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Research paper

# Phylogenetic analysis of near full-length sequences of the *Desmodus rotundus* genetic lineage of rabies virus

Angélica Cristine de Almeida Campos[a,*], Camila Malta Romano[b], Fernando Lucas Melo[c], Danielle Bastos Araújo[a], Elenice Maria Sequetin Cunha[d], Debora Regina Veiga Sacramento[e], Edison Luiz Durigon[a], Silvana Regina Favoretto Lazarini[a,f]

[a] Department of Microbiology, Institute of Biomedical Sciences, University of Sao Paulo, Sao Paulo, SP 05.508-900, Brazil
[b] Institute of Tropical Medicine, University of São Paulo, São Paulo, SP 05.403-000, Brazil
[c] University of Brasilia, Brasilia-DF 70.910-900, Brazil
[d] Biological Institute of Sao Paulo State, Sao Paulo, SP 04.014-002, Brazil
[e] Genomic Molecular Engineering, Sao Paulo, SP 01.332-000, Brazil
[f] Pasteur Institute of Sao Paulo State, Sao Paulo, SP 01.311-000, Brazil

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ABSTRACT

The World Health Organization (WHO), reports that rabies causes tens of thousands of deaths every year killing humans, non-human primates and other animals. Rabies continues to be a public health issue, despite the existence of effective vaccines. The dogs remain the primary reservoir and transmitter of rabies to humans globally. In the Americas, bats are regarded as the second most common source of rabies virus to humans. The vampire bat *Desmodus rotundus* has been identified as a natural reservoir of rabies virus (RABV) in this region. The complete genome of the RABV variant maintained by populations of vampire bats *D. rotundus* has rarely been reported. In this study, we sequenced and analyzed the genome of a RABV variant detected in *D. rotundus*. The sample, collected from an endemic area in São Paulo State, was phylogenetically compared with the genome of the standard sample for species *Rabies virus* as well as other samples belonging to terrestrial and bat-associated cycles of rabies transmission, available in GenBank. Distinct patterns linked to the genetic lineage were identified. These data can aid in the understanding of the molecular epidemiology of this virus and the epidemiological importance of this species in the transmission of the RABV.

## 1. Introduction

Rabies virus (RABV) is a lethal lyssavirus species that has been recognized since the Antique Age with the first reports of it in the Laws of Eshnunna (1930 BCE). The oldest reports involving hematophagous bats and RABV were described in the Americas by colonial Spanish communities in Panama in the 16th century (Steele and Fernadez, 1991). The first recorded suspected case of RABV transmitted to humans by a bat occurred in Santa Catarina, Brazil (Carini, 1911). Despite significant investment in to the study, prevention, and control of the virus, rabies remains globally distributed. Domestic dogs and wild animals are known to be reservoirs and transmitters.

Lyssaviruses have been detected in bats globally and bats are identified as a host reservoir for 12 of 14 recognized *Lyssavirus* species. The bat-adapted *Lyssaviruses* have different characteristics (WHO, 2013), like symptoms, from the rabies virus identified in carnivores. The vampire bat (*Desmodus rotundus*) ranges from in the northern of México through the northern coast of Chile, the central coast of Argentina, and the Uruguayan coast. Vampire bats are found at sea level and at altitudes of greater than 3000 m (Flores-Crespo, 2000). Fossils from Florida and Cuba dating back 2500 years, provide evidence of vampire bats has been found, indicating their presence in the Pleistocene period (Arellano-Sota, 1988). Often, epidemiologic studies of RABV are based on reports from rabies surveillance programs. In Brazil, the prevalence of the RABV in bat populations ranges between 3% to 11% (Almeida et al., 2005; Birney and Rising, 1967; Burnett, 1989; Richardson et al., 1966; Schowalter, 1980; Steece et al., 1982).

In Brazil, RABV has been isolated from 41 of 167 bat species described (Castilho et al., 2008; Sodré et al., 2010; Uieda et al., 1996). Previous analyses have confirmed dogs and vampire bats act as primary reservoirs (Favoretto et al., 2002; Ito et al., 2001a, 2001b), and until 2003 epidemiological cycle of transmission of RABV to humans was

* Corresponding author.
*E-mail address:* camposac@usp.br (A.C. de Almeida Campos).

