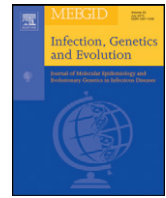




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Short communication

## Genetic diversity of bats coronaviruses in the Atlantic Forest hotspot biome, Brazil



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### ABSTRACT

Bats are notorious reservoirs of genetically-diverse and high-profile pathogens, and are playing crucial roles in the emergence and re-emergence of viruses, both in human and in animals. In this report, we identified and characterized previously unknown and diverse genetic clusters of bat coronaviruses in the Atlantic Forest Biome, Brazil. These results highlight the virus richness of bats and their possible roles in the public health.

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### 1. Introduction

Brazil harbours 15% of the world's bat diversity and carries 178 distinct bats species (Nogueira et al., 2014). Out of these, a total of 113 species exist only in the Atlantic Forest Biome (AFB), which is the second largest rain forest of the South America, and is one of the unique regions with highest biodiversity in the world (Paglia et al., 2012).

Bats are historically unique and widespread mammals playing essential roles in the emergence and re-emergence of viruses of both veterinary and public health importance. Although viruses of diverse genetic backgrounds can co-exist asymptotically in bats, majority of these viruses are single stranded RNA viruses (Calisher et al., 2006). Coronaviruses (CoVs) are enveloped, positive-sense, single-stranded RNA viruses in the family *Coronaviridae*, and are usually associated with respiratory, enteric, hepatic and neurological pathologies of varying severity (Woo et al., 2012). Coronaviruses are classified into four genera; *Alphacoronavirus* ( $\alpha$ -CoV) and *Betacoronavirus* ( $\beta$ -CoV) have been exclusively identified in mammals, whereas *Gammacoronavirus* and *Deltacoronavirus* are mainly detected in avian species (Woo et al., 2012; ICTV, 2015). Based on the genetic relatedness, the  $\beta$ -CoVs can further be subdivided into four clades: A to D (Drexler et al., 2014).

All CoVs that can potentially infect human were originated from animal reservoirs, and four of such CoVs are believe to be transmitted to

human through bats including  $\alpha$ -CoVs (229E and NL63) and highly pathogenic  $\beta$ -CoV (Severe Acute Respiratory Syndrome and Middle East Respiratory Syndrome) (Bolles et al., 2011; Chan et al., 2015; Corman et al., 2013; Huynh et al., 2012). Recently, a number of novel bats CoVs have been identified, primarily from African, Asian and European bats (Calisher et al., 2006; Chu et al., 2006; Drexler et al., 2014), as well as from South American countries including Costa Rica, Panama, Ecuador, Mexico and Brazil (Corman et al., 2013; Goes et al., 2013). Collectively, these studies indicate the co-existence of bats and viruses at the interface of viral evolution and bats ecology.

A limited number of studies have been conducted in Brazil to map the nature and breath of bats in harbouring viral populations. In previous studies, a total of five distinct CoV lineages have been detected in just 10% of local bats (15 species), and most of these are belonging to  $\alpha$ -CoV (Brandao et al., 2008; Corman et al., 2013; Goes et al., 2013). These attributes, and the existence of a large number of human beings (120 million) in the Atlantic Forest Biome, Brazil, clearly highlight the potential of bats in not only carrying zoonotic viruses but also in possible transmission of viruses to human beings.

To ascertain the diversity of bats CoVs circulating in the Brazilian bats, a total of 401 intestine tissues from 17 bat species were collected from 2010 to 2014 encompassing bats with distinct diet habits (Table 1, Fig. 1A, B). Bats from urban (N = 192) and rural (N = 10) areas were received from 14 municipalities of the Northwestern State of São Paulo by the Rabies Laboratory of Universidade Estadual Paulista (UNESP), Araçatuba-SP. Additionally, 99 and 100 samples were collected from Iguazu National Park, and from two distinct disturbed landscape sites, respectively, and provided by our collaborators of the Zoology Department at UNESP, Rio Claro-SP. All activities were

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**Table 1**  
Bat species tested for CoV RNA in different ecological regions of Brazil.

