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Genetic diversity of bat rabies viruses in Brazil

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Summary

Thirty-three Brazilian bat rabies viruses (RVs) were studied by sequence analysis and were compared against sequences of bat-related RVs from other regions of the Americas. Phylogenetic analysis revealed that bat-related RVs formed several monophyletic lineages and that these were associated with bat species. Brazilian bat RVs were found to include nine major lineages, one of which grouped with RVs isolated from *Lasiurus* spp. from different regions of the Americas. These results suggest that there is considerable diversity among Brazilian bat RV variants and that some of these RV variants may be associated with bats from other countries.

Introduction

Rabies virus (RV), which belongs to genotype 1 of the genus *Lyssavirus* within the family *Rhabdoviridae*, causes lethal neurological diseases in mammals. Bats inhabit most regions of the world and are known to frequently act as lyssavirus vectors [1, 17]. The RVs associated with bats are widespread in the Americas, and it is believed that genetic differentiation in RVs occurred in response to their association with particular host species [19]. In Brazil, the transmission of rabies by vampire bats (*Desmodus rotundus*), a known principal RV transmitter, has caused serious problems in the public health sector and livestock industry [4, 16]. In addition, several RV variants have been isolated from both insectivorous and frugivorous bats in the country [3, 12, 23]. While no cases of human rabies associated with non-hematophagous bats have been reported in Brazil to date, most of the human rabies cases in the USA are associated with insectivorous bats [18, 22], which suggests that non-hematophagous bats in Brazil have a similar associated risk potential for latent rabies transmission.

The authors previously reported that Brazilian bat RVs had been considered to be divided into four distinct genetic RV-variant lineages, and that these were associated with *Eptesicus* spp., *Nyctinomops*

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Table 1. Brazilian bat rabies virus isolates using in this study

Sample name	Host species	Isolated State[c]	Isolated city	Year	Accession no.
BR-A1[a]	*Artibeus* sp.	RJ	Paracambi	2002	AB297627
BR-A2[a]	*Artibeus* sp.	RJ	Mesquita	2004	AB297628
BR-AF1[a]	*Artibeus fimbriatus*	RJ	Rio de Janeiro	2002	AB297629
BR-AL1	*Artibeus lituratus*	SP	Itapira	1998	AB117969
BR-AL2	*Artibeus lituratus*	SP	São José do Rio Preto	1998	AB117970
BR-AL3	*Artibeus lituratus*	SP	Novo Horizonte	1998	AB117971
BR-AL4	*Artibeus lituratus*	SP	Dracena	2002	AB201802
BR-AL6[a]	*Artibeus lituratus*	RJ	Rio de Janeiro	2001	AB297630
BR-AL7[a]	*Artibeus lituratus*	SP	Vargem Grande Paulista	2004	AB297631
BR-AP1	*Artibeus planirostris*	SP	São José do Rio Preto	1998	AB117972
BRvmbt33	*Desmodus rotundus*	SP	Taubate	Unknown[d]	AB083806
BRvmbt34	*Desmodus rotundus*	SP	Pindamonhangaba	1998	AB083807
BR-DR1	*Desmodus rotundus*	SP	Lindóia	2000	AB201803
BR-DR2	*Desmodus rotundus*	SP	Lindóia	2000	AB201804
BR-DR3	*Desmodus rotundus*	SP	São José do Barreiro	2001	AB201805
BR-DR5[a]	*Desmodus rotundus*	RJ	Valença	2002	AB297632
BR-DR6[a]	*Desmodus rotundus*	RJ	Laje do Muriae	1998	AB297633
BR-DR7[a]	*Desmodus rotundus*	RJ	Itaperuna	1997	AB297634
BR-DR8[a]	*Desmodus rotundus*	SP	Tambaú	2003	AB297635
BR-DR9[a]	*Desmodus rotundus*	SP	Guarulhos	2000	AB297636
BR-DR10[a]	*Desmodus rotundus*	GO	Cocalzinho de Goiás	2005	AB297637
BR-DR11[a]	*Desmodus rotundus*	GO	Cocalzinho de Goiás	2005	AB297638
BR-DR12[a]	*Desmodus rotundus*	GO	Cocalzinho de Goiás	2005	AB297639
BR-DR13[a]	*Desmodus rotundus*	GO	Guapó	2005	AB297640
BR-DR14[a]	*Desmodus rotundus*	GO	Uruaçú	2005	AB297641
BR-DR16[a]	*Desmodus rotundus*	GO	Niquelândia	2005	AB297642
BR-DR18[a]	*Desmodus rotundus*	GO	Uruaçú	2006	AB297643
BR-DR19[a]	*Desmodus rotundus*	GO	Nova Iguaçú de Goiás	2006	AB297644
BR-DR20[a]	*Desmodus rotundus*	GO	Cocalzinho de Goiás	2006	AB297645
BR-DR21[a]	*Desmodus rotundus*	GO	Cocalzinho de Goiás	2006	AB297646
BR-EF1	*Eptesicus furinalis*	SP	São José do Rio Preto	1998	AB201811
BR-EF2	*Eptesicus furinalis*	SP	Olímpia	2001	AB201812
BR-EF3	*Eptesicus furinalis*	SP	São José do Rio Preto	2001	AB201813
BR-EF4	*Eptesicus furinalis*	SP	Catanduva	2002	AB201814
BR-Pbt1	*Molossus molossus*	PB	Patos	2003	AB206414
BR-Pbt2	*Molossus molossus*	PB	Patos	2003	AB206415
BR-Pbt3	*Molossus molossus*	PB	Patos	2003	AB206416
BR-Pbt4	*Molossus molossus*	PB	Patos	2003	AB206417
BR-MM1	*Molossus molossus*	SP	Jales	1999	AB201815
BR-MM2	*Molossus molossus*	SP	Ilha Solteira	2002	AB201816
BR-NL1	*Nyctinomops laticaudatus*	SP	São José do Rio Preto	1998	AB201806
BR-NL2	*Nyctinomops laticaudatus*	SP	São José do Rio Preto	1999	AB201807
BR-NL3	*Nyctinomops laticaudatus*	SP	Ipiguá	2001	AB201808
BR-NL4[a]	*Nyctinomops laticaudatus*	RJ	Rio de Janeiro	2004	AB297647
BR-TL1[a]	*Tadarida laticaudata*	RJ	Rio de Janeiro	1990	AB297648
BR-TL2[a]	*Tadarida laticaudata*	RJ	Rio de Janeiro	1990	AB297649
BR-BAT3[a]	Non-hematophagous bat[b]	SP	Campinas	2004	AB297650
BR-BAT13[a]	Non-hematophagous bat[b]	SP	Presidente Prudente	2003	AB297651
BR-BAT15[a]	Non-hematophagous bat[b]	SP	Osvaldo Cruz	2003	AB297652
BR-BAT16[a]	Non-hematophagous bat[b]	SP	Álvares Machado	2006	AB297653

(*continued*)

Table 1 (*continued*)

Sample name	Host species	Isolated State[c]	Isolated city	Year	Accession no.
BR-BAT22[a]	Non-hematophagous bat[b]	SP	Presidente Prudente	2004	AB297654
BR-BAT26[a]	Non-hematophagous bat[b]	SP	Panorama	2005	AB297655
BR-BAT27[a]	Non-hematophagous bat[b]	SP	Presidente Prudente	2006	AB297656
BR-BAT28[a]	Non-hematophagous bat[b]	SP	Osvaldo Cruz	2005	AB297657
BR-BAT29[a]	Non-hematophagous bat[b]	SP	Presidente Prudente	2004	AB297658
BR-BAT31[a]	Non-hematophagous bat[b]	SP	Dracena	2004	AB297659

[a] The samples analyzed in this study.
[b] These bats were unclassified.
[c] State abbreviations are as follows: *GO* Gioás state; *PB* Paraíba state; *RJ* Rio de Janeiro state; *SP* São Paulo state.
[d] No information is avaliable from a reliable source.

spp., *Molossus* spp. and *D. rotundus* [12]. The present study corroborates these findings and provides new evidence regarding various epidemiological characteristics of bat rabies in Brazil.