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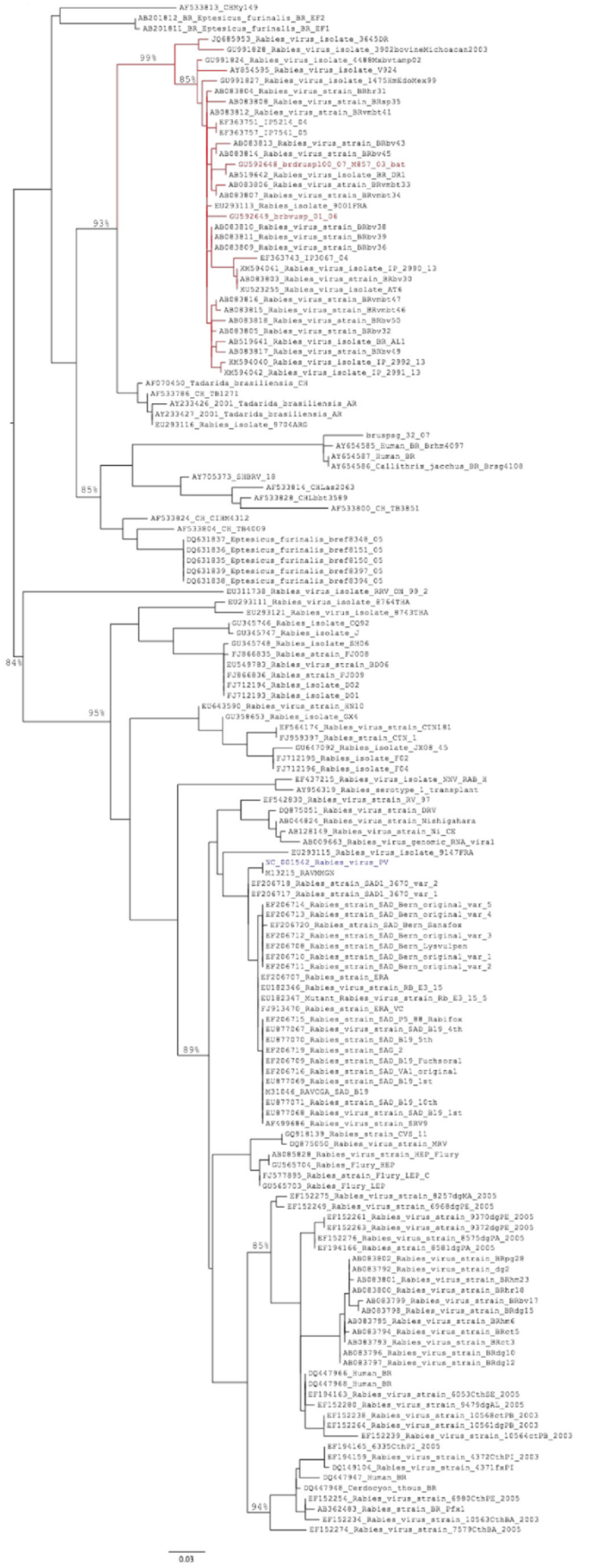
associated primarily with dogs and cats (72.5%). The epidemiological pattern of transmission changed during two outbreaks of RABV in people were traced back to vampire bats in 2004 in the cities of Portel-Pará (15 cases) and Viseu-Pará (6 cases). Additional outbreaks in 2005 in Pará and Maranhão states associated with vampire bats and a decrease in rabies cases transmitted by dogs put the focus on vampire bats as the primary transmitter of rabies to humans (Brasil. Ministério da Saúde, 2006; Brasil. Ministério da Saúde, 2004; Rosa et al., 2006). A study by Fahl et al. (Fahl et al., 2012) identified the existence of two different genetic lineages in vampire and frugivorous bats conducted in Brazil, suggesting one spillover event in *Artibeus* spp. from *D. rotundus* species. Phylogeographic analyses of the origin of the *D. rotundus* genetic lineage of RABV in Trinidad (Seetahal et al., 2013) indicated that Brazil and Uruguay are the most likely sources.

Data on the complete genome of the RABV variant maintained by populations of *D. rotundus* vampire bats are limited. This indicates the need for complementary studies on rabies ecology and epidemiology in chiropterans. The epidemiological importance of vampire bats in the transmission of RABV is unquestionable and genotyping and phylogenetic studies are essential to the understanding the viral genetic diversity in different hosts. The aim of this work was to sequence and conduct a genetic analysis of of a near full-length of the RABV lineage maintained by *D. rotundus* bat populations between 2008 and 2011.

## 2. Material and methods

Samples from a naturally infected bat and bovid were obtained from a rabies surveillance service. The vampire bat died subsequent to arrival at the laboratory. The bovid had clinical signs of rabies and RABV was diagnosed in the brain tissue by the Rabies Diagnosis Laboratory accredited by the Brazilian Ministry of Health and/or Brazilian Ministry of Agriculture. No samples were obtained from, nor were animals used in, an experimental study. Authorization was provided by the Ethics Committee for Animal Research from the Institute of Biomedical Sciences of Universidade de São Paulo - ICB/USP on 29 June 2009 registered as number 48, sheet 73 and book 2. All subsequent experiments were conducted in cell cultures.

The sample virus (brdrusp100/07) was isolated from a *D. rotundus* vampire bat captured in an urban area of São José do Barreiro City, São Paulo State, near the Atlantic Forest in a subtropical area in the Southeastern of Brazil in 2001. To confirm the antigenic variant the viruses was antigenically characterized by indirect immunofluorescence using a panel provided by the Centers for Disease Control and Prevention (CDC), Atlanta, Georgia, USA and distributed by the Pan-American Health Organization-PAHO (Centro Panamericano de Fiebre Aftosa-PANAFTOSA, 2000). The RABV isolated from bat and the virus obtained from the RABV positive bovid (brbvusp01/06 was used as the control. The viruses were passaged once in BHK-21 cells. RNA extraction, cDNA synthesis, and amplification reactions were conducted according Campos et al. (Campos et al., 2011). Sequencing reactions were performed using the primer-walking method described by Tordo et al. (Tordo et al., 1986a) (Noel ) and Ito et al. (Ito et al., 2001a,b), using the degenerate primers described by Campos et al. (Campos et al., 2011) on ABIPrism 3100 (Applied Biosystems) using the Sanger method. The isolate sequences were aligned with BioEdit using reference sequence (NC_001542) available in the public database, GenBank (www.ncbi.nlm.nih.gov/genbank). Sites under positive selection were investigated in the nucleoprotein multiple alignment (147 sequences, 269 nucleotides) using the Single likelihood ancestor counting model (SLAC) available at the DataMonkey website (http://datamonkey.org/). For all genes sequenced, maximum likelihood phylogenetic trees were reconstructed using GARLi, with branch support accessed through bootstrap replicates (1,000). Nucleotide sequences were translated in to amino acids and the level of similarity was investigated using MEGA7. The level of conservation among sequences was measured as the percentage of similar or identical sites observed




(caption on next page)

**Fig. 1.** Partial nucleoprotein maximum likelihood phylogenetic tree. Genetic analysis of 149 sequences from the 320 nucleotide carboxy-terminal region. Tree reconstructed using a GARLi heuristic search and "GTR + gamma+I" algorithm. Bootstrap (10,000 bootstrap replicates) values greater than 50% are depicted in the figure.

through multiple alignment (pair wise and overall).