Species	No of samples tested/no positive (%)	Sex - no of samples tested/no positive (%)		Location - no of samples tested/no CoV positive (%)			Bats family	Bats diet types
		Male	Female	Undist. Forest	Fragm. Forest	Urban area		
<i>Artibeus fimbriatus</i>	3/0	3/0	0/0	3/0	0/0	0/0	Phyllostomidae	Frugivorous
<b><i>Artibeus lituratus</i></b>	126/3 (2.4%)	79/0	44/3 (6.8%)	60/2 (3.3%)	60/1 (1.6%)	6/0	Phyllostomidae	Frugivorous
<i>Artibeus obscurus</i>	1/0	1/0	0/0	0/0	0/0	1/0	Phyllostomidae	Frugivorous
<i>Artibeus planirostris</i>	4/0	4/0	0/0	2/0	1/0	1/0	Phyllostomidae	Frugivorous
<b><i>Carollia perspicillata</i></b>	44/3 (6.8%)	30/2 (6.7%)	14/1 (7.1%)	12/0	32/3 (9.4%)	0/0	Phyllostomidae	Frugivorous
<i>Desmodus rotundus</i>	10/0	8/0	2/0	0/0	10 <sup>a</sup> /0	0/0	Phyllostomidae	Hematophagous
<i>Eptesicus furinalis</i>	5/0	3/0	2/0	0/0	0/0	5/0	Vespertilionidae	Insectivorous
<b><i>Eumops glaucinus</i></b>	27/1 (3.7%)	11/0	16/1 (6.2%)	0/0	0/0	27/1 (3.7%)	Molossidae	Insectivorous
<b><i>Glossophaga soricina</i></b>	3/1 (33.3%)	2/1 (50%)	1/0	0/0	0/0	3/1 (33.3%)	Phyllostomidae	Nectivorous
<i>Lasiurus cinereus</i>	1/0	1/0	0/0	0/0	0/0	1/0	Vespertilionidae	Insectivorous
<i>Molossus molossus</i>	80/0	42/0	38/0	0/0	0/0	80/0	Molossidae	Insectivorous
<b><i>Molossus rufus</i></b>	56/2 (3.6%)	37/1 (2.7%)	19/1 (5.2%)	0/0	0/0	56/2 (3.6%)	Molossidae	Insectivorous
<b><i>Myotis nigricans</i></b>	8/1 (12.5%)	7/1 (14.3%)	1/0	0/0	0/0	8/1 (12.5%)	Vespertilionidae	Insectivorous
<b><i>Myotis riparius</i></b>	1/1 (100%)	1/0	1/1 (100%)	0/0	0/0	1/1 (100%)	Vespertilionidae	Insectivorous
<i>Platyrrhinus lineatus</i>	3/0	1/0	2/0	0/0	0/0	3/0	Phyllostomidae	Frugivorous
<b><i>Sturnira lilium</i></b>	28/3 (10.7%)	19/0	9/3 (33.3%)	22/2 (9.1%)	6/1 (16.6%)	0/0	Phyllostomidae	Frugivorous
<i>Vampyressa pusilla</i>	1/0	1/0	0/0	0/0	1/1 (100%)	0/0	Phyllostomidae	Frugivorous
<b>Total</b>	<b>401/15 (3.7%)</b>	<b>251/5 (2%)</b>	<b>149/10 (6.7%)</b>	<b>99/4 (4%)</b>	<b>110/5 (4.5%)</b>	<b>192/6 (3.1%)</b>		

Undist. Forest (Undisturbed Forest), Fragg. Forest (Fragmented Forest); bold font indicates species positive for CoV RNA.

<sup>a</sup> Sample obtained from rural area.

authorized and approved by the Ethic Committee of Institute of Biomedical Research from University of São Paulo (56-18-03/2014) and bats species were identified based on the morphological characteristics including head-boby, forearm size, and dental arch as described by Vizotto and Tadei (1973); Gregorin and Taddei (2002) and Miranda et al. (2011).

Nucleic acids extracted from 30 mg of intestine tissue of individual bat using NucliSENS® easyMAG® were subjected to first strand cDNA synthesis with random hexamers and RT-PCR High-Capacity cDNA Archive Kit (Applied BioSystems). The cDNA preparations were screened by pancoronavirus Nested PCR assay targeting the RNA-dependent RNA polymerase (RdRp) gene (Chu et al., 2011). Positive samples were sequenced and final sequences were submitted to the GenBank (KT717380-KT717394) (Supplementary table). A dataset, consisting of sequence generated in this study and all publically available bats CoVs, was phylogenetically analysed by neighbour-joining method in MEGA6 software using Kimura's two-parameter correction and 10,000 bootstrap values (Tamura et al., 2013).