Materials and methods

The 33 RVs used in the study were obtained from vampire bats (*D. rotundus*, n = 15), frugivorous bats (*Artibeus* spp., $n = 5$), insectivorous bats (*Nyctinomops laticaudatus*, $n = 1$; *Tadarida laticaudata*, $n = 2$) and 10 unclassified non-hematophagous bats (Table 1). Samples were diagnosed as rabies positive by the immunofluorescence antibody assay and mouse inoculation test [5, 14]. In addition, 23 RVs were collected from vampire bats ($n = 5$), frugivorous bats ($n = 5$), and insectivorous bats (*Eptesicus furinalis*, $n = 4$; *Molossus molossus*, $n = 6$; *N. laticaudatus*, $n = 3$) as reported previously [11, 12, 23, 24]. The extraction and purification of viral RNA, RT-PCR and sequencing were performed as described previously [12]. In addition, the BRABN-C4 primer (5′-ATGTTTGT(C/T)TTGTAATTGCC-3′: 564–545), BRABN-C5 primer (5′-GGGTT(C/T)ATAAAGCA(A/G)ATAAA-3′: 800–819) and BRABN-S7 primer (5′-TTATGAGGA(C/T)TGCTCAGGG-3′: 766–784) were designed and used for sequencing. Nucleotide sequences were determined for 1394 nt of the nucleoprotein (N) gene, corresponding to positions 89–1482 of the PV strain. Multiple alignments were performed using Clustal W [25]. Phylogenetic analyses using distance matrix (neighbor joining) were conducted using MEGA version 3.1 [15]. For neighbor joining, corrected nucleotide substitutions were calculated using the Kimura two-parameter model. The statistical significance of the phylogenies constructed was estimated by bootstrap value analysis with 1000 pseudoreplicate data sets. The Mokola virus (Mokola) (Accession No. Y09762) and the Australian bat lyssavirus (ABL) (Accession No. AF418014) were used as outgroups. Detailed information on bat-related RVs isolated from other regions is presented in Table 2. The TREEVIEW program was used to present the graphical output [20], and sequence identities of the nucleotides and deduced amino acids were calculated using BioEdit software [10].

Results

The phylogenetic tree based on 1394 nt of the N gene revealed that the Brazilian bat RV isolates could be separated into nine distinct genetic lineages with high bootstrap values (Fig. 1). Lineages IV, V, VI, VIII and IX were identified as new RV variants in Brazil, but the vector species of the virus were uncertain. Lineage I consisted mainly of *D. rotundus* and *Artibeus* spp. and was further divided into the two sub-lineages Ia and Ib. Sub-lineage Ia consisted of RV isolates from the states of São Paulo and the neighboring state of Rio de Janeiro, while sub-lineage Ib consisted of RV isolates from the state of Goiás. All RV isolates from *Artibeus* spp. belonged to sub-lineage Ia. Lineages II and VII primarily consisted of RV isolates from *M. molossus* and *E. furinalis*, respectively. Lineage III consisted of RV isolates from *N. laticaudatus*, *T. laticaudata* and *M. molossus* from the states of Paraíba, Rio de Janeiro and São Paulo, respectively (Fig. 2). The sequence identities of nucleotides and deduced amino acids among the Brazilian bat RV isolates were greater than 84.3% and 93.5%, respectively.

The phylogenetic tree based on the 217 nt of the N gene that was used to compare bat-related RVs from Brazil with those from other countries revealed the existence of several monophyletic clusters in the

Table 2. Bat-related rabies virus samples obtained from GenBank

Sample name	Country	Species	Accession no.	References
ARBV1	Argentina	Bovine	AY233446	Cisterna et al. [2]
ARBV2	Argentina	Bovine	AY233444	
ARBV3	Argentina	Bovine	AY233437	
ARBV4	Argentina	Bovine	AY233430	
ARHIM	Argentina	*Histiotus montanus*	AY233448	
ARMY	Argentina	*Myotis* sp.	AY233449	
ARMYN	Argentina	*Myotis nigricans*	AY233450	
ARTB1	Argentina	*Tadarida brasiliensis*	AY233426	
ARTB2	Argentina	*Tadarida brasiliensis*	AY233427	
ARTB3	Argentina	*Tadarida brasiliensis*	AY233428	
USATB1	USA	*Tadarida brasiliensis*	AF396063	Favi et al. [6]
CHLAB	Chile	*Lasiurus borealis*	AF396035	
CHMYC	Chile	*Myotis chiloensis*	AF396064	
BOCT	Bolivia	Feline	AY340783	Favi et al. [7]
BODG1	Bolivia	Canine	AY340782	
BODG2	Bolivia	Canine	AY340771	
USALASN1	USA	*Lasionycteris noctivagans*	DQ445382	Franka et al. [9]
USALASN2	USA	*Lasionycteris noctivagans*	DQ445380	
USAPS1	USA	*Pipistrellus subflavus*	DQ445349	
USAPS2	USA	*Pipistrellus subflavus*	DQ445354	
CAEF1	Canada	*Eptesicus fuscus*	AF351828	Nadin-Davis et al. [19]
CAEF2	Canada	*Eptesicus fuscus*	AF351829	
CAEF3	Canada	*Eptesicus fuscus*	AF351830	
CAEF4	Canada	*Eptesicus fuscus*	AF351831	
CALASN1	Canada	*Lasionycteris noctivagans*	AF351834	
CALASN2	Canada	*Lasionycteris noctivagans*	AF351837	
CALASN3	Canada	*Lasionycteris noctivagans*	AF351840	
CALAB1	Canada	*Lasiurus borealis*	AF351844	
CALAB2	Canada	*Lasiurus borealis*	AF351856	
CALAC1	Canada	*Lasiurus cinereus*	AF351845	
CALAC2	Canada	*Lasiurus cinereus*	AF351846	
CALAI	Canada	*Lasiurus intermedius*	AF351843	
CAMYC	Canada	*Myotis californicus*	AF351836	
CAMYE	Canada	*Myotis evotis*	AF351835	
CAMYL	Canada	*Myotis lucifugus*	AF351838	
TRDR	Trinidad	*Desmodus rotundus*	AF351852	
USALAI	USA	*Lasiurus intermedias*	AF394878	Rohde et al. [21]
USALAC1	USA	*Lasiurus cinereus*	AF394883	
USALAC2	USA	*Lasiurus cinereus*	AF394884	
USALAB1	USA	*Lasiurus borealis*	AF394885	
USALAB2	USA	*Lasiurus borealis*	AF394886	
USAMYC	USA	*Myotis californicus*	AF394871	
USAMYE	USA	*Myotis evotis*	AF394874	
USAMYA	USA	*Myotis austroriparius*	AY039225	
USANCH	USA	*Nycteceius humeralis*	AY208164	
USAPLT1	USA	*Plecotus townsendii*	AF394877	
CHHI1	Chile	*Histiotus* sp.	AF533775	Yung et al. [26]
CHHI2	Chile	*Histiotus* sp.	AF533776	
CHHI3	Chile	*Histiotus* sp.	AF533778	
CHLA1	Chile	*Lasiurus* sp.	AF533782	

(*continued*)

Table 2 (*continued*)

Sample name	Country	Species	Accession no.	References
CHLA2	Chile	*Lasiurus* sp.	AF533810	
CHLA3	Chile	*Lasiurus* sp.	AF533814	
CHMY1	Chile	*Myotis* sp.	AF533813	
CHMY2	Chile	*Myotis* sp.	AF533833	
CHTB1	Chile	*Tadarida brasiliensis*	AF533786	
CHTB2	Chile	*Tadarida brasiliensis*	AF533787	
CHTB3	Chile	*Tadarida brasiliensis*	AF533792	
MELA1	Mexico	*Lasiurus* sp.	DQ416121	Velasco-Villa et al. [27]
MELA2	Mexico	*Lasiurus* sp.	DQ416120	
MELAC1	Mexico	*Lasiurus cinereus*	DQ416119	
MELAC2	Mexico	*Lasiurus cinereus*	DQ416118	
MEDR1	Mexico	*Desmodus rotundus*	DQ416104	
MEDR2	Mexico	*Desmodus rotundus*	DQ416103	
MEDR3	Mexico	*Desmodus rotundus*	DQ416049	
MEBV1	Mexico	Bovine	DQ416078	
MEBV2	Mexico	Bovine	DQ416099	
MEAJ	Mexico	*Artibeus jamaicensis*	DQ416085	
METB1	Mexico	*Tadarida brasiliensis*	DQ416110	
METB2	Mexico	*Tadarida brasiliensis*	DQ416111	
METB3	Mexico	*Tadarida brasiliensis*	DQ416113	
PEHM	Peru	Human	AF045166	Warner et al. [28]
BRLAB	Brazil	*Lasiurus blossevillii*	AY739722	–
USAEF1	USA	*Eptesicus fuscus*	AY039228	–
USAEF2	USA	*Eptesicus fuscus*	AY170278	–
USAEF3	USA	*Eptesicus fuscus*	AY170281	–
USAEF4	USA	*Eptesicus fuscus*	AY170296	–
USANM	USA	*Nyctinomops macrotis*	AY170304	–
USAPH1	USA	*Pipistrellus hesperus*	AY170250	–
USAPH2	USA	*Pipistrellus hesperus*	AY170251	–
USAPH3	USA	*Pipistrellus hesperus*	AY170252	–
USAPLT2	USA	*Plecotus townsendii*	AY170244	–
USATB2	USA	*Tadarida brasiliensis*	AY170231	–
USATB3	USA	*Tadarida brasiliensis*	AY170236	–

