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BRIEF REPORT

Detection of multiple viruses in oropharyngeal samples from Brazilian free-tailed bats (*Tadarida brasiliensis*) using viral metagenomics

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Abstract

In this study, we analyzed the viral population in oropharyngeal samples from *T. brasiliensis* using a viral metagenomic approach. Genomes corresponding to members of the families *Circoviridae, Genomoviridae, Herpesviridae, Paramyxoviridae, Coronaviridae*, and *Astroviridae* were detected. This study provides the first preliminary understanding of the oropharyngeal virome of *T. brasiliensis*, which may guide the discovery and isolation of novel viruses in the future and highlights the need for continuing investigations in this regard.

Bats (order Chiroptera) are one of the most diverse and widely distributed groups of mammals, representing ~ 20% of all known mammalian species [1]. Accompanying this diversity, it would not be unexpected to find an equally diverse microbiome within bat species. Bats are recognized as sources of viruses that can potentially cause disease in humans and animals; a myriad of viruses has been identified in bats of different species worldwide. Therefore, such species may be natural reservoirs for a large variety of potentially zoonotic viruses, such as lyssaviruses, paramyxoviruses, and filoviruses as well as the recently emerged severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [2].

Since nearly 80% of viral diseases that infect humans are zoonotic, field surveys in search for viruses must be conducted by monitoring such populations in their usual – or adopted – habitats [3]. Therefore, knowledge about the ecology of the potential reservoir species and main routes of interspecies transmission is central to any preventive campaign.

T. brasiliensis is one of the most widely distributed mammalian species in the Western Hemisphere and is highly adapted to urban environments. In Brazil, this insectivorous species is known to harbor some important pathogens, such as rabies virus and coronaviruses. However, the actual amplitude of the virome of this bat species remains undetermined.

In the present study, oropharyngeal swabs samples from 155 healthy Brazilian free-tailed bats were collected. The choice of this sample collection was based on the fact that this procedure is widely accepted as sampling method for

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the identification of respiratory viruses in many animal species. All specimens used in this study were identified to the species level based on anatomical and morphological characteristics. Samples were collected in four municipalities within the state of Rio Grande do Sul, Brazil. Swabs were maintained in phosphate-buffered saline (PBS), pooled, filtered (0.45 µm), and centrifuged at 100,000 × g. The viral material was then treated with nucleases as described previously [4, 5]. DNA and RNA were isolated using a PureLink™ Viral RNA/DNA Kit (Thermo Fisher Scientific). DNA was enriched by multiple displacement amplification [4], and RNA was enriched by following the V8AA protocol [6]. High-throughput sequencing (HTS) was performed using an Illumina MiSeq System. All specimens used in this study were identified to the species level based on anatomical and morphological characteristics by a trained zoologist.

A total of 1,404,764 reads were generated by HTS. These were assembled into 10,712 contigs using SPAdes 3.5 and analyzed using BLASTn/BLASTx. A substantial proportion of the assembled reads had no significant similarity to any of the sequences deposited in the GenBank database (69%, Supplementary Fig. 1). The viral component of the reads, as deduced from its similarity to known viral genome sequences in databases, represented 6.6% of the total assembled sequences (221/10,712 contigs). Most of the viral hits were from bacteriophages (77.4%), in agreement with most metagenomic studies in several hosts and environments. Contigs assigned to phages were out of the scope of the present study and were not investigated further. In order of abundance, the eukaryotic viral contigs identified here were as follows: *Mimiviridae* and other giant viruses > *Phycodnaviridae* > *Herpesviridae* > *Polydnaviridae* > *Iridoviridae* > *Circoviridae* > *Genomoviridae* (Supplementary Fig. 1). Sequences from the families *Phycodnaviridae, Polydnaviridae, Mimiviridae,* and *Iridoviridae* probably originated from bats' dietary habits and were therefore not included in subsequent analyses.

It is important to point out that no sequences related to RNA viruses were detected; the authors believe that some failure occurred in the complex workflow of viral metagenomics. Thus, we decided to investigate three important viral families by PCR using broadly reactive primers. Viral RNA was extracted as above and subjected to reverse transcription, and using the resulting cDNA, well-established protocols were applied to detect genomes of members of the families *Paramyxoviridae* [7], *Coronaviridae* [8, 9], and *Astroviridae* [10]. Despite the lack of detection of viral RNA sequences by HTS, when carrying out by RT-PCR, gene fragments from members of the families *Paramyxoviridae*, *Coronaviridae* and *Astroviridae* were detected in the swab samples. Below, the detected viruses are described and discussed in detail.

i) Circoviridae: Viruses of the family *Circoviridae* are known to infect a wide range of vertebrates. The virions consist of naked nucleocapsids of about 20 nm in diameter, with a circular single-stranded DNA (ssDNA) genome of approximately 2.0 kb [11].