Coronaviruses were detected in 15 bat intestines samples from eight bat species with distinct diet habit, demonstrating a marked potential of CoVs distribution among bat species in AFB that harbours 9% of world's bat diversity. All CoVs-positive bat species were geographical distributed in the Neotropics and in anthropogenic area apparently affected by fragmented forests, and bats-abundance urbanized areas. The detection of CoVs varied by bat species; undisturbed forest remnant (4%), fragmented forests (4.5%) and urban areas (3.1%) (Table 1).

Phylogenetics analyses indicated the circulation of 13  $\alpha$ -CoV and 2  $\beta$ -CoV in AFB bats (Fig. 1C). This is of primary interest as majority of bat CoVs surveillance studies conducted in the New World bats detected only  $\alpha$ -CoVs (Corman et al., 2013; Drexler et al., 2014; Osborne et al., 2011). A higher resolution analysis indicated a distinct distribution and diversity of  $\alpha$ -CoV and  $\beta$ -CoV lineages (Fig. 1D).

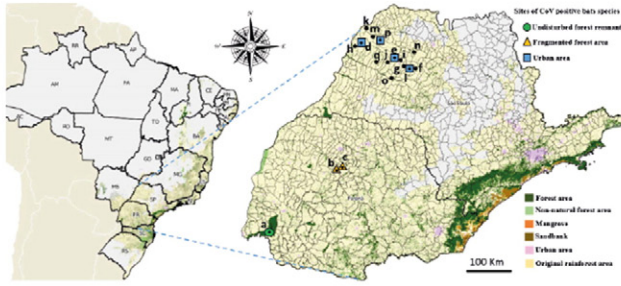
$\alpha$ -CoV sequences obtained from bats of same genus presented high nucleotide sequence similarity (e.g. *Artibeus*, *Glossophaga*, *Carollia*, *Molossus*, *Myotis* and *Sturnira*) (Fig. 1D and Supplementary table), even with sequences detected in other studies from bats of geographically distant regions. This relation can be exemplified by the high similarity of the CoV RNA partial sequences (91,9%) detected in *Carollia perspicillata* species from Fenix-PR, Brazil (KT717385), and Fyzabad, Trinidad and Tobago (EU769557) (Carrington et al., 2008), located at a distance of at least 3900 km. Similar results were previously reported

for a variety of bat CoVs and are taken as evidence of co-evolution of CoV genotypes and specific host genera (Drexler et al., 2014; Corman et al., 2013; Anthony et al., 2013).

Moreover, two previously uncharacterized  $\alpha$ -CoV from *Myotis nigricans* and *Myotis riparius* and one  $\beta$ -CoV cluster were identified in studied bats (Fig. 1C and D). CoV sequences from *Myotis* bats genera presented high nt sequence similarity (98.9%) and were most closely related to bat CoVs (KC110771) collected from Brazilian *Molossus molossus* and *Tadarida brasiliensis*. A distinct cluster of  $\alpha$ -CoVs were also detected in *Sturnira* bat that grouped with  $\alpha$ -CoVs lineage 1, a group with an evolutionary history of recombination and cross-species transmission between domestic and livestock animals, such as feline, canine and swine  $\alpha$ -CoVs (Lorusso et al., 2008). This clade presents nt similarity between 73.9% and 74.4% with transmissible gastroenteritis virus (DQ811789) and feline coronavirus (AF124987) depending on the *Sturnira* lineage (Supplementary table). This branching pattern may possibly explain the common-ancestral origin of  $\alpha$ -CoVs lineage 1 species from bats and other animal species. However, extensive evolutionary studies on complete genome sequences of these isolates are required to provide information on the virus origin and divergence.