bat species examined (Fig. 3). BR-BAT15, 16 and 22, which belonged to lineage IX, formed a monophyletic cluster with RV isolates from *Lasiurus* spp. in Canada, Chile, Mexico and the USA (bootstrap value of 77%). A high nucleotide identity of greater than 99% was also observed among the RV isolates in lineage IX and those from *Lasiurus cinereus* in Canada, Mexico and the USA. With a nucleotide identity of greater than 96%, lineage VIII was closely related to the RV isolate from *Lasiurus blossevillii* in Brazil and shared a common ancestor with clusters containing *Lasiurus* spp. and *Lasionycteris noctivagans* RV isolates (bootstrap value of 80%). Lineage III shared a common ancestor with the RV isolate from *Nyctinomops macrotis* and had a nucleotide identity of greater than 96% (bootstrap value of 89%).

The cluster consisting of vampire bat-related RV isolates was divided into two sub-clusters with a high level of certainty (bootstrap value of 79%). One sub-cluster was comprised of vampire bat-related RV isolates from Brazil, Argentina, Bolivia, Mexico, Peru and Trinidad. The other consisted of RV isolates from Mexican vampire bats and was closely related to RV isolates from *Tadarida brasiliensis* in the USA and Mexico. The RV isolates from Brazilian frugivorous bats did not group with RV isolates from Mexican frugivorous bats.

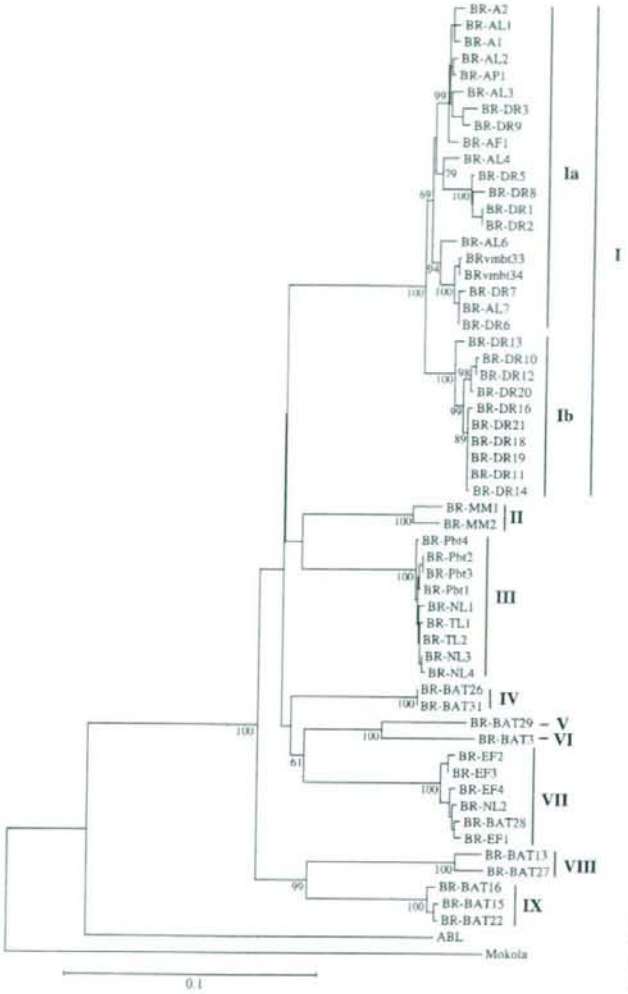

Fig. 1. Phylogenetic tree using NJ method (Kimura two-parameter model) based on 1394 nt of the N gene (89–1482) of Brazilian bat RVs. Bootstrap values above 60% are shown at the branch node. A scale bar indicates the genetic distance represented by the horizontal branches

Discussion

Phylogenetic analysis revealed the existence of nine major bat RV variants in Brazil, with some of these being associated with bat RV isolates from other countries. The long migrations of *Lasiurus* spp. and its wide distribution in the Americas [17] may have contributed to the inclusion of Brazilian bat RV isolates from lineage IX in this study grouping with the RV isolates from *Lasiurus* spp. from other countries. In addition, the Brazilian bat RV isolates were most closely related to RV isolates from *L. cinereus*, which has been implicated in the widespread distribution of the viruses in the Americas, an inference made because of the high levels of genomic sequence identity within *L. cinereus* RV isolates from several countries [27]. These results suggest that *L. cinereus*-associated RV variants are widely distributed in several regions of the Americas, including Brazil.

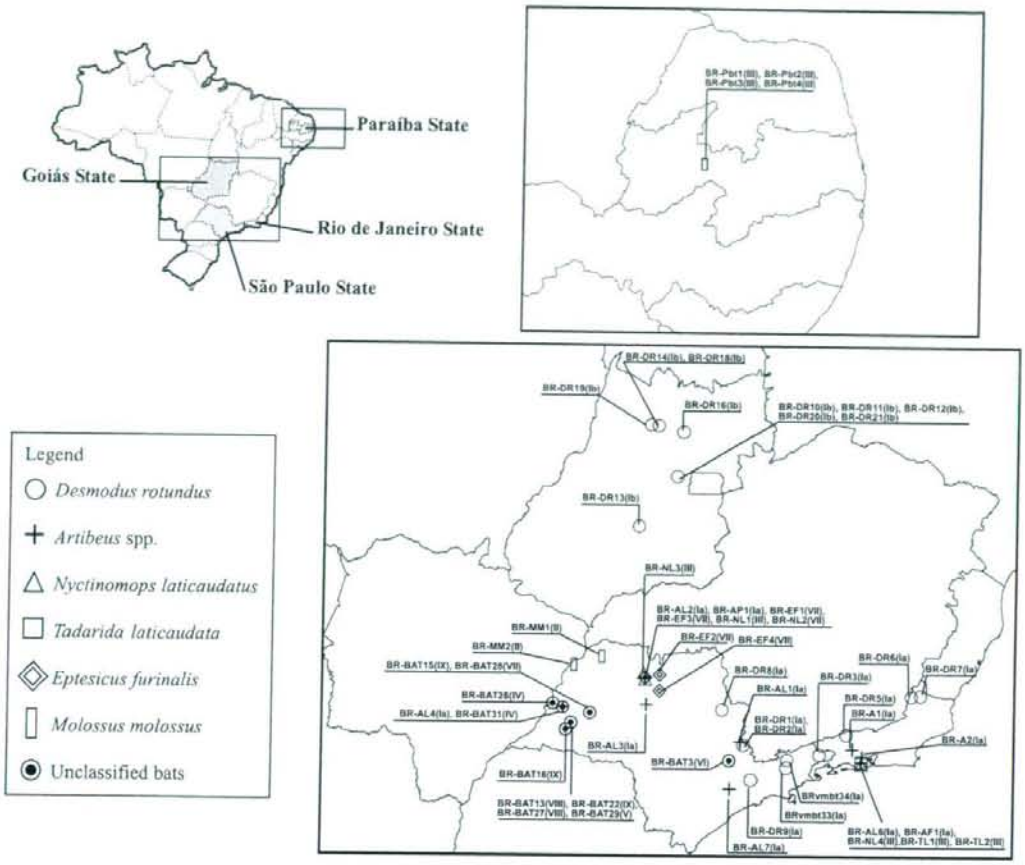

Fig. 2. Geographic distribution of bat RV isolates in Brazil. The symbols illustrate the host bat species. Roman numerals

Lineage III, which was previously characterized as a *Nyctimonops* spp.-related RV variant [12], consisted of RV isolates from *N. laticadatus*, *T. laticaudata* and *M. molossus* from several regions of Brazil. These results indicate that cross-species transmission events of the virus associated with lineage III may have occurred, and that an as yet unidentified host may have carried the viruses into different regions of Brazil. Further analysis is necessary in order to determine which bat species is the rabies transmitter in this lineage.