Conserved sequences are simply similar or identical regions (region, sites, domains) that can be observed through a multiple alignment. We analyzed several regions that showed different levels of conservation, thus determined by how much variability (nucleotides or amino acid changes) was observed in a given region. Highly conserved regions/domains should not have more than one amino acid change. Phylogenetic trees were visualized in FigTree software. The sample used as the main reference was PV strain (NC 001542), the first and most commonly used reference genome for RABV.

## 3. Results

The antigenic characterization confirm that samples represents antigenic variant AgV3, related with *Desmodus rotundus*. Initial genetic characterization was conducted by analyzing a 320-nucleotide (nt) fragment from the nucleoprotein gene and confirmed that the two virus sequences in this study clustered with the *D. rotundus* genetic lineage RABV (Fig. 1). The clustering was also observed when the entire genome was analyzed (Figure S1 – Supporting Information). The complete sequence and alignment of nucleoprotein (1353 nt, 450 aa +1 stop codon - GenBank accession number GU592648, Figure S1), phosphoprotein (894 nt, 297 aa +1 stop codon - GenBank accession number JF523200, Figure S2), matrix protein (609 nt, 202 aa +1 stop codon - GenBank accession number JF523201, Figure S3), and glycoprotein (1575 nt, 524 aa - 19 aa signal peptide +452 aa ectodomain +53 aa endodomain +1 stop codon - GenBank accession number JF523202, Figure S4) genes, as well as the GL intergenic region and partial L (Table 1 and Figures S5, S6, S7 and S8 – Supporting Information) gene resulted in a distinct genealogic pattern for each gene (for details see Supporting Information). We identified protein differentiation patterns for the bat-associated transmission cycle and the specific RABV variant related to haematophagous bat. We identified sites of synonymous and non-synonymous mutations for each of the analyzed proteins and *dN/dS* analysis did not detect any positively selected sites for any gene.

The most common amino acid was serine (S) and the rarest was tryptophan. The most frequent amino acid substitutions were lysine (K) to arginine (R), threonine (T) to alanine (A), and valine (V) to isoleucine (I). These data were analyzed using MacClade software. In the antigenic site I, at the carboxy-terminal region of the nucleoprotein (residues 374 to 383), an amino acid substitution was identified that was exclusive to the *D. rotundus* genetic lineage. The substitution of residues 377 to 379, within the antigenic site (the target region of the host immune system). The domain LGKAPDLNKAYKS between residues 43 and 55 were divergent only in the bat sample, but was highly conserved in the reference sequences. A high degree of conservation was found between 12 domains, 7–21 residues in length (Fig. 2).

**Table 1**
Partial L gene results from sequencing reactions.

| Large protein | # of nucleotides | # o f amino acids | PV reference sample |
|---|---|---|---|
| Fragment 2a | 711 nt | 236 aa | 7241 to 7951 |
| Fragment 3a | 357 nt | 119 aa | 8765 to 9122 |
| Fragment 4a | 801 nt | 267 aa | 9423 to 10,223 |
| Fragment 4b | 657 nt | 219 aa | 10,320 to 10,977 |
| Fragment 4c | 387 nt | 129 aa | 11,466 to 11,852 |
| Total | 2913 nt | 970 aa +1 stop codon | |

Phylogenetic analysis of the phosphoprotein gene demonstrated that the studied sample clustered within into one distinct clade related to the *D. rotundus* genetic lineage, confirming the standard classification by nucleoprotein.

The phosphoprotein amino acid analysis identified 51 amino acid substitutions relative to PV (NC_001542). Twenty-nine amino acid substitutions were nonconservative, while three residues were specific to the *D. rotundus* genetic lineage (asparagine at residue 61, serine at residue 68, and glycine at residue 112). The most frequent amino acid in the phosphoprotein was serine (S) and the rarest was cysteine (C). The most frequently occurring amino acid substitutions were from asparagine (N) to glycine (G), from lysine (K) to alanine (A), and from valine (V) to leucine (L). Within the *D. rotundus* genetic lineage clade, three regions were relatively conserved: amino acids 80–125, 179–221, and 258–294 (except for residues 85, 91, and 189 in sample AF369359 - *T. brasiliensis*), whereas the region between residues 41 and 195 had the highest number of amino acid substitutions, with two small conserved sites within this region (residue 43 to 47, PIEVD, and 113 to 118, RFLKIW).

The M gene also clustered within the *D. rotundus* genetic lineage, as did the N and P genes. The genetic analysis of the matrix protein showed a 21-amino-acid substitution including eight non-conservative substitutions. The most common amino acid in matrix protein was leucine and the least common was histidine. The most frequently occurring amino acid substitutions were valine (V) to isoleucine (I), isoleucine (I) to valine (V), leucine (L) to proline (P), and alanine (A) to valine (V).

Five residues of the glycoprotein gene were specific to the *D. rotundus* genetic lineage, $Ser_{213}$, $Arg_{367}$, $Glu_{427}$, $Cys_{475}$, and $Asp_{521}$ and three sites were common to the RABV genetic lineage related to *D. rotundus* and *T. brasiliensis*, $Ala_{478}$, $Arg_{489}$, and $Ser_{493}$. Only at residue 218 did other samples (from GeneBank) from the Brazilian bat-associated RABV transmission cycle contain the arginine to lysine substitution (Fig. 3). Leucine was the most common amino acid and glutamine was the rarest. The most frequent amino acid substitutions were isoleucine (I) to threonine (T) and serine (S) to threonine (T). The conserved residues, $Ser_{194}$, $Arg_{348}$, $Glu_{408}$, $Cys_{456}$, and $Asp_{502}$, were observed by Sato et al. (Sato et al., 2004) in Brazilian samples related to hematophagous bats were also detected in this study and these sites are specific to the *D. rotundus* genetic lineage and can be considered a genetic signature.

