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Braz J Infect Dis, v. 16, n. 6, p. 545-551, 2012

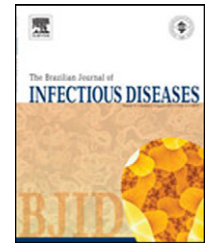
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The Brazilian Journal of INFECTIOUS DISEASES

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Original article

Desmodus rotundus and Artibeus spp. bats might present distinct rabies virus lineages

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ARTICLE INFO

Article history:

Received 18 May 2012

Accepted 26 July 2012

Available online 10 November 2012

Keywords:

Rabies

Bat

Artibeus spp.

Desmodus rotundus

Phylogeny

Molecular epidemiology

ABSTRACT

In Brazil, bats have been assigned an increasing importance in public health as they are important rabies reservoirs. Phylogenetic studies have shown that rabies virus (RABV) strains from frugivorous bats *Artibeus* spp. are closely associated to those from the vampire bat *Desmodus rotundus*, but little is known about the molecular diversity of RABV in *Artibeus* spp. The N and G genes of RABV isolated from *Artibeus* spp. and cattle infected by *D. rotundus* were sequenced, and phylogenetic trees were constructed. The N gene nucleotides tree showed three clusters: one for *D. rotundus* and two for *Artibeus* spp. Regarding putative N amino acid-trees, two clusters were formed, one for *D. rotundus* and another for *Artibeus* spp. RABV G gene phylogeny supported the distinction between *D. rotundus* and *Artibeus* spp. strains. These results show the intricate host relationship of RABV's evolutionary history, and are invaluable for the determination of RABV infection sources.

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Introduction

Rabies is a worldwide neglected fatal encephalitis,^{1,2} which is listed amongst the ten major infectious causes of human deaths worldwide, estimated at 55,000 per year.³ This infection is efficiently preventable by vaccination,⁴ but treatment costs of rabid or exposed patients, diagnostic procedures, and vaccines make it a significant challenge for public health systems in endemic countries.^{5,6}

The disease is caused by rabies virus (RABV) (family *Rhabdoviridae*, genus *Lyssavirus*),⁷ an enveloped virus with a length of 110–250 nm and a diameter of 75 nm,

usually transmitted by the saliva of an infected mammal. The RABV genome has a negative-stranded non-segmented ssRNA with 11,932-nucleotides that encodes the five structural proteins N (nucleoprotein), P (phosphoprotein), M (matrix), G (envelope glycoprotein), and L (large, RNA-dependent RNA-polymerase).^{8,9} Between 2004 and 2005, 62 people died in the Brazilian Amazon of rabies transmitted by *Desmodus rotundus* vampire bat, a primary reservoir of rabies in Latin America.^{10,11} Regarding frugivorous bats of the genus *Artibeus*, rabies has been reported in the species *A. fimbriatus*, *A. jamaicensis*, *A. lituratus*, and *A. planirostris*.¹² *Artibeus* spp. bats have been assigned an increasing importance in public health, as they are considered a rabies reservoir for humans in urban areas

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<http://dx.doi.org/10.1016/j.bjid.2012.07.002>

in Brazil, which is aggravated by the increasing population of these bats and the fact that they share roosts with *D. rotundus*.¹²⁻¹⁴

From 2003 to 2008, the Instituto Pasteur in Brazil tested 18,007 non-hematophagous bats for rabies, 252 of which were found to be positive. Also, from 2005 and 2007, 56 out of 160 non-hematophagous bats that tested positive for rabies were classified as *A. lituratus*.

Phylogenetic studies based on the N gene have suggested that RABV lineages from *Artibeus* sp. are not divergent from those from *D. rotundus*,¹⁵ all belonging to the same genic¹⁶ and antigenic variant 3 (AgV3).¹⁷ Nonetheless, these studies have provided only inconclusive results, as they were based on a very restricted sampling regarding geographic area and sample number, mainly in the case of *Artibeus* spp. In addition, these studies were based on a single gene sequences for phylogenetic reconstructions.