In this study, one circular contig related to members of the family *Circoviridae* was identified, representing the genome of a virus that was named "Tadarida brasiliensis circovirus 1" (TbCV-1, GenBank KT783484). TbCV-1 displays the common genomic architecture of *Circoviridae* members. Two inversely arranged open reading frames (ORF) that putatively encode the replicase (Rep) and capsid (Cap) proteins were observed. In the 5' intergenic region, a stem-loop structure was predicted. At the apex of this secondary DNA structure, a conserved nonanucleotide sequence (TAG TATTAC) was found. In the Rep-associated protein, motifs involved in rolling-circle replication (RCR) (motif I, CFTINN; motif II, PHLQG; motif III, YCSK) as well as the SF3 helicase family motifs (Walker A, GEPGSGKS; Walker B, VLDDF; motif C, ITSN) were identified. The closest relative to TbCV-1 is a bat circovirus recovered from *Rhinolophus ferrumequinum* bats (RfCV-1) in China. TbCV-1 and RfCV-1 show 75.5% genome sequence identity. Phylogenetically, TbCV-1 groups with RfCV-1 (species *Bat associated circovirus 3*) in the genus Circovirus, family *Circoviridae* (Fig. 1a). TbCV-1 genome sequence was reported at the end of 2015 [5] and later officially named "bat associated circovirus 4" (BatACV-4).

ii) Genomoviridae: *Genomoviridae* is a family of recently discovered ssDNA viruses found initially in fungi [12]. These viruses have a genome ranging from 2.1 to 2.3 kb, containing two opposite open reading frames that code for a Cap protein and a spliced Rep.

Here, the genome of a new genomovirus (TbGV) was identified. Its genome is 2,139 nt in length, arranged as a circular molecule with a GC content of 51%. A stem-loop structure with the nonamer TAATAAAAT is present at nt position 20-28 (Fig. 1b). The TbGV genome encodes two putative proteins: a spliced Rep-associated protein and a coat protein, separated by a 116-nt-long untranslated region. The putative Rep-associated protein possesses three RCR motifs (LLTYA, HLHTFV and YACKD). Analysis of the C-terminal region of the spliced Rep also detected three ATP-dependent helicase motifs: Walker A (GPSRMGKT), Walker B (IFDDI), and motif C (WLAN). Phylogenetic inferences based on the Rep aa sequence confirmed that this genome belongs to a member of the genus *Gemykibivirus*, clustering with SL1 genomovirus (KP133075) and human associated gemykibivirus 2 (MK513443), both of which were recovered from humans (Fig. 1).

Regarding species classification, based on the analysis of distribution of the pairwise sequence identity across genomes, a threshold of 78% was adopted here, as