The distribution of RVs associated with non-migratory bats tends to reflect the geographic distributions of the reservoir bat populations [19]. Natural barriers such as mountain ranges promote the genetic divergence of these viruses, which result in the same pattern being observed in bat-related RVs from vampire bats [13, 27]. In this study, the segregation of sub-lineages Ia and Ib, both of which consisted of RV isolates from vampire bats, corresponded to the geographic distribution of the viruses, suggesting that these viruses may be maintained among geographically distinct vampire bat populations. Rabies transmission events between vampire and frugivorous bats have frequently been reported [12, 23, 27]. In this study, RV isolates from frugiv-

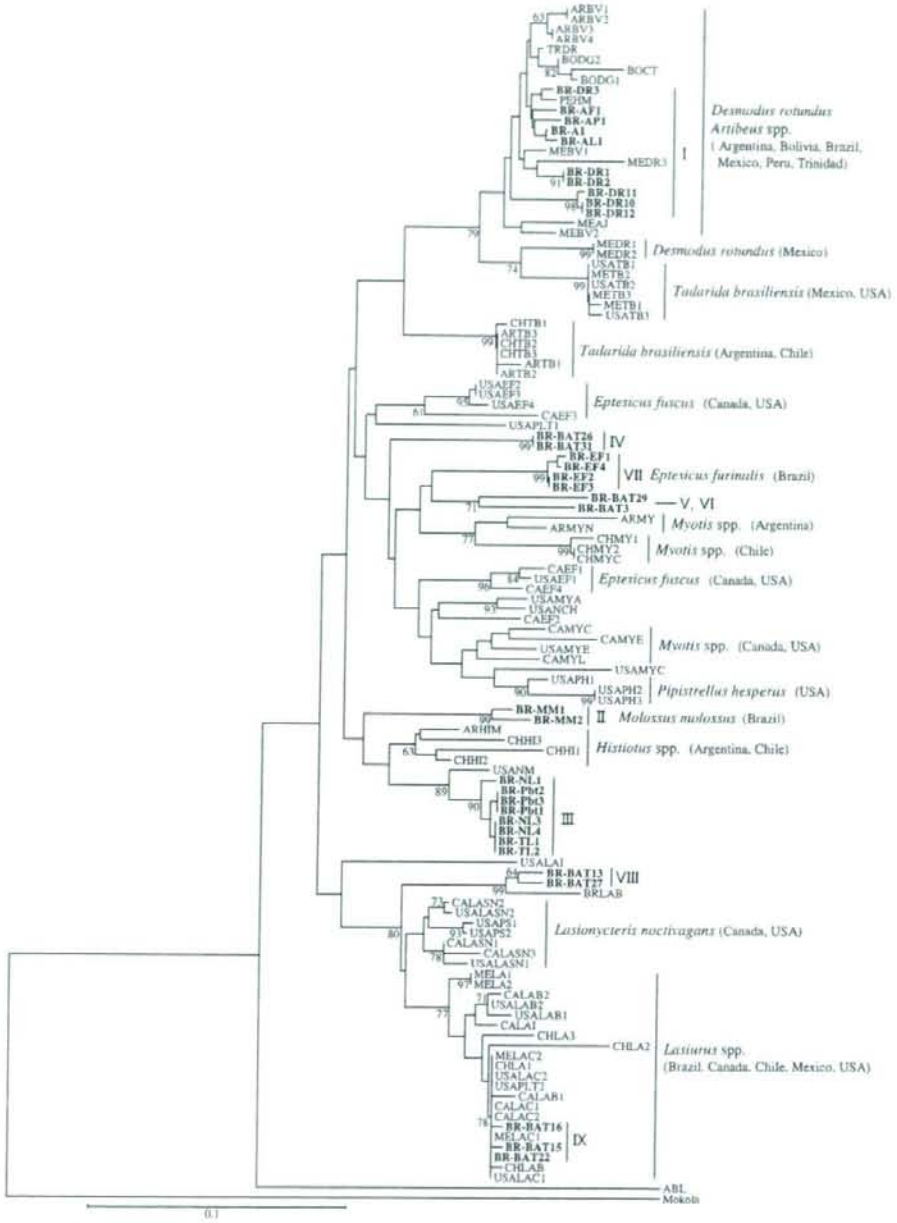

Fig. 3. Phylogenetic tree using NJ method (Kimura two-parameter model) based on 217 nt of the N gene (1177–1393). Bold in font indicates Brazilian bat RV samples analyzed in this study. Bootstrap values above 60% are shown at the branch node. A scale bar indicates the genetic distance represented by the horizontal branches. Roman numerals correspond to the phylogenetic lineages shown in Fig. 1. The geographical origins of bat RV isolates are shown in parentheses

orous bats grouped with RV isolates from vampire bats, and the distributions of these viruses overlapped in the states of São Paulo and Rio de Janeiro. Vampire and frugivorous bats have also been observed to utilize the same roost in Mexico [8]. These findings suggest that RV transmission from vampire bats to frugivorous bats might occur as a result of similarities in the life histories of these bats.

The present study examined the genetic diversity of Brazilian bat RVs and their relationships with RV isolates from other regions of the Americas. These findings showed that RV variants exhibit epidemiological characteristics that are reflected in aspects of the ecology such as migratory patterns and range, of the reservoir bats. Consequently, risk evaluation measures directed at preventing the transmission of rabies, as well as the development of control measures that will benefit public health, need to consider the importance of bat ecology. Additionally, since the transmitters of some bat-related RV variants are not yet known, further epidemiological analyses are necessary in order to elucidate the infection cycles associated with bat rabies.

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FULL PAPER *Virology*

Genetic Analysis of Phosphoprotein and Matrix Protein of Rabies Viruses Isolated in Brazil

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ABSTRACT. To investigate the genetic characteristics of phosphoprotein (P) and matrix protein (M) genes of variable rabies virus (RV) prevalent in Brazil, the authors genetically characterized the P and M genes from 30 Brazilian RV field isolates. Phylogenetic analysis based on the P and M genes revealed the presence of six RV variants that consisted primarily of three insectivorous bats, the vampire bat, dog and fox in Brazil. Specific amino acid substitutions corresponding to these phylogenetic lineages were observed, with Asp_{42} and Glu_{62} in the P protein found to be characteristic of Brazilian chiroptera- and carnivora-related RVs, respectively. Amino acid sequence motifs predicted to associate with a viral function in the P and M proteins were conserved among Brazilian RV variants.
KEY WORDS: Brazil, genetic analysis, matrix protein, phosphoprotein, rabies virus.

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Rabies virus (RV) is a member of the *Lyssavirus* genus, which belongs to the *Rhabdoviridae* family. Lyssavirus is characterized as having seven genotypes (GTs) comprising, rabies virus (GT 1), Lagos bat virus (GT 2), Mokola virus (GT 3), Duvenhage virus (GT 4), European bat lyssavirus type (EBL) 1 (GT 5), EBL 2 (GT 6) and Australian bat lyssavirus (GT 7). Lyssaviruses have approximately 12-kb of unsegmented negative-stranded genomic RNA for encoding the genes of the nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and polymerase (L).