The ability to determine the source of infection and the epidemiology of rabies cycles are paramount for accurate decision-making in public health, mainly regarding vaccination strategies and animal population control. This study aimed to evaluate the possibility of distinction between RABV genetic lineages related to *D. rotundus* and *Artibeus* spp. bats based on N and G genes sequences.

Materials and methods

Rabies virus strains

Twenty *Artibeus* spp. RABV strains were obtained from first-passage isolates in mice inoculated with 20% suspensions of *A. lituratus* and *Artibeus* spp. central nervous systems (CNS); 15 *D. rotundus*-related strains were obtained directly from naturally infected cattle brain tissues (Table 1). These 35 strains were collected in nine municipalities from São Paulo State, Southeastern Brazil (Fig. 1), between 2004 and 2005. All samples were diagnosed positive for rabies by direct immunofluorescence test (DIFT) targeted to the viral nucleoprotein.¹⁸

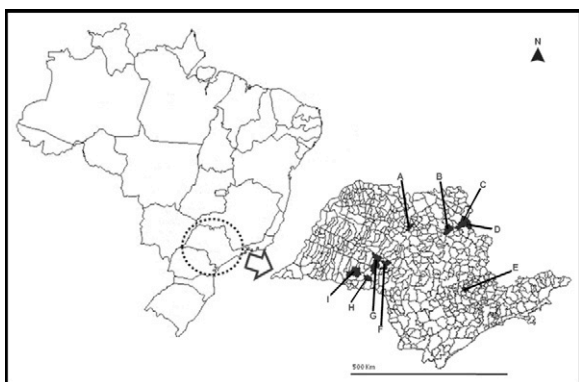


Fig. 1 – A map of São Paulo State (minor map) and Brazil (larger map), showing the cities (in black) where the 35 bats used in this study were collected. A) Catanduva, B) Ribeirão Preto, C) Altinópolis, D) Santo Antônio da Alegria, E) Monte Mor, F) Garça, G) Marília, H) Platina, and I) Paraguaçu Paulista.

The nucleoprotein and the glycoprotein sequences generated in this study have been assigned the GenBank accession numbers JF682392 - JF682426 and JF682427-JF682461, respectively.

Amplification and sequencing of the nucleoprotein and glycoprotein genes

Total RNA from the 35 RABV isolates CNS samples tested and the from positive and negative controls were extracted with TRIzolTM (Invitrogen – Carlsbad, CA, USA) method, following the manufacturer's instructions. The challenge virus standard (CVS) fixed strain of RABV isolated in mice brain and nuclease free-water were used as positive and negative controls, respectively.

Reverse transcription polymerase chain reaction (RT-PCR) to partially amplify the N and G genes was performed according to the protocol described by Carnieli et al.,¹⁹ using the primers described by Orciari et al.²⁰ for the N gene and those described by Sato et al.²¹ for the G gene (Table 2). The PCR products were purified from the PCR reactions using the QIAquickTM Gel Extraction KitTM (Qiagen – Valencia, CA, USA), according to the manufacturer's instructions. The products with nonspecific bands were purified using 1% agarose gel and the QIAquick[®] kit. After the purification step, the DNA samples were visually quantified in 2% agarose gel with Low Mass DNA Ladder (Invitrogen – Carlsbad, CA, USA), following the manufacturer's instructions.

The DNA sequencing reaction mixture consisted of 4 μ L of BigDye 3.1TM (Applied Biosystems – Foster City, CA, USA), 3.2 pmol of both sense and antisense primer for each gene in separate reactions, 30–60 ng of target DNA and DNase-free water to a final reaction of 10 μ L. The reaction was performed in a Mastercycler Gradient thermal cyclerTM (Eppendorf, NY, USA) with 35 cycles at 96 °C for 10s, 50 °C for 5s, and 60 °C for 4min, with a ramp of 1 °C/s between each temperature. Sequencing reaction products were purified with SephadexTMG-50 fine beads (GE Healthcare Biosciences) in 96-well multiscreen HV plates. After purification, the sequences were resolved in an ABI-3130TM Automatic Sequencer (Applied Biosystems – Foster City, CA, USA).

