

Fig. 5 気候要因による吸血コウモリ由来ウシ分離株の地理的分布  
記号はFig. 3に対応している。

Nishigahara	-19:	MVPQALLVP	ILGFSSCFQK	FFIYITPDTL	GFNSPIDIHH	LSCPNNLVE	DEGCTNLSGF	SKXELKGVVI	SAIKVNGFTC	61
RC-HL	-19:		L						T	61
CVS	-19:	V	F.L.L.L	L						61
FluryHEP	-19:	V	F.A.L	VSPL						61
SHBRV-18	-19:	I	F.L	IP.L.L				S	M	61
BR-BAT29	-19:	I	F.L.L	IS.L.S					SS	61
BR-BAT3	-19:	I	F.L.L	IS					N	61
BR-BAT28	-19:	I	F.L	IS					A.NS	61
BR-EA1	-19:	I	F.L	IS					A.NS	61
BR-EF1	-19:	I	F.L	IS					A.NS	61
BR-EF2	-19:	I	F.L	IS					A.NS	61
BR-EF3	-19:	I	F.L	IS					A.NS	61
BR-EF4	-19:	I	T.F.L	IS					A.NS	61
BR-NL2	-19:	I	F.L	IS					A.NS	61

Nishigahara	62:	TGVVTEAETV	INFGVYVIT	FKRKHFRPTP	DACRAAYNWK	MAGDPRVYES	LHSPYDPVHW	LRTVKTKES	LVIISPSVAD	141
RC-HL	62:									141
CVS	62:						QN	R	I	T
FluryHEP	62:						N			T
SHBRV-18	62:			M	HD	I	D	QN		
BR-BAT29	62:			M						
BR-BAT3	62:						N			
BR-BAT28	62:			I			N		T	N
BR-EA1	62:			M			N		T	N
BR-EF1	62:			M			N		T	N
BR-EF2	62:			M			N		T	N
BR-EF3	62:			M			N		T	N
BR-EF4	62:			M			N		T	N
BR-NL2	62:			M			N		T	N

Nishigahara	142:	LDPYDNLHS	RVFPSG&CSG	ITVSSVYCST	NHDYTVNMP	SLRLGTSCDI	FINSRGRAS	KG&ATCGFVD	ERGLYKSL&G	221
RC-HL	142:									221
CVS	142:	K	G	T	I	DP.P.P		N.N		221
FluryHEP	142:	K	G.N	T	I	N	H	D		221
SHBRV-18	142:	K	L	T.P	I	VEA		GR		221
BR-BAT29	142:	K	I.G.L	I.T	I	EA		G.M		221
BR-BAT3	142:	K	L	T	I	KA		G.V		221
BR-BAT28	142:	K	G.M	TF	I	NA		G.I		221
BR-EA1	142:	K	G.M	A.TF	I	NT		G.I		221
BR-EF1	142:	K	G.M	TF	I	NA		G.I		221
BR-EF2	142:	K	G.M	TF	I	NA		G.I		221
BR-EF3	142:	K	G.M	TF	I	NA		G.I		221
BR-EF4	142:	K	G.M	TF	I	NA		G.I		221
BR-NL2	142:	K	G.M	TF	I	NA		G.I		221

Fig. 6a G蛋白アミノ酸配列のマルチプルアラインメント 点線ボックスは (-19 to 0 and 440-461)シグナルペプチドおよび膜貫通ドメインを示す。ボックスは以下の順に抗原サイトを示す; 抗原サイト II (34-42 および 198-200), 抗原サイト I (231), VI (264), III (330-338), and "a" (342). N-結合型グリコシレーションサイトは下線で示されている。333位は太字、▼は168F変異、▽は194T変異、★は蛇毒神経毒結合アセチルコリン受容体に類似のアミノ酸残基を示す。

