with pandemic potential and worrying frequency. Among them is the virus causing the current pandemic, SARS-CoV-2 [11].

The aim of this study was to detect, by molecular methods, the presence of influenza virus A and coronaviruses in non-hematophagous bats collected in the state of São Paulo, Brazil.

Materials and methods

Specimen collection

Between April and July 2015, hundred and five bats were obtained in 22 municipalities in the state of São Paulo. Dead animals received by Pasteur Institute for rabies diagnosis were selected and kept in refrigeration for a maximum of 24 h before the intestine and lungs were obtained. These organs were kept at −80 °C until the extraction of nucleic acids. The individuals were submitted to the detection of rabies antigens by direct immunofluorescence antibody test (FAT) on brain smears [12, 13]. All individuals were negative for rabies antigen.

Bat species identification

Genetic identification of bats species was made by total DNA extraction using the kit RTP® DNA/RNA Virus Mini Kit (Stratec Biomedical AG) and PCR amplification of around 710-bp fragment of the mitochondrial gene cytochrome oxidase subunit I (COI), employing the universal primers LCO 1490 and HCO 2198 (Table 1), followed by sequencing of the fragment by Sanger method, as previously described [14].

Virus RNA detection from tissue samples

Fragments (50–100 mg) of the intestine and lungs were submitted to RNA extraction using the TRYzol™ reagent (Invitrogen), following the manufacturer’s recommendations. The extracted RNA was used for genomic amplification as follows. Aiming at the detection of influenza A RNA, cDNA was synthesized using the High-Capacity Reverse Transcription Kit (Applied Biosystems™), with the universal influenza Uni12 primers (Table 1) [15]. Following cDNA synthesis, amplification by RT-PCR was obtained using the previously described M52C and M253R primers (Table 1) [16].

For the identification of coronavirus genomes, the cDNA was obtained using random primers, following a pancoronavirus semi-nested PCR was performed, targeting a fragment of 602 and 440 bp of the RNA-dependent RNA polymerase gene (RdRp) [17–20]. As an internal control, a PCR was performed for the gene that encodes beta-actin, using the ACTB-F and ACTB-R primers (Table 1).

Positive control and PCR limit of detection

Positive controls were used for each set of 20 tissue samples. As positive controls, RNA from influenza A H1N1 virus, previously isolated from swine lung and confirmed by sequencing (99.12% of identity with swine influenza A virus H1N1 accession: MK367354.1), and RNA from a SARS-CoV-2 virus isolate (99.09% of identity with human SARS-CoV-2 accession: MW266954.1) were amplified following the same protocol as described above. Both the IAV amplicon (245 bp long) and the coronavirus amplicon (from the second round of amplification, 440 bp long) were cloned. Amplicons were inserted into a pCR2.1 TOPO TA plasmid (Invitrogen) and chemically transformed into competent *Escherichia coli* NEB® 10-beta cells (New England

| Table 1 | Oligonucleotides used in this study |
|---------|----------------------------------|
| Name    | Primer (5′-3′) | Amplicon (bp) | Amplification target | References |
| LCO 1490| GGTCAAACAAATCATAAAGATATTGG       | 710          | COI                  | [14]       |
| HCO 2198| TAAACTTCCAGGTGACCAAAAAATCA       | NA          | cDNA Influenza virus | [15]       |
| Uni 12  | AGCG/AAAAACAGG                   | 127         | Beta-actin bat       | Unpublished |
| ACTB-F  | TCACTGGYATGGARTCCTGT        | 245         | Influenza virus A matrix protein gene | [16]       |
| ACTB-R  | CCKGGGTACATGGTGGTYCC          | 440         | Coronavirus RNA–dependent RNA polymerase (RdRp) | [19–21]  |
| M52C    | CATCAACCCGAGGTGCAAAAAG/TGT CTA | 602         | Coronavirus RNA–dependent RNA polymerase (RdRp) | [19–21]  |
| M253R   | AGGCATTGTTGGACAAAG/TGT CTA    | 440         |                     |            |
Biolabs®). Plasmid DNA was purified by the alkaline lysis method [21] and cleaved with the EcoRI enzyme (Jena Bioscience™). Clones containing the target sequences were confirmed by PCR and sequencing. To determine the PCR detection limit, eleven ten-fold serial dilutions of plasmid DNA containing $3.53 \times 10^8$ to $3.53 \times 10^{-2}$ copies with the H1N1 insert and $4.59 \times 10^8$ to $4.59 \times 10^{-2}$ copies with SARS-CoV-2 insert were prepared.