Phylogenetic analyses

To construct the genealogic trees, nucleotide and putative amino acids sequences for the 35 RABV strains of N and G genes were aligned by the CLUSTAL/W multiple alignment algorithm method using the BioEdit program,²² and then by manually checking the alignments for each set of aligned sequences. A score was assigned to each of the nucleotides shown on the electropherograms for each of the sequencing reactions using the online Phred application. Only positions with a Phred score > 20 were used.²³ The final sequence for each strain was obtained using the Contig Assembly Program (CAP) in BioEdit v.5.0.9²² and submitted to BLASTn for homology confirmation.

Phylogenetic trees of the RABV isolates were built using the Neighbor-joining algorithm and the maximum composite likelihood (MCL) evolutionary model implemented in Mega 4.1 (© 1993–2008)²⁴ with 1,000 bootstrap replicates.

Table 1 – GenBank accession numbers for the reference sequences of N gene and G gene used in phylogenetic analysis in this study showing strain, the specific-host from which the AgV3 RABV lineages were isolated, city, and year in which the samples were obtained.

GenBanknumber		Strain	City	Specific-host	Year
N gene	G gene				
JF682392	JF682427	05/Art3250	Catanduva	<i>Artibeus</i> spp.	2005
JF682393	JF682428	05/Art4578	Monte Mor	<i>Artibeus lituratus</i>	2005
JF682394	JF682429	05/Art3598	Ribeirão Preto	<i>Artibeus lituratus</i>	2005
JF682395	JF682430	05/Art3738	Ribeirão Preto	<i>Artibeus lituratus</i>	2005
JF682396	JF682431	05/Art4850	Ribeirão Preto	<i>Artibeus</i> spp.	2005
JF682397	JF682432	05/Art4932	Ribeirão Preto	<i>Artibeus</i> spp.	2005
JF682398	JF682433	05/Art5459	Ribeirão Preto	<i>Artibeus lituratus</i>	2005
JF682399	JF682434	05/Art6734	Ribeirão Preto	<i>Artibeus lituratus</i>	2005
JF682400	JF682435	05/Art6956	Ribeirão Preto	<i>Artibeus</i> spp.	2005
JF682401	JF682436	05/Art7045	Ribeirão Preto	<i>Artibeus</i> spp.	2005
JF682402	JF682437	05/Art7436	Ribeirão Preto	<i>Artibeus lituratus</i>	2005
JF682403	JF682438	05/Art8688	Ribeirão Preto	<i>Artibeus lituratus</i>	2005
JF682404	JF682439	05/Art8921	Ribeirão Preto	<i>Artibeus lituratus</i>	2005
JF682405	JF682440	05/Art10509	Ribeirão Preto	<i>Artibeus</i> spp.	2005
JF682406	JF682441	05/Art7270	Paraguaçu Paulista	<i>Artibeus lituratus</i>	2005
JF682407	JF682442	05/Art7547	Paraguaçu Paulista	<i>Artibeus lituratus</i>	2005
JF682408	JF682443	05/Art8456	Paraguaçu Paulista	<i>Artibeus lituratus</i>	2005
JF682409	JF682444	05/Art11206	Paraguaçu Paulista	<i>Artibeus lituratus</i>	2005
JF682410	JF682446	05/Art8639	Marília	<i>Artibeus lituratus</i>	2005
JF682413	JF682445	05/Art7848	Marília	<i>Artibeus</i> spp.	2005
JF682411	JF682460	05/Bov6314	Garça	Bovine	2005
JF682412	JF682461	05/Bov7535	Platina	Bovine	2005
JF682414	JF682447	05/Bov451	Altinópolis	Bovine	2005
JF682415	JF682449	05/Bov3924	Altinópolis	Bovine	2005
JF682416	JF682451	05/Bov10339	Altinópolis	Bovine	2005
JF682417	JF682450	04/Bov8967	Altinópolis	Bovine	2004
JF682418	JF682448	04/Bov2196	Altinópolis	Bovine	2004
JF682419	JF682452	05/Bov2579	Santo Antônio da Alegria	Bovine	2005
JF682420	JF682453	04/Bov3441	Santo Antônio da Alegria	Bovine	2004
JF682421	JF682454	04/Bov3833	Santo Antônio da Alegria	Bovine	2004
JF682422	JF682455	04/Bov4698	Santo Antônio da Alegria	Bovine	2004
JF682423	JF682456	04/Bov6930	Santo Antônio da Alegria	Bovine	2004
JF682424	JF682457	04/Bov11044	Santo Antônio da Alegria	Bovine	2004
JF682425	JF682458	04/Bov11817	Santo Antônio da Alegria	Bovine	2004
JF682426	JF682459	04/Bov11818	Santo Antônio da Alegria	Bovine	2004