RV has an almost global distribution and infects a wide range of mammalian species in which it causes a lethal form of encephalopathy. In Brazil, the principal RV transmitters to humans and domestic animals - dogs and vampire bats - have caused serious problems in the public health sector and the livestock industry [6, 19]. In addition, RVs have been isolated from other animal species [8]. Previously, phylogenetic analyses targeting the N and G genes revealed that there were several RV variants which varied depending on the host species, which included vampire bats, insectivorous bats, dogs and foxes. Furthermore, RV isolates from cattle and frugivorous bats (*Artibeus* (*A.*) sp.) have frequently been typed as vampire bat-related RVs in Brazil [19, 25, 26, 41, 42, 44, 45]. Variability has also been reported in the G protein, a major contributor to the pathogenicity of the virus, in which several amino acid substitutions at antigenic sites associated with the pathogenicity and immunogenicity of the virus have been identified in Brazilian RV variants [41]. However, differences in the pathogenicity and antigenic characteristics of these Brazilian RV variants are not yet known. Recently, in addition to the G protein, both P and M proteins have also been reported to be associated with the pathogenicity of the virus [43]. The P protein forms a ribonucleoprotein (RNP) with N and L proteins, which then wraps around the viral RNA, and plays an important role in transcription and replication in conjunction with the L protein [3, 5, 11]. In addition, the P protein acts to counteract the host's interferon (IFN) responses in infected cells [1, 2]. The M protein is responsible for recruiting RNPs to the cell membrane, as well as their condensation into tightly coiled 'skeleton'-like structures [36]. In addition, the M protein is involved in viral assembly and budding, and is associated with regulating the balance between viral transcription and replication [9, 10, 20]. Furthermore, the M protein acts as a major inducer of apoptosis in neuronal cells [23].

However, although P and M proteins have been demonstrated to have several important functions in viral infection, these investigations have all been conducted using laboratory-adapted strains. In addition, few studies on P and M proteins in wild-type RV strains have been reported to date [30, 31, 32, 33], and no reports have genetically characterized the P and M genes in Brazilian RV isolates. In this study, we conducted genetic analyses of P and M genes of several RV field isolates prevalent in Brazil.

MATERIALS AND METHODS

Viruses: Thirty RV isolates were collected from brain specimens of frugivorous bats (*A. lituratus, A. planirostris*), insectivorous bats (*Eptesicus (E.) furinalis, Molossus (M.) molossus, Nyctinomops (N.) laticaudatus*), vampire bats (*Desmodus (D.) rotundus*), cattle, cats, dogs, and foxes

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Table 1. Brazilian rabies virus isolates used in this study

Isolate No.	Species of origin	Geographic origin		Year of isolation	Accession No.	
		City	State		P gene	M gene
BR-AL1	*Artibeus lituratus*	Itapira	São Paulo	1998	AB291108	AB291078
BR-AL2	*Artibeus lituratus*	São José do Rio Preto	São Paulo	1998	AB291109	AB291079
BR-AL3	*Artibeus lituratus*	Novo Horizonte	São Paulo	1998	AB291110	AB291080
BR-AP1	*Artibeus planirostris*	São José do Rio Preto	São Paulo	1998	AB291111	AB291081
BRbv303	Cattle	Casimire de Abrue	Rio de Janeiro	2002	AB291112	AB291082
BRbv306	Cattle	Vassouras	Rio de Janeiro	2004	AB291113	AB291083
BRct112	Cat	Morrinhos	Goiás	1999	AB291114	AB291084
BRct116	Cat	Itaberaí	Goiás	1999	AB291115	AB291085
BRct333	Cat	Gonçarves Dias	Maranhão	2003	AB291116	AB291086
BRdg96	Dog	Itaguaçu	Goiás	2001	AB291120	AB291090
BRdg97	Dog	Itaguaçu	Goiás	2001	AB291121	AB291091
BRdg101	Dog	Sta Tereza	Goiás	2001	AB291117	AB291087
BRdg317	Dog	Rio de Janeiro	Rio de Janeiro	1985	AB291118	AB291088
BRdg335	Dog	Barra do Corda	Maranhão	2003	AB291119	AB291089
BR-DR1	*Desmodus rotundus*	Lindóia	São Paulo	2000	AB291122	AB291092
BR-DR2	*Desmodus rotundus*	Lindóia	São Paulo	2000	AB291123	AB291093
BR-DR3	*Desmodus rotundus*	São José do Barreiro	São Paulo	2001	AB291124	AB291094
BR-EF1	*Eptesicus furinalis*	São José do Rio Preto	São Paulo	1998	AB291125	AB291095
BR-EF2	*Eptesicus furinalis*	Olimpia	São Paulo	2001	AB291126	AB291096
BR-EF3	*Eptesicus furinalis*	São José do Rio Preto	São Paulo	2001	AB291127	AB291097
BR-EF4	*Eptesicus furinalis*	Catanduva	São Paulo	2002	AB291128	AB291098
BR-Pfx1	*Dusicyon* sp.	Patos	Paraíba	2002	AB291133	AB291103
BR-Pfx3	*Dusicyon* sp.	Patos	Paraíba	2001	AB291134	AB291104
BR-Pfx4	*Dusicyon* sp.	Patos	Paraíba	2002	AB291135	AB291105
BR-Pfx5	*Dusicyon* sp.	Patos	Paraíba	2002	AB291136	AB291106
BR-Pfx6	*Dusicyon* sp.	Patos	Paraíba	2002	AB291137	AB291107
BR-MM1	*Molossus molossus*	Jales	São Paulo	1999	AB291129	AB291099
BR-MM2	*Molossus molossus*	Ilha Solteira	São Paulo	2002	AB291130	AB291100
BR-NL1	*Nyctinomops laticaudatus*	São José do Rio Preto	São Paulo	1998	AB291131	AB291101
BR-NL3	*Nyctinomops laticaudatus*	Ipiguá	São Paulo	2001	AB291132	AB291102

Table 2. Primers used for RT-PCR and sequencing of the P and M genes

Primer	Sequence	Position[a)]	Sense
Psense1	CGAATCATGATGAATGGAGG	1292–1311	+
Psense2	CAAATAGTCAGACAAATGA	1820–1838	+
Psense3	CAAATGGTCAGACGAATGA	1820–1838	+
RVP860–879	TGCAAGACGACCTGAACCGT	2273–2292	+
P781–802	TGTGT(A/G)CTGGGATGGGT(C/T)GCTT	2294–2315	+
Panti1	TCATTTTATCAGTGGTGTTG	2499–2480	–
Panti2	AAGTTCCTCATGTTCTTCTTGC	2653–2632	–
Panti3	AAGCTCTCAGCAATCTGGTGAGC	2139–2117	–
Panti4	TTATACAAGAATATCCCTGA	2190–2171	–
DG129	GATGATGTATGTCAATCGGG	3438–3419	–
RVG62–81	TGGTATCGTGTAGACGGGGA	3398–3379	–
GfoM1	CTTGAGTGGAG(A/G)GA(C/T)TTGTCGTA	3827–3805	–

a) Nucleotide positions are numbered according to the PV sequences (M13215)

(*Dusicyon* sp.), that had been diagnosed as rabies positive by the immunofluorescence antibody assay and the mouse inoculation test [7, 27] (Table 1).

RT-PCR and sequencing: RT-PCR and sequencing were performed as previously described [41]. The primers used for RT-PCR and sequencing for complete P and M genes are shown in Table 2. Reference data used in the phylogenetic analysis are shown in Table 3.

Phylogenetic analysis: Phylogenetic trees were generated using the neighbor-joining method of Saitou and Nei [40, 46]. Mokola virus was used as an outgroup. Bootstrap values were calculated using 1,000 replicates, and homologies and multiple alignment between nucleotide and deduced amino acid sequences were identified using BioEdit software [14].