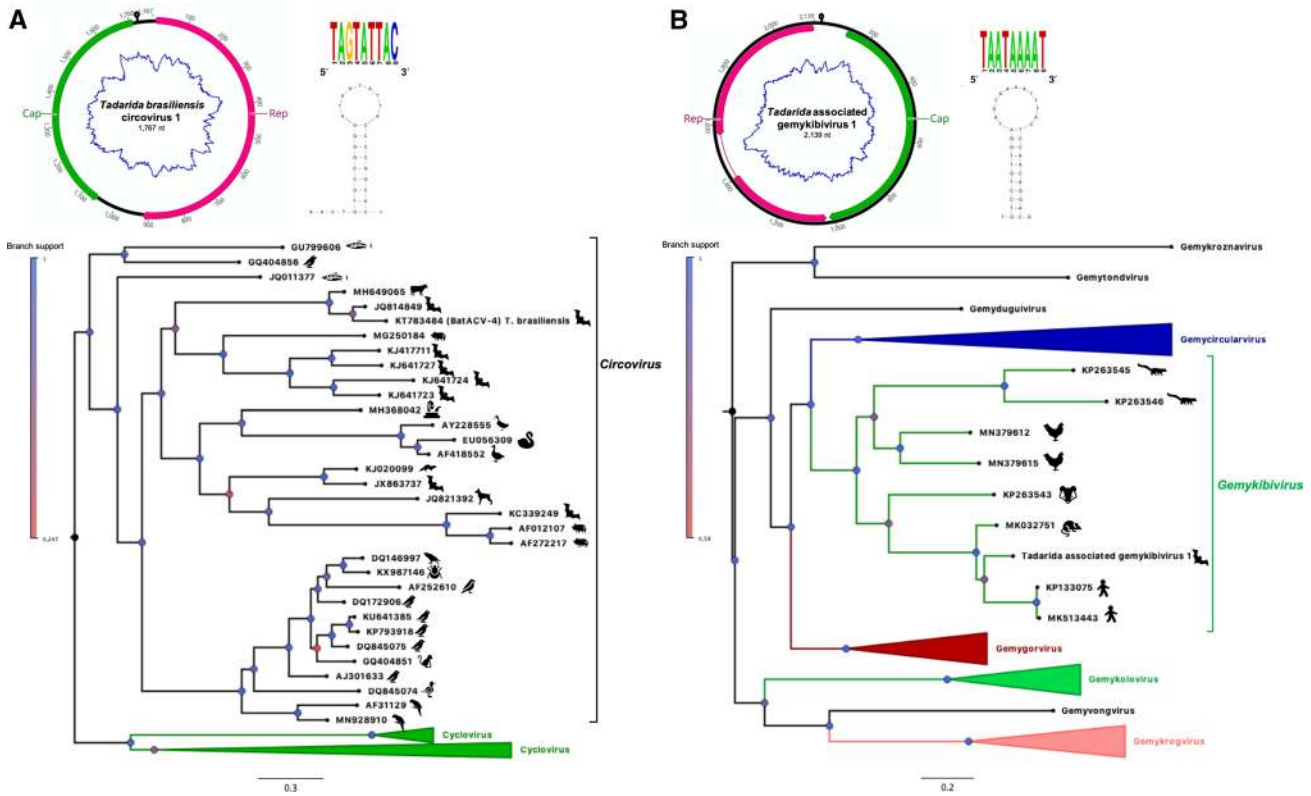

Fig. 1 Genome organization and phylogenetic tree of novel members of the families *Circoviridae* and *Genomoviridae*. (**a**) Genome organization and phylogenetic tree of TbCV-1. (**b**) Genome organization and phylogenetic tree of TbGV-1. Maximum-likelihood (ML) phylogenetic trees were constructed using PhyML [22]. Statistical significance analysis of tree topologies was performed using the approximate likelihood branch support test (aLRT)

recommended for species demarcation [13]. The TbGV genome sequence was then compared with similar genome sequences retrieved from GenBank. TbGV shares 61.4% nucleotide sequence identity with human associated gemykibivirus 2 (MK513443), this was the highest degree of similarity obtained. Therefore, it is proposed that TbGV be a representative of a new species in the genus *Gemykibivirus*, herein named "Tadarida associated gemykibivirus 1" (TbGV-1; GenBank MT512493).

iii) Herpesviridae: It is likely that every vertebrate species can be infected with several herpesviruses [14]. Here, eight contigs related to members of the family *Herpesviridae* were detected. Of these, two showed the highest sequence similarity to γ-herpesviruses (related to Myotis gammaherpesvirus 8, which was serendipitously identified in an established bat cell line derived from *Myotis velifer*). Other herpesvirus contigs identified clustered with β-herpesviruses (closely related to bat betaherpesvirus B7D8, isolated in

Table 1 Herpesvirus sequences identified in Brazilian free-tailed bats

Contig ID/length (nt)	Closest relative[¥]/*Herpesviridae* subfamily	Nt identity	E-value
Contig 108/3,156	Myotis gammaherpesvirus 8 (KU220026)/γ	63%	2e-47
Contig 615/1,391	Saimiriine herpesvirus 2 (X64346)/γ	71%	7e-10
Contig 1374/925	Bat betaherpesvirus B7D8 (JQ805139)/β	74%	6e-15
Contig 1673/843	Bat betaherpesvirus B7D8 (JQ805139)/β	71%	9e-19
Contig 3169/639	Bat betaherpesvirus B7D8 (JQ805139)/β	70%	2e-20
Contig 6960/481	Murid betaherpesvirus 1 (MH118556)/β	70%	1e-3
Contig 7857/465	Bat betaherpesvirus B7D8 (JQ805139)/β	73%	6e-24
Contig 8793/451	Saimiriine herpesvirus 4 (FJ483967)/β	74%	4e-7