Additionally, 38 homologous sequences recovered from GenBank were included in the phylogenetic trees of the N and G genes (29 and none, respectively) and European bat *Lyssavirus* 1 (another specie in the *Lyssavirus*) was used as an outgroup.

The minimum, maximum, and mean nucleotide (with the MCL model) and amino acids (with the Poisson correction) identities for the clusters for the N and G gene sequences were calculated using Excel (©1985–2003 Microsoft Corporation) based on the identity matrices calculated with the BioEdit program. The changes in the amino acids observed in the

samples analyzed were studied using Mega 4.1 (© 1993–2008)²⁴ and BioEdit v.7.0.0²² programs.

Results

After editing, the N gene was 1,281-nucleotides long, located between nucleotides 68 and 1,350 in relation to the N gene CVS reference strain (GenBank number AF406696) and had a putative amino acid sequence with 427 amino acids.

Table 2 – Primers for RT-PCR and genetic sequencing of RABV N and G genes.

Primers	Orientation	Sequence	Gene
21G	Sense	5' ATGTAACACCTCTACAATG 3'	N
304	Antisense	5' TTDACGAAGATCTTGCTCAT 3'	N
GA 3222-40	Sense	5'CGTGTCATTTTTRTCARAGT 3'	G
GB 4119-39	Antisense	5' GGAGGGCACCATTGGMTC 3'	G
GS 3994	Sense	5'GGMTTGTGGATGAAAGRGC 3'	G
GantiBR2072	Antisense	5' TGCTGATTGCRCTCATT 3'	G