Hydropathic profiles: The hydropathic profiles were characterized using the Kyte and Doolittle parameter (GENETYX Version 6.0.3, Software Development, Tokyo,

Table 3 Rabies virus isolates used in this study

Isolate No.	Species of origin	Country	Accession No	
			P gene	M gene
058.BBB	*Eptesicus fuscus*	Canada	AF369338	–
WCS1	Skunk	Canada	AF369285	AF360848
WCS2	Skunk	Canada	AF369286	AF360849
3694.MYO	*Myotis* sp.	Canada	AF369349	–
6832.RB	*Lasiurus borealis*	Canada	AF369351	–
FL.RAC	Raccoon	U.S.A.	AF369294	AF360856
FT2891.DG	Dog	U.S.A.	AF369308	–
115.DG	Dog	India	AF369309	–
119.DG	Dog	India	AF369310	–
IR5.DG	Dog	Iran	AF369311	–
KY2877.SK	Dog	U.S.A.	AF369292	–
M4.VB	Cattle	Mexico	AF369366	–
M29.DG	Cat	Mexico	AF369313	–
NY.RAC	Raccoon	U.S.A.	AF369293	AF360857
ONT1.RFX	Red fox	Canada	AF369265	AF360850
ONT5.RFX	Red fox	Canada	AF369269	AF360854
P4.VB	Cattle	Paraguay	AF369364	–
V027.DG	Dog	Tunisia	AF369322	–
V037.MG	Mongoose	Botswana	AF369300	–
V077.SHB	*Lasionycteris noctivagans*	Canada	AF369346	–
V078.BBB	*Eptesicus fuscus*	Canada	AF369341	–
V084.BBB	*Eptesicus fuscus*	Canada	AF369340	–
V089.LBB	*Myotis lucifugus*	Canada	AF369343	–
V103.HB	*Lasiurus cinereus*	Canada	AF369347	–
V113.DG	Dog	Sri Lanka	AF369320	–
V118.DG	Dog	Sri Lanka	AF369321	–
V121.DG	Dog	Nepal	AF369317	–
V211.SK	Skunk	U.S.A.	AF369287	–
V213.SK	Skunk	U.S.A.	AF369289	–
V216.SK	Skunk	U.S.A.	AF369291	–
V230.BBB	*Eptesicus fuscus*	U.S.A.	AF369342	–
V235.FTB	*Tadarida brasiliensis*	U.S.A.	AF369359	–
V264.MG	Mongoose	S. Africa	AF369302	–
V265.BFX	Bat-eared fox	Tanzania	AF369296	–
V280.FX	Red fox	France	AF369278	–
V461.DG	Dog	Nigeria	AF369326	–
V464.DG	Dog	Nigeria	AF369328	–
V660.FX	Fox	Israel	AF369280	–
SHBRV-18	*Lasionycteris noctivagans*	U.S.A.	AY705373	AY705373
USASK	Skunk	U.S.A.	–	AY170396
ZAMRAV5100	Dog	Zambia	AB285215	AB285215
PV	Unknown	Unknown	M13215	M13215
ABL	Human	Australia	AF418014	AF418014
Mokola	Unknown	Unknown	Y09762	Y09762

Japan).

RESULTS

The ORF of the P gene contained 894 nucleotides encoding 297 amino acids in the most of the RV isolates studied, while isolates BR-Pfx3 and BR-Pfx4 contained 906 nucleotides encoding 301 amino acids, as well as raccoon RV isolates from the U.S.A. (NY.RAC and FL.RAC), and BR-Pfx5 contained 891 nucleotides encoding 296 amino acids, as well as dog RV isolates from Asia (I15.DG, V113.DG and V118.DG) (Fig. 1). The similarities between the nucleotide and amino acid sequences within the Brazilian RV isolates were greater than 77.5% and 80.6%, respectively.

A phylogenetic tree based on partial P genetic data revealed two large genetic clusters (Fig. 2). One cluster consisted of chiroptera-related RV isolates and could further be divided into several monophyletic lineages according to host bat species. The other cluster consisted mainly of carnivora-related RV isolates, and was further divided into several lineages according to geographic origin and the host species. Some skunk and raccoon RV isolates from North America were placed in outlying lineages of the chiroptera-related RV cluster.

Brazilian RV isolates consisted of six RV variants which were associated with specific host species; the three insec-

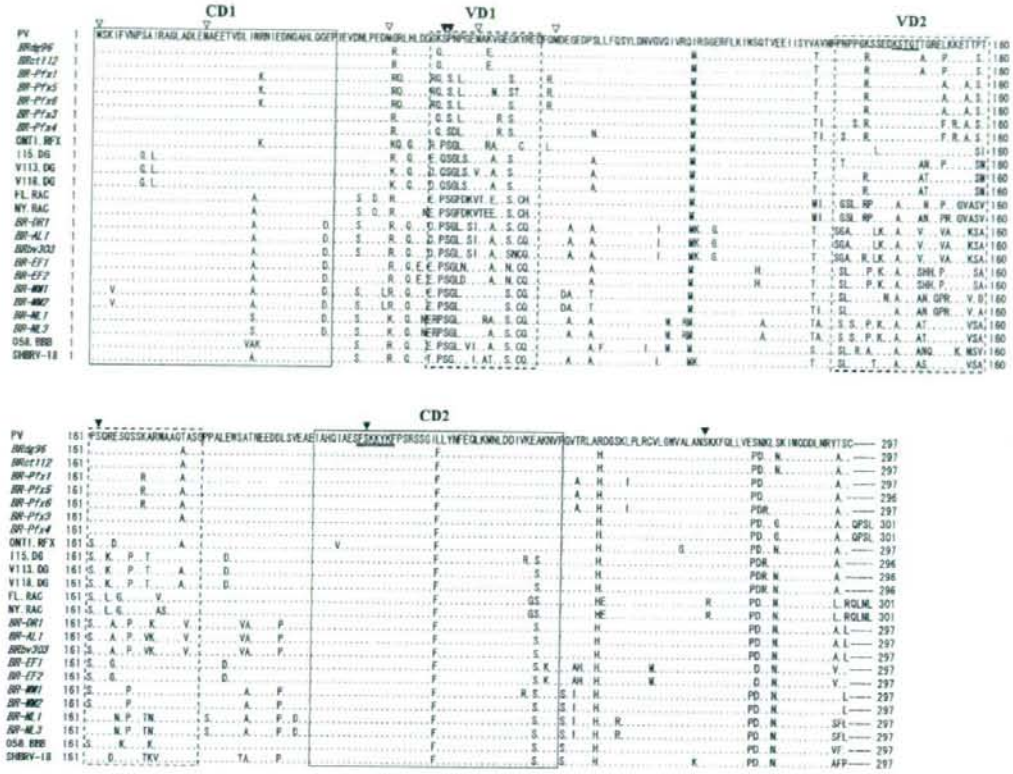

Fig. 1. Multiple alignments of P protein amino acid sequences. Italic font indicates the samples for which nucleotide sequences were determined in this study. Dots indicate amino acids that are in agreement with the sequence in the first line. Boxes with continuous lines delimit conserved domains (CD1 and 2), while those with dashed lines delimit variable domains (VD1 and 2). Black triangles indicate the positions of serine residues identified as phosphoacceptors in the P protein of the CVS strain. White triangles indicate the positions of methionine residues and confirmed translation initiation in the CVS strain. Continuous and double-continuous underlining show the LC8 binding motif and the lysine-rich motif, respectively.

tivorous bat RV lineages consisted of *N. laticaudatus, M. molossus* and *E. furinalis* and the vampire bat-related RV lineage consisted of *D. rotundus, Artibeus* sp. as well as cattle RV isolates (Fig. 2). In addition, Brazilian carnivora-related RVs formed a monophyletic cluster and were divided into dog- and fox-related RV lineages.

Based on multiple alignments of P protein amino acid sequence data, two conserved domains (CD1 and 2) comprising positions 1–50 and 201–245, respectively, and two variable domains (VD1 and 2) comprising positions 61–80 and 134–180, respectively, have been identified [30, 32]. These conserved and variable domains were observed in the Brazilian RV isolates of this study (Fig. 1). Specific amino acid substitutions corresponding to the phylogenetic lineages were observed throughout the P protein, as the result that Asp_{42} and Glu_{62} were found to be retained among Brazilian chiroptera- and carnivora-related RV isolates, respectively. The distribution of amino acid substitutions was particularly concentrated on the VD2, in which the hydropathic profiles associated with specific RV variants were observed in the hydrophilic region (data not shown).