The large protein had 2,910 nucleotides mapped (44.6%) in five discontinuous fragments (708 nt in fragment 2a-Figs. 4, 357 nt in fragment 3a, 801 nt in fragment 4a, 657 nt in fragment 4b, and 387 nt in fragment 4c). Although the L gene is considered to be a conserved region in RABV and easy to sequence, we were unable to sequence the whole fragment. The sequencing of large protein gene was our major challenge and the principal limitation of our study. Therefore, we present results from the discontinuous fragments. We detected 102 substitutions and 66 (64.7%) were non-conservative substitutions when compared with the PV reference sample (NC_001542). Seventeen amino acid residues were identified as being specific to the hematophagous bat genetic lineage, while another 13 residues (24, 27, 61, 122, 170, 1144, 1342, 1374, 1487, 1556, 1653, 1665, and 2101) were related to the bat-associated cycle, and 3 residues (21, 88, and 107) were common to genetic lineages related to *D. rotundus* and *T. brasiliensis*. Fourteen residues had non-conservative substitutions ($Phe_8$, $Glu_{139}$, $Lys_{217}$, $Gly_{683}$, $Thr_{1092}$, $Arg_{1139}$, $Ala_{1255}$, $Val_{1617}$, $Cys_{1619}$, $Val_{1852}$, $Ala_{1889}$, $Ala_{2057}$ $Ala_{2058}$ and $Arg_{2089}$), while three residues had conservative substitutions ($Arg_{645}$, $Val_{1214}$, and $Asp_{1336}$). At 18 sites, there were identified conserved domains with seven or more amino acid residues were identified.

## 4. Discussion

Molecular techniques can be used to verify the vast diversity in

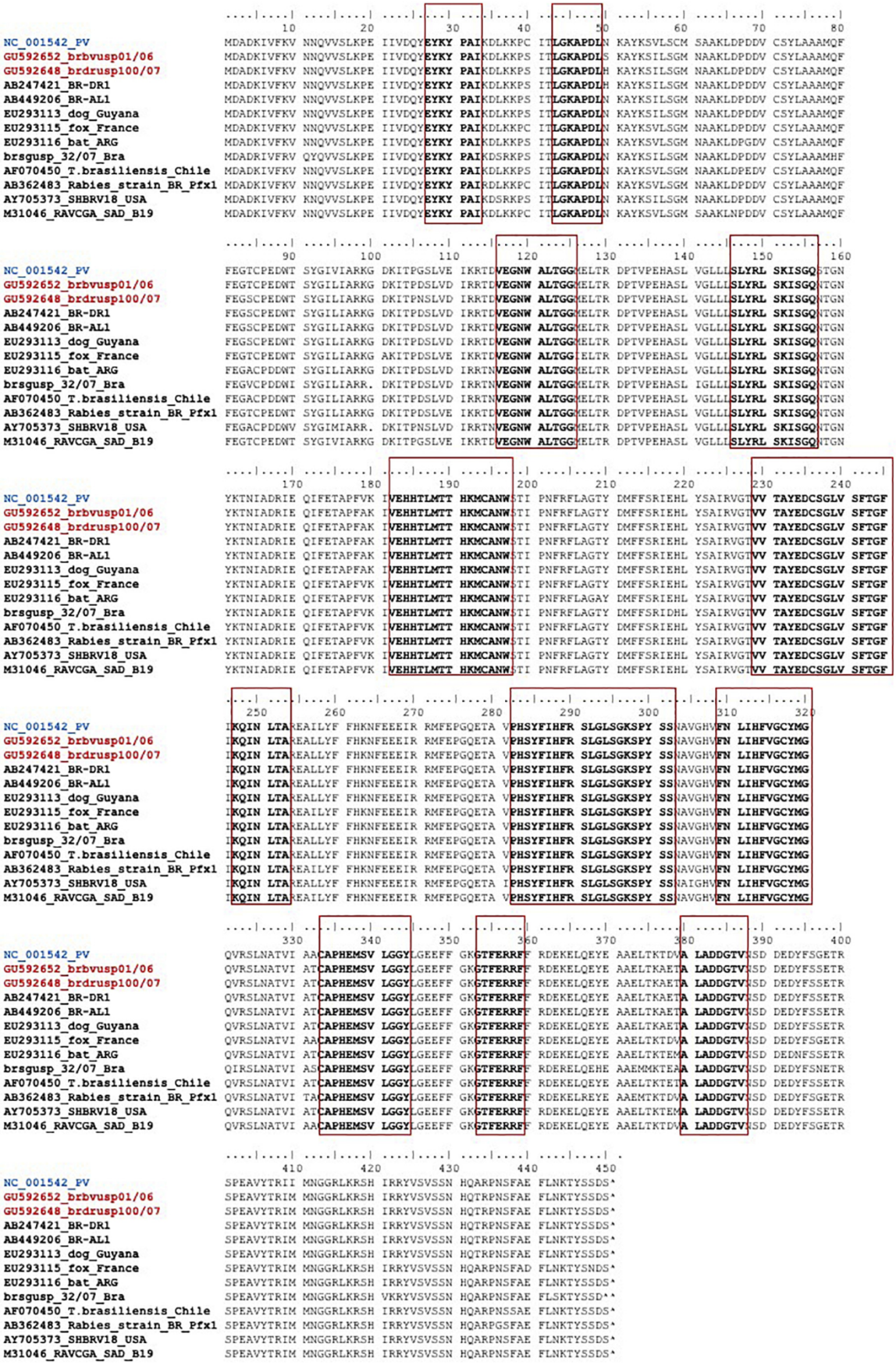



**Fig. 2.** Twelve highly conserved domains in the nucleoprotein gene with lengths vary from seven to 21 residues.


**Fig. 3.** Amino acid change in Glycoprotein antigenic sites IIb, IIa and III between Rabies virus standard samples and transmission cycle representatives: PV (NC_001542), brbvusp01/06 (GU592652) and brdrusp100/07 (bovine and Brazilian *Desmodus rotundus* bat), EU293113 (Guyana dog), EU293115 (France fox), EU293116 (insectivorous bat *Tadarida brasiliensis* from Argentine) and brsgusp32/07 (Brazilian marmoset *Callithrix jacchus*).