[¥]Based on the best BLASTn hit (May/2020). Sequences described in this study have been deposited in the GenBank database under accession nos. MT512494-MT512495 (γ-HV) and MT683419-MT683424 (β-HV)

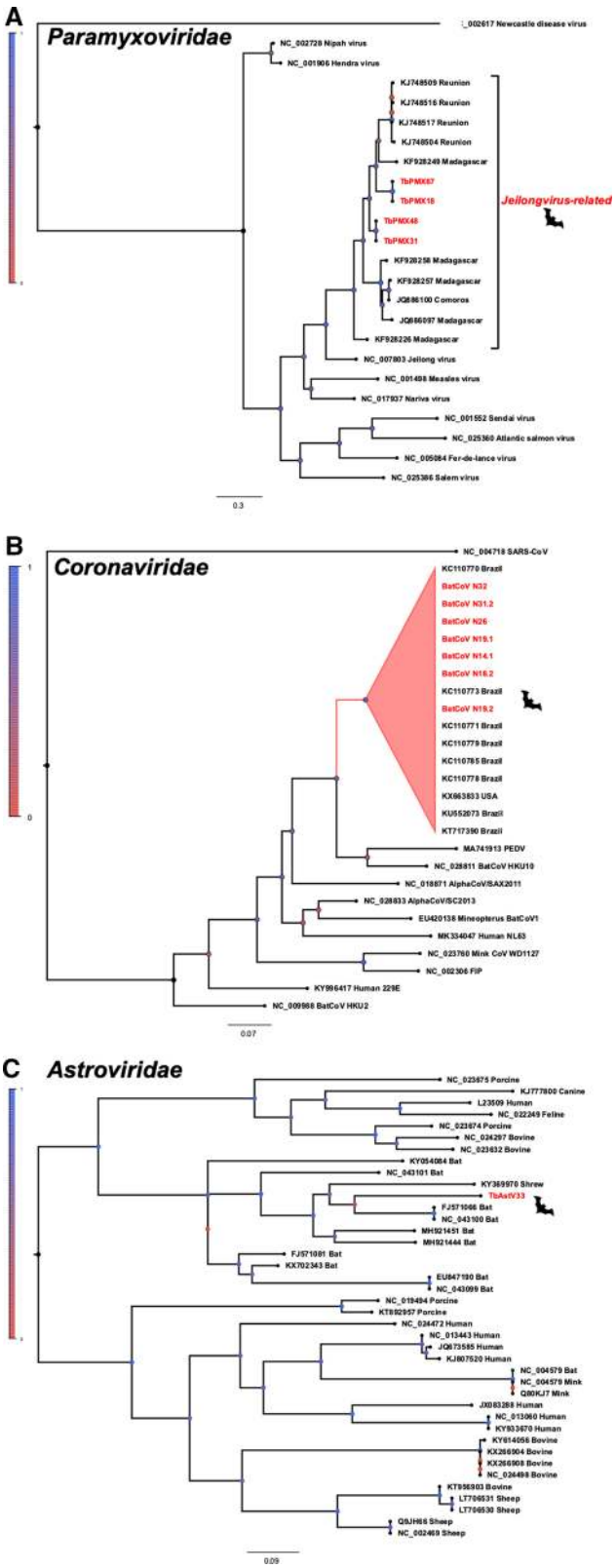

Fig. 2 Maximum-likelihood phylogenetic trees of RNA viruses ▶ detected in *T. brasiliensis* bats. (**A**) *Paramyxoviridae*. (**B**) *Coronaviridae*. (**C**) *Astroviridae*. Phylogenetic trees were generated using aligned aa sequences of the RNA-dependent RNA-polymerase (RdRp) protein. ML phylogenetic trees were constructed using PhyML [22] with the aLRT support test. Sequences described in this study are indicated in red (GenBank accession nos. MT671960-MT671963, PMX; MT671952- MT671958, CoV; MT671959, AstV)

Australian *Miniopterus fuliginosus* bats). Detailed contig descriptions are shown in Table 1. Although the herpesvirus nucleotide sequences reported in this study do not constitute full genome sequences, this initial characterization contributes to our knowledge of the virome of *T. brasiliensis.*

iv) Paramyxoviridae: Members of the family *Paramyxoviridae* are responsible for some of the most significant human and domestic animal viral diseases, such as measles, distemper, mumps, parainfluenza, and Newcastle disease. Bats were identified as potential reservoirs of important paramyxoviruses. It has been hypothesized that bats may have been the hosts of the ancestors of members of the families *Paramyxoviridae* and *Pneumoviridae* [15].