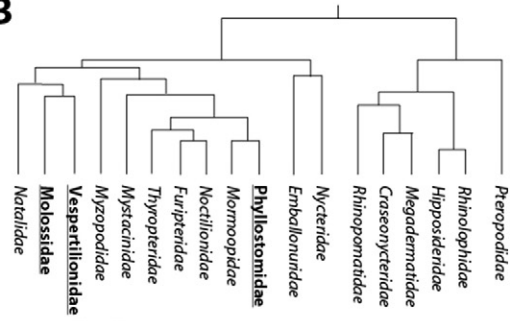
Out of two  $\beta$ -CoVs, the virus from *Eumops glaucinus* clustered within MERS-CoV containing lineage C. The second  $\beta$ -CoV detected in *Artibeus lituratus* shown highest similarity (96.4%) with CoV reported from Costa Rica (Corman et al., 2013) and lower similarity (69.3%) with MERS-CoV (Supplementary table). These sequences showed sequence similarities with  $\beta$ -CoV detected in *Pipistrellus* bats that poses high homology with human MERS-CoV (JX869059). Notably to observe that the *Eumops* bat positive for CoV was found on an urban area and was predated by a domesticated cat. It was not possible to rule out any event of virus transmission to human, however, this highlight the potential of domesticated animals in virus transmission and disease dynamics at the virus-animal-human interface. This is of special importance due to the established role of animals in the transmission of viruses to human being (Johnson et al., 2015). Taken together, these results represent the first detection of lineage C  $\beta$ -CoV in South American bats. Despite of close relationship between lineage C  $\beta$ -CoVs in Asia, Africa and Europe, cumulative data indicate that this lineage showed a more diversified host family distribution in Americas in *Mormoopidae*, *Phyllostomidae* and *Molossidae* bat species (Anthony et al., 2013; Corman et al., 2013; Goes et al., 2013).

**A**

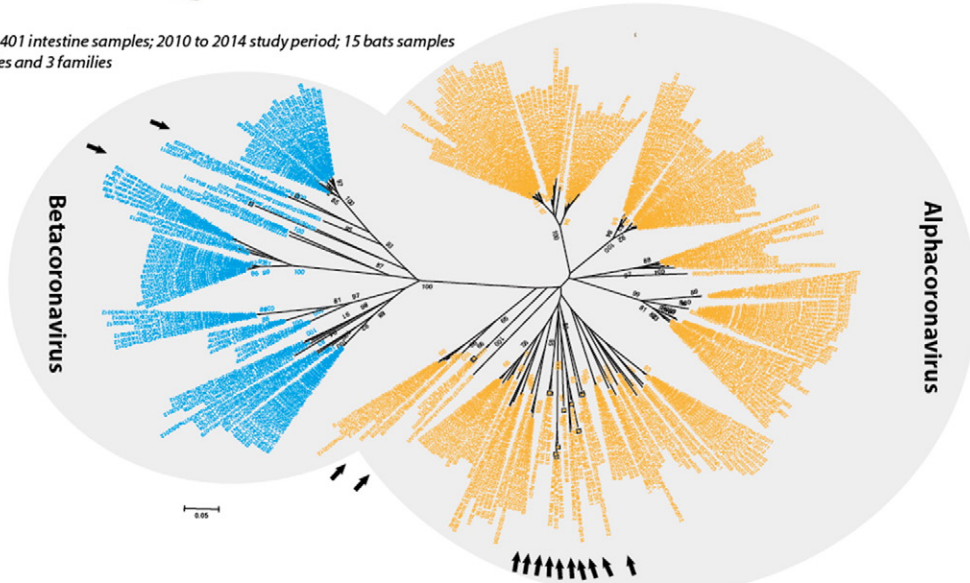


**São Paulo**  
3 families; 17 species; 401 intestine samples; 2010 to 2014 study period; 15 bats samples positive in 8 bat species and 3 families