nucleotide sequences among different RABV isolates. This diversity has been demonstrated by studies conducted in several regions, including Canada (Nadin-Davis et al., 1994; Nadin-Davis et al., 1993), France (Sacramento et al., 1991), Venezuela (de Mattos et al., 1996), Mexico (De Mattos et al., 1999; Loza-Rubio et al., 1999; Velasco-Villa et al., 2002), Chile (Yung et al., 2002), Bolivia (Favi et al., 2003), Argentina (Cisterna et al., 2005), and Brazil (Carnieli et al., 2009a; Carnieli et al., 2009b; Carnieli et al., 2008; Carnieli et al., 2006; Favoretto et al., 2006; Favoretto et al., 2001; Kobayashi et al., 2005) as well as in Africa, Asia, Europe, and the Americas (Markotter et al., 2008; McElhinney et al., 2008; Singer et al., 2009; Velasco-villa et al., 2017).

Genetic characterization was performed using the nucleoprotein gene (Fig. 1 and Supplementary Figure S1), an abundant protein that is the most synthetized and conserved among all RABV proteins (Banerjee, 1987). The Nucleoprotein is 450aa in length with 21 amino acid substitutions when compared with the PV reference sample (NC_001542). This confirms that the nucleoprotein is the most conserved protein in RABV (Marston et al., 2007). Ten of the amino acid substitutions (47.6%) were non-conservative substitutions. N gene amplification enables reliable identification of the lyssavirus species based on nucleotide sequences (Sacramento et al., 1991) and has been studied in all *Lyssavirus* viral species. These data are available in GenBank and permit a large number of genetic diversity studies of the RABV circulating in major countries (Badrane and Tordo, 2001; Bourhy et al., 1993; Kissi et al., 1995). In our analysis of the antigenic site I, at the carboxy-terminal region of the nucleoprotein (residues 374–383), amino acid substitution, exclusive to the *D. rotundus* genetic lineage was identified in residues 377 to 379. This demonstrates that vampire bats have a distinct lineage of RABV and suggests viral immunological strategies to infect host cells.

The phosphoprotein (see Figure S2) has previously been described in the PV reference sample (Tordo et al., 1986a; Noel Tordo et al., 1986b) and other samples from GenBank (Kobayashi et al., 2007; Nadin-Davis et al., 1997). The initial portion of the sequence was comprised of the methionine start codon and residue 46 was highly conserved, as have been observed previously by Kobayashi et al. (Kobayashi et al., 2007). However, in the *D. rotundus* genetic lineage two specific amino acid residues asparagine and glutamic acid (29 and 42) were changed to alanine and aspartic acid respectively.

The lysine-rich domain FSKKYKF was described by Kobayashi et al. (Kobayashi et al., 2007) and is an important binding component of the C-terminal N protein region were conserved in the *D. rotundus* genetic lineage. The alanine residue at 145 was conserved within the bat-associated RABV transmission cycle and the serine residue was conserved within the viruses from the terrestrial mammal RABV transmission cycle, as also observed by Kobayashi et al. (Kobayashi et al., 2007). In this study, we observed that the methionine (residue 20) and serine (residues 210 and 271) residues were conserved in the phospho-acceptor target described by Nadin-Davis et al. (Nadin-Davis et al., 2002). The residues $Ser_{68}$ and $Gly_{112}$ were identified as amino acid substitutions characteristic of the *D. rotundus* genetic lineage (genetic signature). Whereas the residues $Ala_{136}$, $Val_{153}$, $Lys_{158}$, and $Val_{178}$ were detected in more samples belonging to the *D. rotundus* clade. Exceptions include samples AF369359 (*T. brasiliensis*, all residues), AF369362 (*D. rotundus*, site 158), and AF369363 (*D. rotundus*, site 178), which had different residues at these sites. The domain MQDDC between residues 287 and 291 was conserved in all samples. The phosphoprotein plays a role in viral replication activity by interacting with viral polymerase (Nadin-Davis et al., 1997); therefore, major attention to the variable region uncovered here is warranted.

The M protein interacts directly with the cytoplasmic region of the glycoprotein. It is associated with cytotoxicity of RABV in BHK-21 cells infected with vaccine lineages (Mita et al., 2008). The M protein is related to viral transcription (Nadin-Davis et al., 1997) and with the