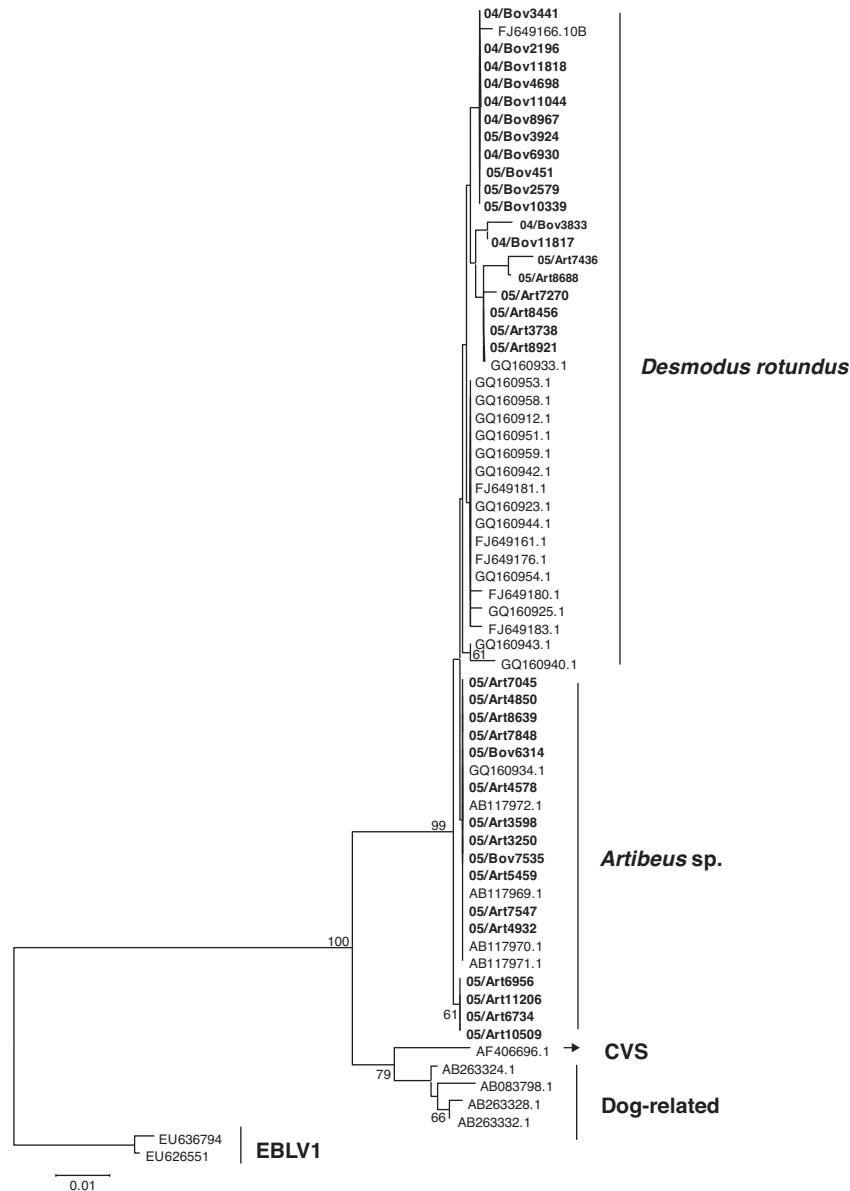


Fig. 2 – RABV N amino acids tree showing clusters specific to *Desmodus rotundus* and *Artibeus* spp. bats. The tree was built with the Neighbor-joining distance algorithm and the Poisson correction with 1,000 bootstrap replicates and European bat *Lyssavirus* (EBLV-1) as outgroup. The bar represents the number of substitutions per site.

Regarding the G gene, sequences were 1,571-nucleotides long, located between nucleotides 8 and 1,579 in relation to the G gene CVS reference strain (GenBank number FJ979833) and had a putative amino acid sequence with 520 amino acids.

The N gene nucleotide phylogenetic tree showed three clusters for the 35 RABV strains included in the study; one cluster for *D. rotundus* strains and two for the *Artibeus* spp. strains. Regarding N protein amino acids tree, the 35 strains segregated in only two clusters, one for *D. rotundus* and one formed mainly by *Artibeus* spp. strains (Fig. 2). Nucleotides and amino acids identities for the N region under analysis between *D. rotundus* and *Artibeus* spp. sequences ranged from 97.4% to 98.7% and 98.1% to 99.7%, respectively.

The G gene nucleotide phylogenetic tree showed two clusters for the 35 RABV strains included in the study; one cluster

for *D. rotundus* strains and one for the *Artibeus* spp. strains. Regarding G amino acids tree, the 35 strains segregated in two clusters, one for *D. rotundus* and one formed by *Artibeus* spp. strains (Fig. 3). Nucleotides and amino acids identities for the G region under analysis between *D. rotundus* and *Artibeus* spp. sequences ranged from 97.0% to 99.1% and 96.1% to 99.0%, respectively.