The lysine-rich motif, FSKKYKF, which was identified as an important component of C-terminal N protein-binding [22], was conserved in amino acids 209–214 in all Brazilian RV isolates analyzed (Fig. 1). The binding site for the cytoplasmic light chain of dynein (LC8), which is involved in viral nucleocapsid axoplasmic transport [21, 39], has been reported as the RSSEDKSTQTTGR sequence of amino acids 139–151 in the P protein of the PV strain [38]. However, Lo *et al.* reported that the consensus sequence (K/R)XTQT is the common target-accepter of LC8 [28]. In this study, the consensus sequence of Brazilian RV isolates was

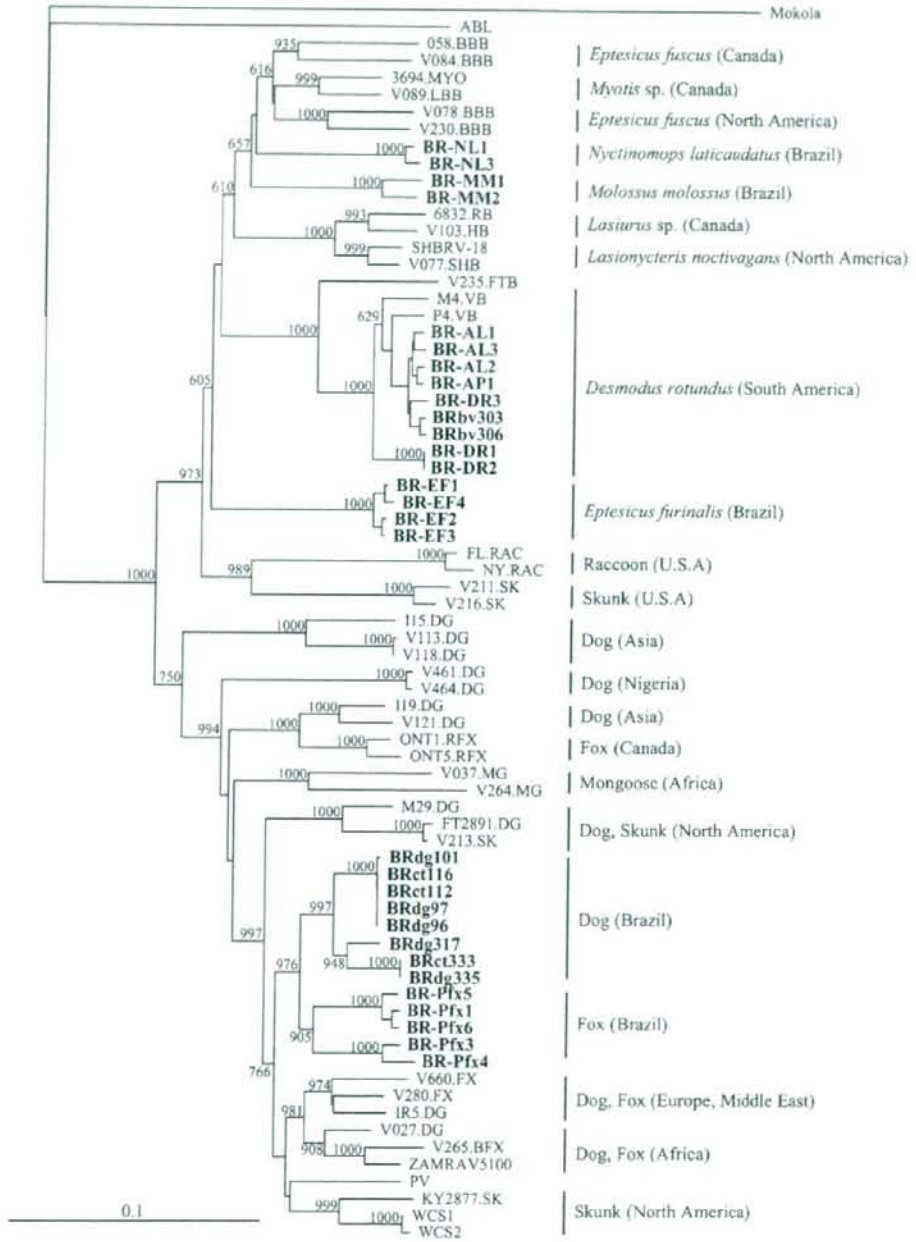

Fig. 2. Phylogenetic tree based on the P gene (1514–2404 compared to PV strain). Bold font indicates the samples for which nucleotide sequences were determined in this study. Bootstrap values are shown at major branches.

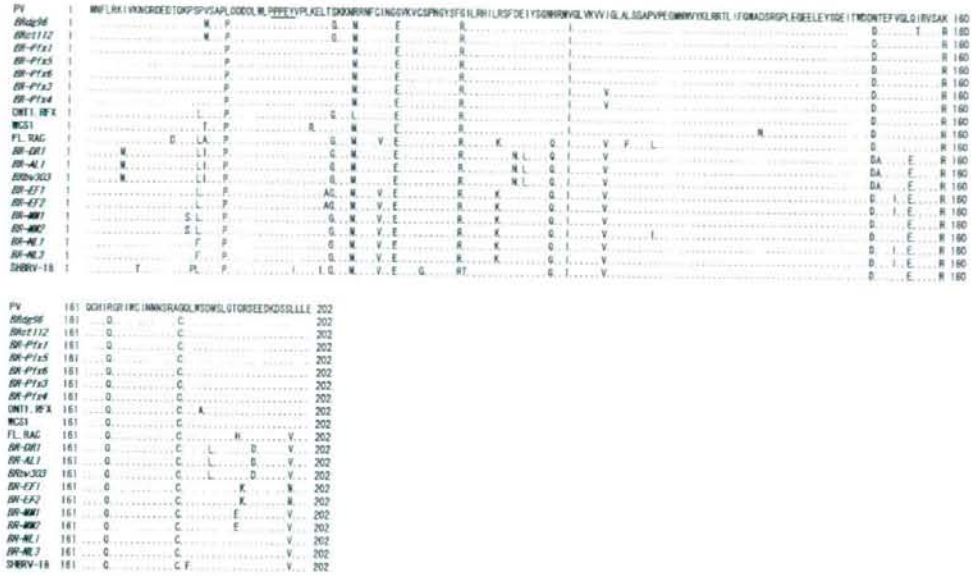

Fig. 3. Multiple alignments of M protein amino acid sequences. Italic font indicates the samples for which nucleotide sequences were determined in this study. Dots indicate amino acids that are in agreement with the sequence in the first line. Continuous underline shows the PPxY motif.

K(S/A)TQT encoded by amino acids 144–148. In addition, Ser_{145} was found to be conserved predominantly carnivora-related RV isolates while Ala_{145} was predominant in chiropteran-related RV isolates.

Phosphoacceptors associated with protein kinase C (PKC) or RV protein kinase (RVPK) were identified as Ser_{63}, Ser_{64}, Ser_{162}, Ser_{210} and Ser_{271} in the P protein of the CVS strain [13]. The phosphoacceptors associated with PKC, Ser_{162}, Ser_{210} and Ser_{271}, were retained in all Brazilian RV isolates, while those of RVPK, Ser_{63} and Ser_{64}, were not. Ser_{63} was retained in Brazilian carnivora-related RVs, and was substituted by Pro_{63} in Brazilian chiroptera-related RVs. Ser_{64} was retained in Brazilian fox- and chiroptera-related RVs, and was substituted by Pro_{64} in Brazilian dog-related RVs. Of the four methionine residues located in-frame of the single P protein sequences, Met_{20}, Met_{53}, Met_{69} and Met_{83}, all have been shown to translate four proteins of various lengths in the CVS strain [4]; Met_{20} and Met_{83} were conserved in all Brazilian RV isolates.

The ORF of the M gene contained 609 nucleotides encoding 202 amino acids in Brazilian RV isolates (Fig. 3). The similarities between the nucleotide and amino acid sequences in the Brazilian RV isolates exceeded 82.4% and 92.5%, respectively. As with the analysis of the P gene, phylogenetic trees based on the ORF of the M gene revealed two large genetic clusters, one chiroptera- and one carnivora-related RV cluster, containing six Brazilian RV lineages associated with the host species and geographic distributions (Fig. 4). Some raccoon RV isolates from the U.S.A. were placed within outlying lineages to the chiroptera- and carnivora-related RV clusters. Specific amino acid substitutions associated with host species were found scattered throughout the sequences of the M protein, for example Met_7, Ile_{22}, Asn_{80}, Leu_{82}, Ala_{148}, Leu_{184} and Asp_{192} were all retained in vampire bat-related RVs (Fig. 3). However, the hydropathic profiles did not differ significantly among the Brazilian RV isolates (data not shown). The proline-rich motif (PPxY motif), which interacts with the WW domain of cellular proteins [15, 16], formed consensus sequence PPEY at amino acids 35–38 in all Brazilian RV isolates.