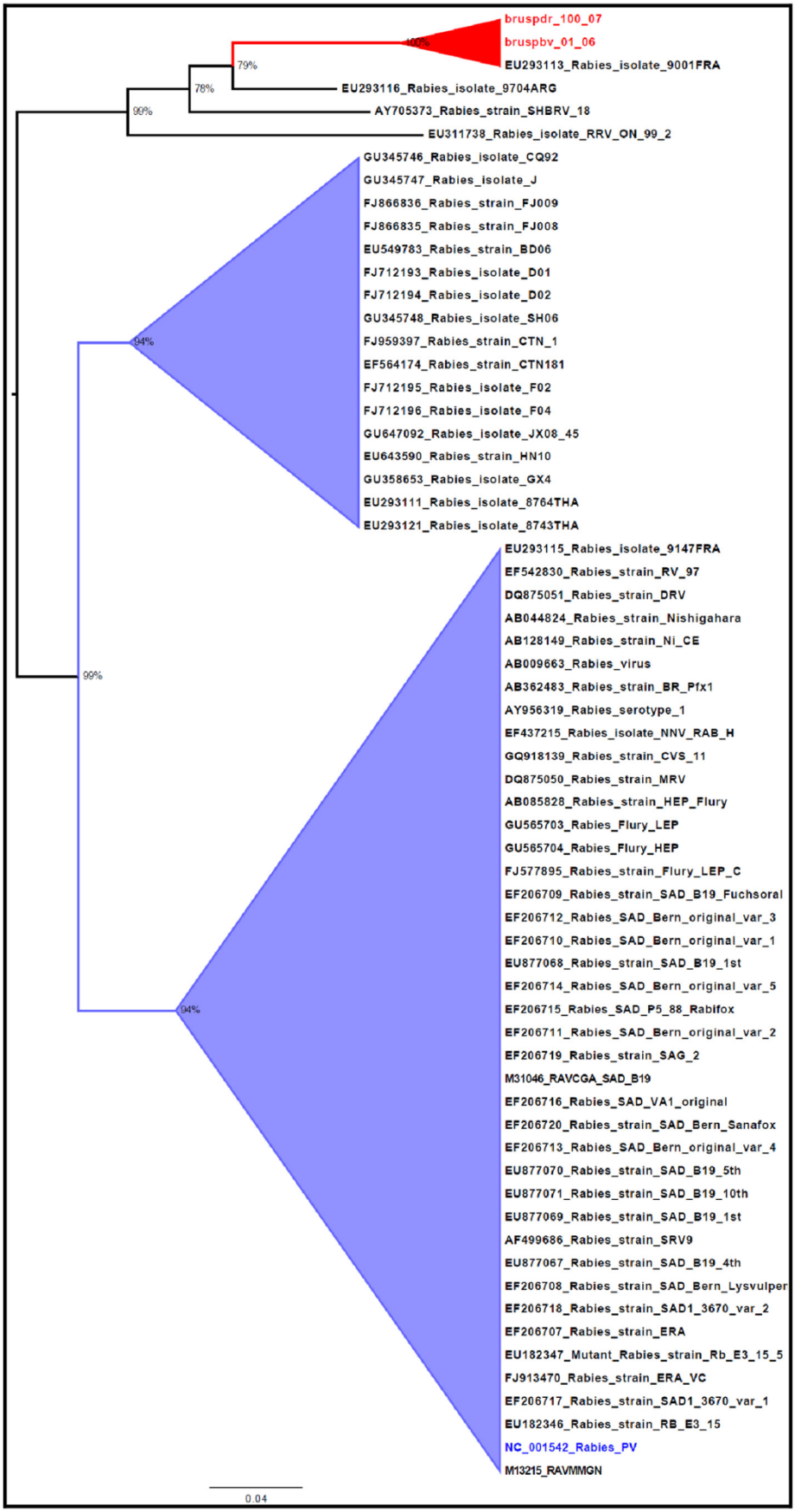



**Fig. 4.** Partial large protein maximum likelihood phylogenetic tree. Genetic analysis of Fragment 2a region from bat sample (brdrusp100/07). Tree reconstructed using a GARLi heuristic search and "GTR + gamma+I" algorithm. Bootstrap (10,000 *bootstrap* replicates) values greater than 50% are depicted in the figure.

glycoprotein associated apoptosis (Faber et al., 2002). When compared with the PV reference sample, four conservative substitutions (residues 82, 148, 184, and 192) were detected only in the clade related to *D. rotundus*. The residues $Leu_{82}$, $Ala_{148}$, $Leu_{184}$, and $Asp_{192}$ suggest a genetic signature of the *D. rotundus* lineage. The amino acid substitutions found at positions 7 (Met), 22 (Ile), 80 (Asn), 82 (Leu), 148 (Ala), 184 (Leu), and 192 (Asp) were already described by Kobayashi et al. (Kobayashi et al., 2007). The residue $Met_7$ was detected in all samples from the hematophagous bat lineage clade and in only one vaccine sample (EF542830, terrestrial cycle). The residue $Asn_{80}$ was identified in the *D. rotundus* genetic lineage and in only two samples (DQ875050_MRV and GQ918139_CVS-11, terrestrial cycle). Despite the residue $Ile_{22}$ being observed in EU293116 (insectivorous bat *T. brasiliensis*) and in some samples related to the *D. rotundus* lineage, the studied sample brdrusp100/07 did not contain this substitution. The residue $Gln_{87}$ was identified only in the bat-associated transmission cycles.

Four domains, DLWLPPPEY (residues 30–38), PNGYSF (residues 64–69), NWVYKLRRT (residues 111–119) and IFQWADSRGPLEGEELE (residues 121–137), in the M protein were highly conserved in all samples. However, residue 126 of the isolate had an aspartic acid substitution, which was present in the conserved domain of our Brazilian bovid sample (brbvusp01/06). The last two domains were within the binding segment of the glycoprotein (residues 115–151). In this study, a proline-rich domain (PPPxY) between residues 34 and 38 was detected. This domain is involved in the budding site, was described by Harty et al. (Harty et al., 2001; Harty et al., 1999) and Kobayashi et al. (Kobayashi et al., 2007). Shimizu et al. (Shimizu et al., 2007) and Mita et al. (Mita et al., 2008) described two positions, residue 29 (Asp) and 95 (Val), in vaccine lineages; however, the wild samples in this study did not have this substitution, and it may be of interest to segregate wild and vaccine lineages.

The matrix protein hydrophobic domain, between residues 89 and 107 (MIGLVKVVV), was highly conserved in the bat-associated transmission cycle RABV variant. According to Tordo et al. (Tordo et al., 1986a; Noél Tordo et al., 1986b), this site is probably responsible for virus-host cell membrane interaction. Despite the important role of the phosphoprotein and matrix protein in RABV, these proteins are underestimated and poorly studied (Nadin-Davis et al., 1997), likely because the three antigenic sites of the virus occurred in nucleoprotein and glycoprotein.