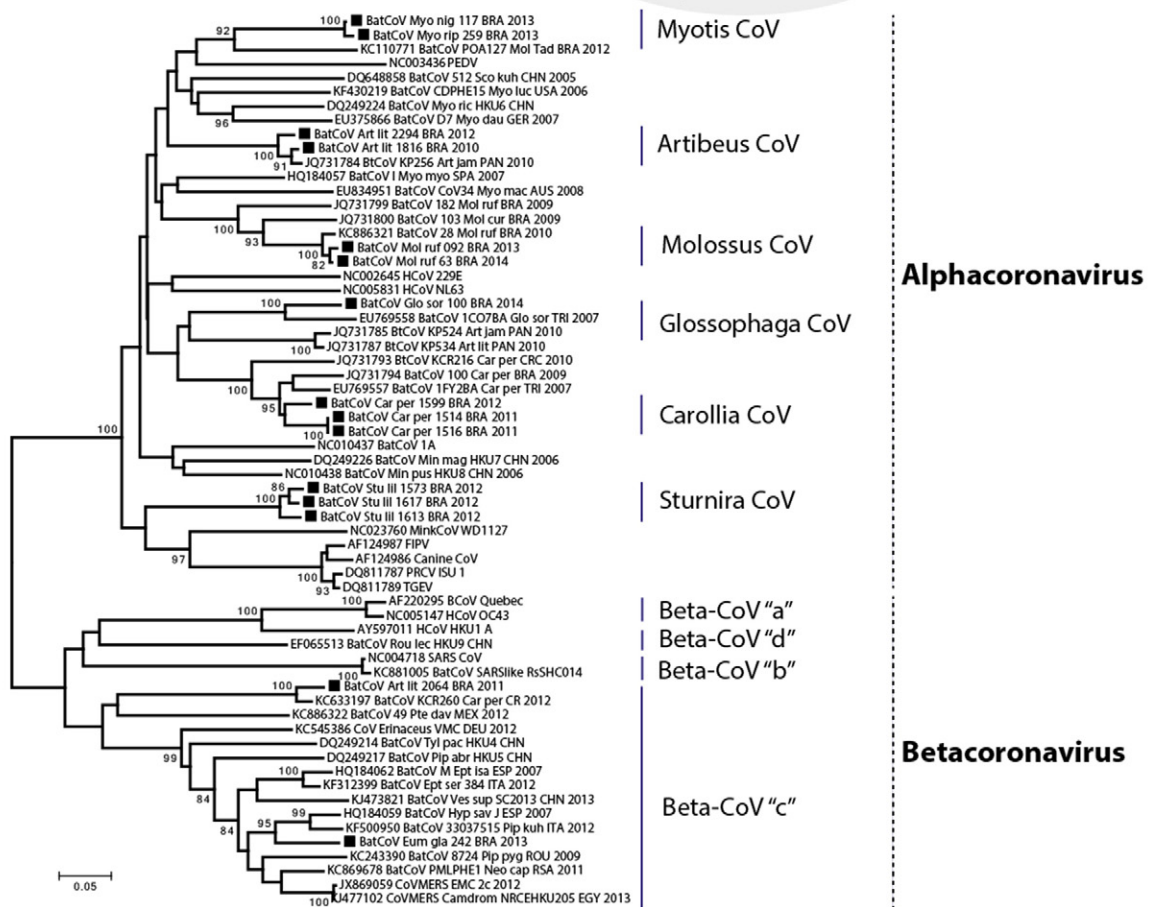
**B**



**C**



**D**





## 2. Conclusions

We present a great diversity of CoV genotypes and clusters in Brazilian bats, highlighting a biogeographic distribution of bats CoVs in the region. It is indispensable in future to investigate the evolutionary events in genetically diverse bats CoVs using complete genome sequences, and their possible transmission potentials to human being. Although it is not possible to calculate the risk of “spill over” events of Brazilian bats CoVs to humans, our results reinforce the need for expanded and continuing surveillance of CoVs in bat fauna, including those in the AFB regions of Brazil.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.meegid.2016.07.034>.

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**Fig. 1.** A: Brazil’s map and sites for bats capture/collection. Bats were collected from 17 sites (a–q) in the Atlantic Forest Biome from two adjacent Paraná and São Paulo states, Brazil. B: Based on morphological characteristics, bats were classified into different families (positive families were bold and underlined). Phylogenetic tree was adapted from Simmons (2005). C: Phylogenetic analysis of bat CoVs detected in the AFB, and reported previously around the world. Brazilian sequences reported in this study are marked with arrows. D: A higher resolution tree of the alpha and betacoronaviruses. Sequences studied in this report are marked with black filled square. Original map available in <http://mapas.sosma.org.br> from Atlas of the Atlantic Forest Remains (Fundação SOS Mata Atlântica/Instituto Nacional de Pesquisas Espaciais–INPE).