**Results**

One hundred and five individuals from 22 municipalities in São Paulo state were evaluated: Araraquara (n: 8), Campinas (n: 39), Caraguatatuba (n: 1), Catanduva (n: 3), Diadema (n: 2), Holambra (n: 2), Itapira (n: 4), Jarinu (n: 1), Louveira (n: 4), Marília (n: 5), Olimpia (n: 1), Ribeirão Preto (n: 19), Rio Claro (n: 3), Santa Cruz das Palmeiras (n: 1), Santa Fé do Sul (n: 1), São Bernardo do Campo (n: 2), São Carlos (n: 1), Sumaré (n: 2), Tatuí (n: 1), Ubatuba (n: 1), Valinhos (n: 2), and São Paulo (n: 2) (Fig. 1).

When the different tissue samples were submitted to RT-PCR and RT-nPCR, no amplification for influenza A virus or coronavirus was observed. All samples amplified the internal control for beta-actin. The lower limit of detection of the RT-PCR used for the amplification of influenza virus A was estimated at 4.59 genomic copies per microliter. The lower limit of detection for coronavirus RT-nPCR was calculated at $3.53 \times 10^3$ genomic copies per microliter.

Bats were classified according to the partial sequences of the mitochondrial gene COI into ten different species, belonging to nine genera and three families. The number of bats, type of sample used for each test, species identified, and the GenBank accession number are described in Table 2.

**Discussion**

Bats have several biological characteristics that make them a fascinating and efficient reservoir/host for a large number of microorganisms of public health interest [2, 3]. Many of these microorganisms are of viral origin, infect bats for thousands of years, and have developed a close relationship of co-evolution with these hosts [22–24]. For this reason, the aim of this study was to evaluate the occurrence of AIV and coronaviruses, two viruses of public health interest, in...
non-hematophagous bats collected in the state of São Paulo, Brazil.

When submitted to the detection of AIV RNA, none of the samples used here was positive, despite the high sensitivity of the RT-PCR (4.59 genomic copies/μL). These results are consistent with results previously obtained by other authors, as it has been shown that the frequency of detection of AIV in different species of bats is frequently very low. In the first study that evidenced the presence of HL17NL10, in 2012, the frequency of positivity was 0.94% (3/316) in bats of the species Sturira lilium in Guatemala [7]. One year after that, the HL18NL11 virus was identified, with a positivity frequency of 0.1% in individuals of the species Artibeus lituratus from Peru [8]. In Brazil, HL18NL11 was first demonstrated, in 2019, in bats of the species A. lituratus with a frequency of 0.4% (2/533), the same bat species in which the virus was reported in Peru [25], but in this study, A. lituratus was negative. The presence of AIV in bats from other countries was also shown. In 2015, 1369 bats from 26 different species from central Europe were negative for AIV genomes [26]. Recently, a relatively high frequency of AIV positivity of 5.4% (33/601) was found in Rousettus aegyptiacus bats in Egypt; this virus was isolated in cell culture, different from those identified in South America, and showed 73% similarity to H9N2 [27].

Although the samples analyzed here were all negative, it is possible that studies with a larger number of individuals of these species and/or different Pan-influenza primer sets would enable the detection of IAVs in geographic regions different from those previously evaluated.

When submitted to a nested PCR targeting the coronavirus RdRp, none of the samples was positive, independent from the bat species or origin. Although such results could have been influenced by the number of individuals examined and the low sensitivity of the RT-PCR used here, the same technique and primers have been used by different authors, who obtained both high and low frequencies of positive animals [18, 28–30]. Unfortunately, it was not possible

| Table 2 Number of bats species, GenBank accession number, and type of sample tested to RT-PCR and RT-nPCR in search for influenza A virus and coronavirus genomes |
|---|---|---|---|---|
| Identification | Number of bats and type of sample tested | GenBank accession number |
| **Family** | **Species** | SI | L | L and SI | Total |
| Phyllostomidae | Artibeus lituratus | 4 | 1 | 4 | 9 | OK398131, OK398132, OK398133, OK398134, OK398135, OK398136, OK665672, OK398136, OM049203 |
| | Glossophaga soricina | 4 | 1 | 1 | 6 | OK398155, OL307715, OK398156, OK398157, OK398158, OM049220 |
| | Platyrrhinus lineatus | 2 | 0 | 0 | 2 | OK398138, OL986401 |
| Molossidae | Eumops perotis | 1 | 0 | 1 | 2 | OK584813, OL307710 |
| | Cynomops planirostris | 10 | 3 | 4 | 17 | OK423448, OK423449, OK423450, OK423451, OK429132, OK429133, OK429134, OK429135, OK560887, OK430886, OL307712, OK493381, OK493382, OM049224, OM049225, OM117613 |
| | Eumops glaucinus | 5 | 3 | 3 | 11 | OK509182, OK509183, OK509184, OK509185, OK509186, OK509187, OK509188, OK509189, OK509190, OM108097, OM049199 |
| | Molossus molossus | 30 | 2 | 14 | 46 | OK425855, OK425857, OK562112, OK425858, OK425877, OK430884, OK430883, OK425866, OK424624, OK424643, OK424645, OK461347, OK44826, OK44827, OK44828, OK424646, OK424647, OL307711, OK444829, OK493350, OK493301, OK493302, OK493303, OK667181 |
| | Molossus rufus | 3 | 1 | 4 | 8 | OK425856, OK425873, OL544971, OK649960, OK649972, OK649962, OK649963, OK649961 |
| | Promops sp. | 1 | 0 | 0 | 1 | OM102956 |
| | Myotis riparius | 1 | 0 | 0 | 1 | OK398129 |
| Vespertilionidae | Myotis sp. | 1 | 0 | 0 | 1 | OK413003 |
| | Lasiurus ega | 0 | 1 | 0 | 1 | OK398126 |
| Total | 62 | 12 | 31 | 105 |
to compare the sensitivity of different reports because in these previous studies the sensitivity of detection was not calculated.