The glycoprotein has a 19-aa signal peptide as described by Tordo et al. (Tordo et al., 1986a; Noel Tordo et al., 1986b) and Sato et al. (Sato et al., 2004). This signal peptide has high variability, likewise, the trans-membrane domain of the glycoprotein, is quite diverse varying between residues 440 and 461. In the samples isolated from *D. rotundus,* substitutions as large as 82 amino acid substitutions were detected, of which 54.9% (45 substitutions) were non-conservative. The antigenic sites, II discontinuous (34–42 and 198–200) and III continuous (330–338), were conserved and one amino acid substitution detected (antigenic site IIa), as also observed by Sato et al. (Sato et al., 2004). Dietzschold et al. (Dietzschold et al., 1983) and Seif et al. (Seif et al., 1985) described that one unique arginine substitution in residue 333, inside antigenic site III, determines the pathogenic rate of RABV. This was confirmed by Faber et al. (Faber et al., 2005), who determined that an $Arg_{333}$ substitution occurred more frequently in laboratory lineages than in wild samples (Faber et al., 2007). In this study, the arginine was conserved confirming the observations of Sato et al. (Sato et al., 2004), contrasting to those of another study by Sato et al. (Sato et al., 2009), in which the same substitution was observed in samples isolates from non-hematophagous bats.

Four conserved domains in the glycoprotein GFTCTGVVTEA (residues 58–68), TYTNFVGYVTTTF (residues 70–82), LRKLVPGFGKAYTI (residues 304–317), and VLIPEMQSSLL (residues 371–381), were detected in this study. $Lys_{330}$ described by Coulon et al. (Coulon et al., 1998) allows the virus to bind to neurons, and two of three glycosylation sites in residues 37 to 39 (Asn-X-Ser residues 56–58 if the signal peptide was considered) and residues 319–321 (Asn-X-Thr residues 338–340 if the signal peptide was considered), described by Anilionis et al. (Anilionis et al., 1981), were conserved in the brdrusp100/07 sample, as in the previous study by Mochizuki et al. (Mochizuki et al., 2011). At one glycosylation site, residues 247–249 (Asn-X-Thr residues 266–268 if a signal peptide was considered), one conserved (Glu267Asp) and one non-conserved (Asn266Asp) amino acid substitution was observed, in addition to one conservative substitution in the lipidation site at residue 479.

The GL intergenic noncoding region previously called pseudogene Ψ, appears similar to the unstable region described by Wu et al. (Wu et al., 2007). Tordo et al. (Tordo et al., 1986a; Noél Tordo et al., 1986b) showed this region to be extremely important to viral infection and it can act to efficiently reduce polymerase transcription, which can reduce viral expression and increase pathogenicity (Marston et al., 2007).

The number of nucleotide mutations in the polymerase gene is expected to be low, since the viral polymerase is a multifunctional protein and the most important protein in viral replication and transcription. Partial fragment analysis was performed and one distinct segregation profile between the bat-associated and terrestrial RABV transmission cycles was observed. The largest sequences available in GenBank, such as those from the study of Ming et al. (Ming et al., 2009), came from either vaccine lineages or from sequences that were clustered with samples related to the terrestrial RABV transmission cycle (Beckert et al., 2009; Nagaraja et al., 2008). A majority of the previously conducted phylogenetic studies performed on the L gene were not capable of identifying the genetic lineage due to the limited number of sequences available in GenBank. Wu et al. (Wu et al., 2007) recommends that the L gene is the best model for a molecular clock analysis due the high level of conservation. In this study, as well as that of Wu et al. (Wu et al., 2007), genealogic analysis of each RABV gene presented the same topology as that of the N gene.

According to Poch et al. (Poch et al., 1990), the polymerase gene of RABV has six conserved blocks when compared with other viruses of the *Mononegavirales* order. Blocks II and III present more functional domains. Block I is between residues 232 and 423 of the PV reference sample, and three residues have been described as strictly or conservatively maintained (Poch et al., 1990). The first and second domains, GNSGYEVIKILEPYVVNSLVQ (residues 291–311) and VYGCYRHWGHPYID (residues 364–377) respectively, could not be sequenced in this study. The third domain represents the functional GHP region, residues 373–375, where the histidine is conformationally exposed. In this study, block II (RNA template ligation, comprising residues 504 and 607) was not sequenced.

In block III (residues 608–831) Poch et al. (Poch et al., 1990) and Schnell and Conzelmann (Schnell and Conzelmann, 1995) described four major motifs (A–D). The amino acid sequence in Motif A (AFHLDYEKWNNHQ; residues 614–626) was 100% conserved (fragment 2a). Motif B (TCWNGQDGGLEGLRQKGWSLVSLLMID; residues 686–712) had one non-conservative substitution from valine to glycine at residue 706. Motif C (VLAQGDNQVL; residues 725–734), which includes the pentapeptide QGDNQ (727–731) essential to L gene expression, was 100% conserved in all bat sequences analyzed. Motif D (FRGNILVPESKRW; residues 797–809) was 100% conserved in all samples.

The role of proline-rich block IV (residues 889–1060), is not yet completely understood, but may involves nucleotide ligation (Marston et al., 2007; Poch et al., 1990). This block was not sequenced from bat sample analyzed.

Block V (between residues 1090 and 1327) includes six domains (Poch et al., 1990). The first three domains, PHPSEML (residues 1116–1122), GYLGS (residues 1164–1168), and DFPLEE (residues 1224–1229) were 100% conserved in all sequences analyzed. The domain VKRAL, residues 1189–1193, was conserved in the bat *D. rotundus* lineage. The domains DFMFQ (1283–1287) and FHWHL (1311–1315) were not sequenced as part of study. According to Marston et al.

(Marston et al., 2007), block V presents a great number of Cys and His invariant residues, which was confirmed by this study.