DISCUSSION

Phylogenetic analyses based on the P and M genes revealed the existence of several Brazilian RV variants corresponding to phylogenetic analyses targeting the viral N and G genes [19, 25, 26, 41, 42, 44, 45]. Phylogenetic analysis showed two clusters consisting of chiroptera- or carnivora-related RV isolates. The chiroptera-related RV cluster was further divided into several lineages depending on host bat species, with four Brazilian chiroptera-related RV lineages observed. While several Brazilian fox RV isolates were characterized as encoding either extended or deleted P proteins, these formed a monophyletic cluster with other Brazilian carnivora-related RVs encoding general P

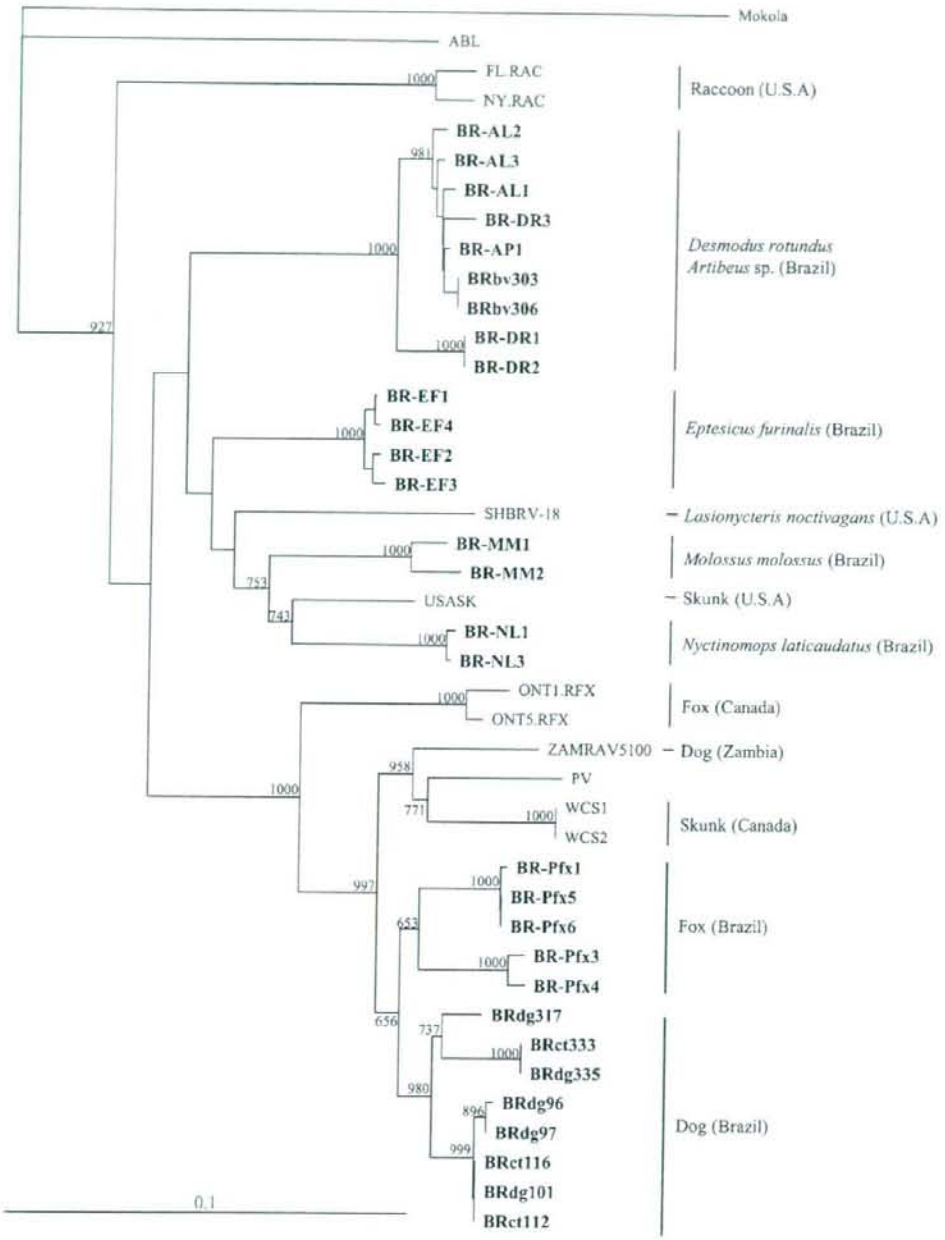

Fig. 4. Phylogenetic tree based on the M gene (2496–3104 compared to PV strain). Bold font indicates the samples for which nucleotide sequences were determined in this study. Bootstrap values are shown at major branches.

protein length, and did not group with carnivora-related RV isolates from other regions that encoded P proteins of different length. In addition, specific amino acid substitutions with respect to host species and geographic origin were found. Taken together, these findings suggest that phylogenetic analyses using these two genes are capable of identifying epidemiological characteristics of Brazilian RVs.

Conservation of the P protein in Brazilian RV isolates was low, with two conserved (CD1 and 2) and two variable domains (VD1 and 2), which corroborates the findings of Nadin-Davis *et al.* [30, 32]. Variable amino acid substitutions characteristic of Brazilian RV variants occurred at high frequencies in the variable domains, and this was reflected in the hydrophilic regions of the observed hydropathic profiles. Additionally, the LC8-binding motif, involved in viral nucleocapsid axoplasmic transport [21, 39], formed consensus sequence K(S/A)TQT in the VD2 region. With the exception of several RV isolates, the Ser and Ala differences were observed in the consensus sequences of isolates from either the carnivora- or chiroptera- related RV isolates, as reported by Nadin-Davis *et al.* [32]. The variable domains thought to be located on the surface structure of the P protein have been suggested to be involved in host/viral interactions and adaptation to the host environment [31, 32]. Our results support this hypothesis and studies of the variable domains are expected to be useful in elucidating the adaptive evolution of the virus.

At least 2 independent site of the P protein that confer N protein-binding activity have been identified; one is located within the N-terminal half of the protein, and another within 50 residues of the C-terminus [3, 11]. The first 19 N-terminal residues of the P protein confer L protein-binding ability, as does the N-terminal region containing the L protein-binding site [5]. Conserved domains contain N and L protein-binding sites and the conserved lysine-rich motif, FSKKYKF, which is an important component in N protein-binding [22, 32]. These two conserved domains containing the lysine-rich motif were observed in all Brazilian RV isolates, supporting the hypothesis that they may be essential to activities such as granule formation and general functioning of the virus.

Phosphorylation of the P protein is essential for transcription to occur in the vesicular stomatitis virus (VSV) [18], which belongs to the *Rhabdoviridae* family. In five serine residues which constitute the phosphoacceptors in the P protein of the CVS strain [13], Ser_{210} and Ser_{271} within the PKC phosphoacceptor target were notably conserved in all lyssavirus genotypes (GT 1 to 7) [32], including the Brazilian RV variants examined in this study. Studies on VSV and paramyxovirus have shown that P gene-encoded multiple proteins are important in the viral replication cycle and pathogenicity of the virus [12, 24, 37]. The four methionine residues located in-frame of single P protein sequences are translated into four proteins of various lengths with no known function in the CVS strain [4]. Nonetheless Met_{20} has been retained in all lyssavirus genotypes [32] and conservation of Met_{20} was observed in all Brazilian RV variants. Notably these conserved amino acid residues in the wild-type RVs are likely to be associated with important viral functions. Further laboratory research of these conserved amino acid residues would facilitate the identification of the biological characteristics of potential functions in wild-type RVs.

The M protein binds to the RNP core, and collaborates with the viral G protein in the infected cells to produce progeny virions by budding at the cell membrane [34, 35]. The M protein is also required for the development of a typical bullet-shaped rhabdovirus [29]. Consequently, the M protein is involved in binding to several viral components. In this study, although the M protein exhibited RV variant-associated amino acid substitutions, the hydropathic profiles conformed closely with each other. In addition, the PPxY motif, which interacts with the WW domains of cellular components [15], was conserved in all Brazilian RV variants. Laboratory-adapted and carnivora RV strains from North America have been characterized as having identical hydropathic M-protein profiles [17, 30], suggesting that the primary structure of the M protein may be important for the retention of viral structure and function.

The present study reveals that Brazilian RV isolates possess sufficient variability with respect to host species and geographic origin, while retaining conserved sequences (motifs) thought to be associated with a viral function in the P and M genes of RV. Studies on wild-type RVs are likely to increase our understanding of the relationship between the virus and the host, as well as the biological characteristics of the virus in the wild. Such genetic data would be helpful in investigating pathogenicity associated with RV.

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