Interestingly, previous reports do show different frequencies of coronavirus positive animals, when bats from different regions/species are investigated. Thus, high positivity for coronaviruses (17–63%) was detected in molecular surveillance studies carried out on bats in the Asian and European continents, especially bats of the genus *Rhinolophus*, *Mini- opterus*, and *Myotis* [18, 28, 29, 31]. However, in a number of studies, the positivity was low [18, 32, 33]. In Brazil, the first report on the detection of coronavirus in bats showed a positivity of 14%, corresponding to one positive bat among seven individuals tested (1/7) [34]. Further studies with Brazilian bats obtained positivity frequencies of 1% (2/297) [35], 1.7% (4/341) [36], 10% (1/10) [37], 2.95% (9/305) [38], 3.7% (15/401) [39], 17.8% (11/64)–2.56% (1/39) [40], 4.50% (7/155) [41], and 3.9% (4/101) [42]. The bat species where coronavirus genomes were identified in Brazil were *Desmodus rotundus*, *Artibeus lituratus*, *Carollia brevicauda*, *Carollia perspicillata*, *Sturnira lilium*, *Molossus molossus*, *Molossus currentium*, *Molossus rufus*, *Eumops glaucinus*, *Glossophaga soricina*, *Myotis nigricans*, *Myotis riparius*, *Cynomops abrasus*, *Cynomops planirostris*, *Platyrrhinus lineatus*, *Phyllostomus discolor*, *Tadarida brasiliensis*, and *Eptesicus* sp. Interestingly, none of the studies developed to date in Brazil was able to detect SARS-CoV–related viruses (from genus *Betacoronavirus*), as further sequencing of amplicons revealed that only alphacoronaviruses seem to circulate in such species. Likewise, their role as potential pathogens for bats is not clear; therefore, it is important to monitor viral pathogens in bats aiming a One Health surveillance and risk of spillover events in Brazil.

The results presented here are far from what one would expect to find in species which are so frequently associated with viruses of public health importance. Very little is known about the infectious dynamics and the way these viruses circulate in bats of different species [43]. Some virus infection in bats have been reported as asymptomatic, as described for rabies virus infection [44]. However, in a larger number of reports, virus infection in bats has been described as asymptomatic infections [24]. Although the signs indicative of disease in bats are controversial, it has been shown that there are seasonal fluctuations in virus replication and, consequently, in viral loads [45]. Biological factors that can influence viral loads and coincide with increased virus circulation are reproductive and nutritional stress, secondary fungal infections, and the presence of a large number of juvenile individuals with reduced maternal immunity [43, 45]. These phenomena in host biology are positively correlated with an increased incidence of virus spillover events in humans [43, 46–49]. It is noteworthy that our knowledge on the biology of New World bats is very limited, and such aspects may also influence virus circulation among these hosts; thus, we believe that further studies aiming at understanding their role as hosts and reservoirs of viruses should be of major priority, to either understand epidemiological cycles of viruses or to take actions aiming at the preservation of these animals.

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**Author contribution** All authors made substantial contributions to the conception of the work, performed research and interpretation of data, wrote the paper, and did critical review.

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**Data availability** Not applicable.

**Code availability** Not applicable.

**Declarations**

**Ethics approval** This study was approved by the Animal Use Ethics Committee of the Pasteur Institute (CEUA-IP), approval 04/2020.

**Consent to participate** Not applicable.

**Consent for publication** Not applicable.

**Conflict of interest** The authors declare no competing interests.

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