Block VI (1705–1710) consists of the less conserved GxGxG motif. This block is preceded upstream by Lys 19–22 residues that have a role in polyadenylation or protein kinase activity (Poch et al., 1990) as well as a highly conserved amino-terminal region (56–61) that was identified in the *D. rotundus* samples. These domains of 4–7 residues are highly conserved in block VI. At residue 8, comparison of the two samples (brbvusp01/06 and EU293113) demonstrates that *D. rotundus* lineage has one change from Tyr (PV-NC_001542) to Phe, as well as conservative substitutions in another 13 residues ($Glu_{139}$, $Lys_{217}$, $Gly_{683}$, $Thr_{1092}$, $Arg_{1139}$, $Ala_{1255}$, $Val_{1617}$, $Cys_{1619}$, $Val_{1852}$, $Ala_{1889}$, $Ala_{2057}$, $Ala_{2058}$, and $Arg_{2089}$) and three conservative substitutions ($Arg_{645}$, $Val_{1214}$, and $Asp_{1336}$). One change from Val to Ile at residue 27 was observed only in samples related to the bat-associated RABV transmission cycle. The residues 24, 61, 122, 170, 1144, 1342, 1374, 1487, 1556, 1653, 1665, and 2101 are related to the bat-associated RABV transmission cycle and the residues 21, 88, and 107 were common to genetic lineages related to *D. rotundus* and *T. brasiliensis*. These findings suggest there is one genetic signature of the *D. rotundus* genetic lineage.

Ming et al. (Ming et al., 2009) described important residues, including $Cys_{70}$, $Val_{84}$, $Ala_{94}$, $Ile_{118}$, $Ser_{153}$, and $Asp_{191}$. Analyzing these residues in the *D. rotundus* genetic lineage we detected one unique amino acid substitution at residue 94 relative to the PV reference sample. Our sample did not contain two consecutive start codons (double Met) as were present in other sequences available in dataBank. The presence of a double Met was described and associated with the American bat genetic lineage by Mochizuki et al. (Mochizuki et al., 2011), but this observation could not be confirmed since samples that clustered with the terrestrial cycle of transmission also present the double Met (EU311738 - Canadian raccoon; FJ712193–5, GU345746, GU345748, GU358653 - dogs; FJ712196, FJ866835, FJ866836 - ferret; EF564174, EU643590, GU345747 - human; and EU549783, FJ959397 - vaccine lineage).

Despite the availability of complete sequences of four viral protein genes and the successful L gene amplification by PCR, sequencing of the polymerase could not be completed. Most studies of viral polymerases available date back 20 to 40 years (Mellon and Emerson, 1978; Poch et al., 1990) and were not specifically focused on RABV (Hamaguchi et al., 1983; Horikami et al., 1992). Recently, some groups have sequenced the polymerase as part of a study to complete the RABV genome (Beckert et al., 2009; Delmas et al., 2008; Ming et al., 2009; Mochizuki et al., 2011; Mochizuki et al., 2009; Nagaraja et al., 2008).

Studies on the conservation, viral evolution, and working mechanisms of the viral polymerase are needed. Streicker et al. (Streicker et al., 2012) showed that the L gene was also implicated in the establishment of new RABV reservoirs in addition to the G gene. Delmas et al. (Delmas et al., 2008) discuss genetic classification based on the N gene alone, as suggested by Kissi et al. (Kissi et al., 1995). Davis et al. (Davis et al., 2006) discuss the dynamics, evolutionary history and origin of RABV in bats on the American continent. Some researchers have identified RABV in non-hematophagous bats prior to rabies epizootics in livestock and pets, suggesting that non-hematophagous bats may be the link between wildlife rabies and urban rabies (Badrane and Tordo, 2001). The detection of the rabies variant maintained by vampire bats *D. rotundus* in dogs and cats demonstrates the bat-associated RABV transmission cycle is not limited to wildlife hosts. In recent years, the epidemiologic profile of rabies in Brazil and Latin America has changed (Queiroz et al., 2012), and this situation is characteristic of regions where rabies maintained by dog populations is controlled, highlighting the existence of lineages maintained by wild animals, mainly bats (Calisher et al., 2006). According to Streicker et al. (Streicker et al., 2012) the number of positively selected changes in the L gene can be the best predictor of the speed of viral emergence of RABV in new hosts, such as bats. Bats are capable to transmit or, in some cases, be the reservoir of this virus and other viruses (Calisher et al., 2006; Shi, 2010), making prophylaxis and epidemiological surveillance essential part of disease control and prevention of further rabies outbreaks.

## 5. Conclusions

The molecular characterization and phylogenetic study of the *D. rotundus* genetic virus lineages, indicate that the bat-associated transmission cycle of differs significantly from lineages detected in the terrestrial transmission cycle, maintained and transmitted by dogs and other terrestrial mammals. Amino acid analysis found in *D. rotundus* lineage indicates that determined residues in the viral polymerase gene can represent genetic signatures of the RABV lineage maintained and transmitted by the common vampire bat *D. rotundus* and could be used to increase the vaccine coverage.

The difference ratio observed in the aminoacid genetic sequences identified in the common vampire bat lineage, when compared with terrestrial lineages, follows the descending order of protein genes: P (phosphoprotein) > G (glycoprotein) > L (L-polymerase) > M (Matrix protein) > N (nucleoprotein), breaking the paradigm of that polymerase needs be a most conserved viral protein..

The large number of substitutions, in particular non-synonymous and non-conservative changes, hint at the dynamics of evolutionary adaptation of the *D. rotundus* genetic lineage RABV and highlights the importance of investigating viral genetic diversity and surveillance. This found demonstrates that vampire bats have a distinct lineage of RABV and suggests viral immunological strategies to infect host cells.

The present study improves the understanding of RABV diversity maintained by vampire bats. Furthermore, data presented here could use to conduct future pathogenicity studies, compare assumed vaccine protection against the lineage described and improve development strategies for rabies virus control.

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## Declaration of Competing Interest

The authors have no conflicts of interest to declare and approve this document.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2020.104179.

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