Discussion

Frugivorous bats of *Artibeus* spp. have an emerging importance for rabies epidemiology in Brazil, mainly in urban centers, and have been reported as carriers of RABV lineages close to those found in the vampire bat *D. rotundus*.^{12,16} In the present

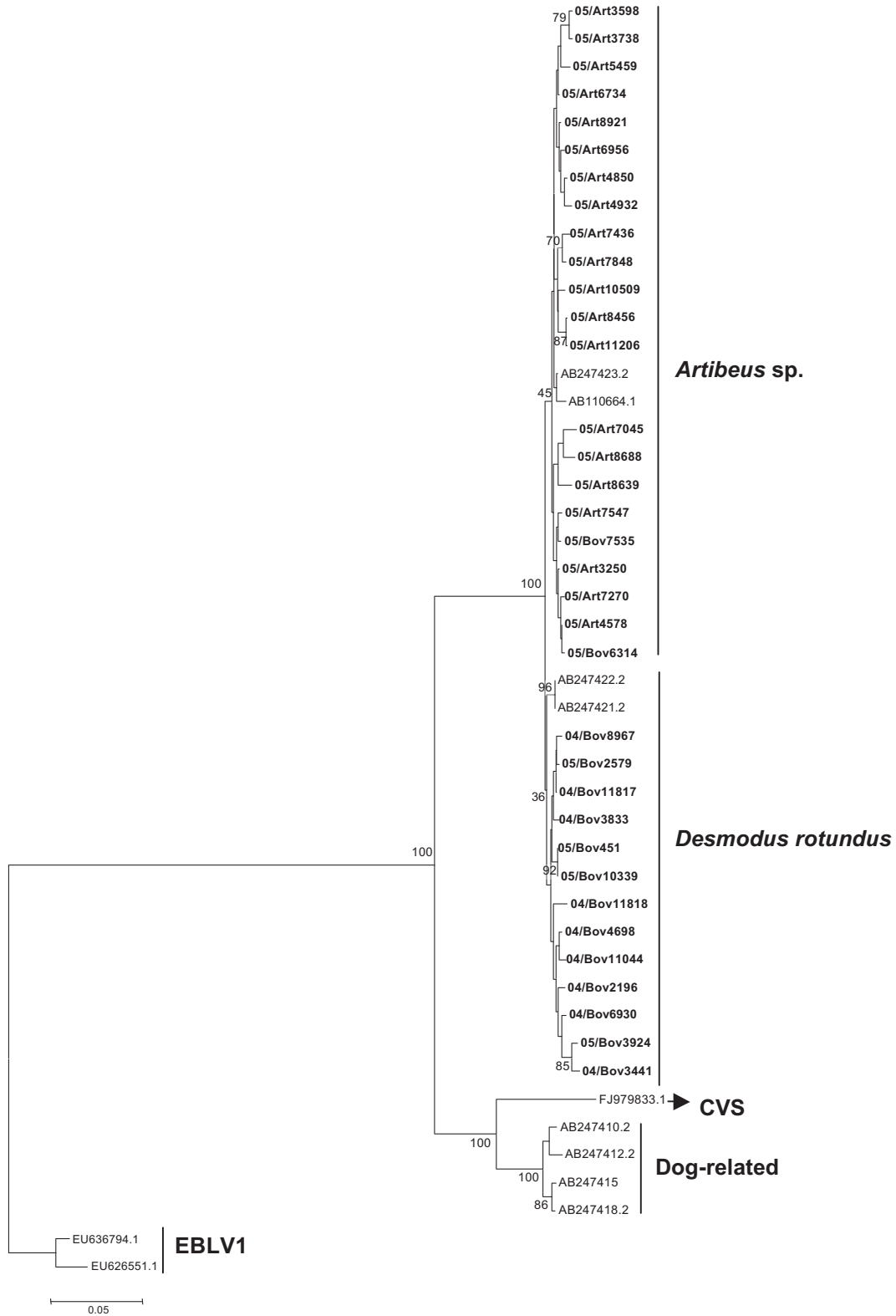


Fig. 3 – RABV G amino acids tree showing clusters specific to *Desmodus rotundus* and *Artibeus* spp. bats. The tree was built with the Neighbor-joining distance algorithm and the Poisson correction with 1,000 bootstrap replicates and European bat Lyssavirus (EBLV-1) as outgroup. The bar represents the number of substitutions per site.

investigation, RABV N and G phylogenies of strains recovered from these bats showed the existence of viral lineages that can be accurately attributed to *D. rotundus* or *Artibeus* spp. bats, a previously unknown fact.