Using a broadly reactive PCR, paramyxovirus genomes were detected in four swab samples (detection rate, 2.6%). To the best of our knowledge, this is the first report of members of the family *Paramyxoviridae* being detected in *T. brasiliensis* bats. The paramyxoviruses detected in *T. brasiliensis* (Tb-PMX) were further sequenced, and a phylogenetic tree was constructed using representative sequences. When compared with each other, all the nucleotide sequences obtained in this study were very similar (80.4-100% identity). Phylogenetically, the Tb-PMX sequences clustered with other paramyxoviruses detected in *Miniopterus* and *Mops* (Molossidae) and *Mormopterus* bats (Vespertilionidae) from Madagascar, Reunion and Comoros (Fig. 2a). Altogether, these sequences form a sister cluster to the recently created genus *Jeilongvirus*, which is composed of virus detected in bats and rodents.

v) Coronaviridae: The currently ongoing pandemic caused by SARS-CoV-2, plus the previous outbreaks of severe acute respiratory syndrome (SARS) and Middle East respiratory syndrome (MERS) have flipped the coin to reveal how devastating and life-threatening coronavirus infections could be. The emergence of SARS-CoV-2 has thrust CoVs into the spotlight again. Multiple lines of evidence support an evolutionary origin of all human CoVs from bats, where viruses are well adapted but prone to aleatory genetic diversity, eventually leading to infections in other animal species with unimaginable consequences. With the ordeals of SARS-CoV, MERS-CoV, and SARS-CoV-2, better wildlife surveillance and response plans should be in place worldwide [16].

In *T. brasiliensis*, coronaviruses were detected in 7 out of 155 samples (detection rate, 4.5%). The *T. brasiliensis* coronavirus (Tb-CoV) sequences were closely related, sharing

99.3-100% nt sequence identity. Phylogenetically, Tb-CoV grouped with members of the genus *Alphacoronavirus*. Tb-CoV formed a cluster with CoV sequences recovered from *T. brasiliensis* from Southern Brazil and the USA and from *Myotis nigricans* and *Cynomops planirostris* from Brazil (Fig. 2b). Tb-CoV-related sequences form a sister cluster with porcine epidemic diarrhea virus (PEDV; subgenus *Pedacovirus*) and bat coronavirus HKU10 (subgenus *Decacovirus*).

In contrast to the enormous diversity of CoV genomes found in Old World bats [17, 18], in this study, only alphacoronaviruses closely related to CoV isolates reported in a previous study in Molossidae species were detected [8]. Based on those results, it has been hypothesized that CoV found in neotropical bats are less diverse than those detected in Old World bats [19].

vi) Astroviridae: In recent years, bats have been found to harbor a great diversity of astroviruses, which raises the inevitable question of its zoonotic potential [20]. Astroviruses have been considered to be strictly species-specific. However, a great genetic diversity has recently been discovered among animal and human astroviruses that might indicate the potential of these viruses to cross species barriers, becoming a suitable viral candidate for studying evolutionary history as they infect a wide variety of species and recombine.

Bat astroviruses have been detected at high rates in feces, where remarkable genetic diversity has been observed [10]. In this study, in the oropharynx, only one out of the 155 samples allowed the recovery of an astrovirus genome (here named TbAstV33). As astroviruses replicate in the gastro-intestinal tract and are transmitted by the fecal-oral route, the relative viral load may be higher in feces than in oropharyngeal swabs [21]. Phylogenetically, TbAstV33 groups within a large cluster formed by sequences from bats and shrews (Fig. 2c). TbAstV33 shows a low degree of nt sequence identity with available sequences from GenBank (83% with NC_043100, an astrovirus recovered from *Taphozous melanopogon* bats in China).

In this work, a large number of viruses were detected in the oropharyngeal tract of *T. brasiliensis* bats. The viral genomes do not display close phylogenetic relationships to viruses detected in humans so far. Nevertheless, with the example of SARS-CoV-2 and many other emergent viruses that have spilled over from wildlife with an impact to human health in the last 20 years, knowledge about the viromes of synanthropic bat species may contribute to contingency plans in the advent of potential viral threats to humans and/or other species.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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