RABV lineages heterogeneity expressed phylogenetically as host-specific lineages is a widely documented epidemiological phenomenon,^{16,19,25,26} and is influenced by geographic barriers rather than by species barriers only.²⁷ Accordingly, the two *Artibeus* spp. clusters found for N gene sequences might also represent regional sub lineages of RABV, a fact already described for *D. rotundus* RABV lineages in Brazil.^{28,29} These observations agree with the proposition that, regarding rabies in bats, host species are as important as geographic variations.³⁰ Regarding viral lineages, genetic variations that occur within a host species are different from those that occur in another, and this variation, coupled with the host's geographical isolation, may explain the RABV genetic differences reported in this work.³¹

Seven strains (05/Art7270, 05/Art8456, 05/Art7436, 05/Art8921, 05/Art8688, 05/Art3738, and 08IacriSP3577B) were classified in the *Artibeus* spp. group for the N nucleotides tree; however they segregated into the *D. rotundus* cluster of the N amino acids tree. This fact suggests the possibility that *Artibeus* spp. strains are still under an adaptation process after the spill-over event from *D. rotundus*, as already suggested by Kissi,³¹ who have already experimentally reported this type of stepped adaptation of RABV. This phenomenon can occur as a consequence of the predominance of synonymous mutations over non-synonymous nucleotides mutations, which leads to greater differences among the nucleotide sequences than among the amino acids sequences.^{32,33}

Regarding the Bov7525 and Bov6314 strains, in cattle, both segregated in the *Artibeus* spp. RABV cluster; this was unexpected, as cattle rabies is related to that of *D. rotundus* and not to that of frugivorous bats.^{34,35} The most plausible explanation is that these two strains have been transmitted from an *Artibeus* spp. to a *D. rotundus*, and then from this bat to the cattle in a rare class of spill-over transmission.

Kobayashi et al.,¹⁶ analyzing the N gene of the RABV isolated from frugivorous bats, insectivorous bats, and *D. rotundus*, reported lineages associated with *Artibeus* spp. bats (frugivorous), *D. rotundus*, and insectivorous bats, suggesting that there are species-specific lineages.²⁶ However, they have not provided significant results to distinguish between RABV isolated from *D. rotundus* and *Artibeus* spp., possibly due to a restricted number of samples and sampling area. In the present study, this problem was compensated by the inclusion of 35 RABV isolates of bats (20 from *Artibeus* spp. and 15 from *D. rotundus*) from a broad geographical area of São Paulo. In addition, this study also analyzed the complete G gene, which provides a more specific distinction between the genetic sequences.

Different hosts pose different challenges for rabies control. This is more complex in bats due to the large number of species, the different ecologic niches that they occupy, and the impossibility to vaccinate these animals. For instance, the population of *D. rotundus* in Latin America can be legally controlled with vampiricide anticoagulants applied on cattle or on the bats themselves.³⁴ Conversely, non-hematophagous bats are under legal protection, and only now

the knowledge of rabies epidemiology in these bats species is increasing.^{35,36}

In this context, the results obtained in this study are valuable, because based on the partial amino acid sequences for the N gene it is possible to differentiate RABV strains from *Artibeus* spp. and *D. rotundus* for the purpose of defining the infection sources in molecular epidemiology. These results show the close host relationship of RABV transitions, and have an invaluable application for determining the sources of rabies infections transmitted mainly to dogs and cats in urban centers.

In conclusion, for rabies virus isolates related to frugivorous bats of the *Artibeus* spp. and to the vampire bat *D. rotundus*, the phylogeny based on sequences of the N and G genes shows segregation patterns in genus-specific agreement in each of these bats. Data from this study suggest that a lineage of RABV is possibly being established in the *Artibeus* spp. genus.

Conflict of interest

All authors declare to have no conflict of interest.

Acknowledgements

The authors are grateful to CAPES (W.O. Fahl's PhD fellowship) and CNPq (P.E. Brandão's PQ-2 fellowship).

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