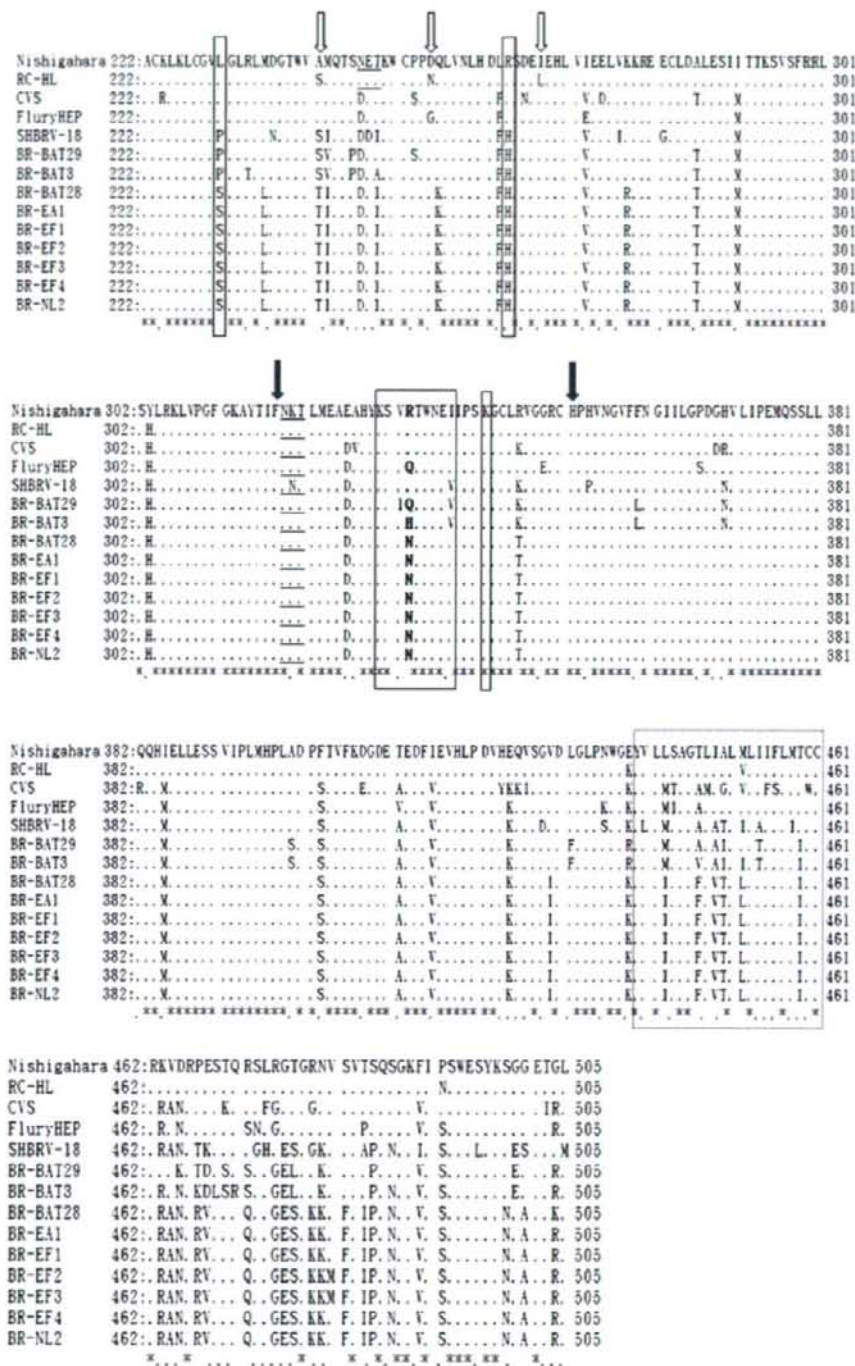


Fig. 6b G蛋白アミノ酸配列のマルチプルアラインメント 点線ボックス(440-461)は膜貫通ドメインを示す。ボックスは以下の順に抗原サイトを示す; 抗原サイトI (231), VI (264), I II (330-338), and "a" (342). N-結合型グリコシル化サイトは下線、333位は太字、空矢印は西ヶ原株の病原性に重要な242, 255および268位を、黒矢印はp75NTRニューロトリン受容体への結合に重要な318Fおよび352Hを示す。

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書 籍 名	出版社名	出版地	出版年	ページ
なし							

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Sato G, Kobayashi Y, Mochizuki N, Hirano S, <u>Ito T</u> , Cunha EM, Ito FH, <u>Sakai T</u> .	A unique substitution at position 333 on the glycoprotein of rabies virus street strains isolated from non-hematophagous bats in Brazil.	Virus Genes	38	74-79	2009



Research article

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## Molecular and geographic analyses of vampire bat-transmitted cattle rabies in central Brazil

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### Abstract

**Background:** Vampire bats are important rabies virus vectors, causing critical problems in both the livestock industry and public health sector in Latin America. In order to assess the epidemiological characteristics of vampire bat-transmitted rabies, the authors conducted phylogenetic and geographical analyses using sequence data of a large number of cattle rabies isolates collected from a wide geographical area in Brazil.

**Methods:** Partial nucleoprotein genes of rabies viruses isolated from 666 cattle and 18 vampire bats between 1987 and 2006 were sequenced and used for phylogenetic analysis. The genetic variants were plotted on topographical maps of Brazil.

**Results:** In this study, 593 samples consisting of 24 genetic variants were analyzed. Regional localization of variants was observed, with the distribution of several variants found to be delimited by mountain ranges which served as geographic boundaries. The geographical distributions of vampire-bat and cattle isolates that were classified as the identical phylogenetic group were found to overlap with high certainty. Most of the samples analyzed in this study were isolated from adjacent areas linked by rivers.

**Conclusion:** This study revealed the existence of several dozen regional variants associated with vampire bats in Brazil, with the distribution patterns of these variants found to be affected by mountain ranges and rivers. These results suggest that epidemiological characteristics of vampire bat-related rabies appear to be associated with the topographical and geographical characteristics of areas where cattle are maintained, and the factors affecting vampire bat ecology.

## Background

Rabies is a fatal infection of the central nervous system caused by being bitten by a rabid animal. The vampire bat, which has a distribution extending from Mexico to Argentina, is an important rabies vector in the region. Outbreaks of rabies in livestock transmitted by vampire bats were first observed between 1906 and 1908 in the State of Santa Catarina in Brazil, when approximately 4000 cattle and 1000 horses and mules died due to paralytic rabies [1]. To date, cattle losses attributed to vampire bat transmitted rabies have had a marked economic impact on the livestock industry in the areas [1-3]. In addition, outbreaks of human rabies transmitted by vampire bats in the Amazon regions of Brazil are an important public health consideration [4-6].

The vampire bat is a non-migratory colonial species that roosts in natural shelters or in shelters associated with human habitation [7]. Since vampire bats feed on mammalian blood, the distribution of vampire bat populations is affected by food availability and the distribution of livestock and cattle in particular [7,8]. Consequently, the occurrence of rabies within vampire bat populations is very closely reflected by the incidence of rabies in cattle [3,9,10].

Vampire bat-related rabies viruses have been genetically typed as being a species-specific variant [11]. To date, several rabies virus variants have been identified based on geographic distributions, which possibly reflects their association with bat ecology [12-14].

Attempts to control the transmission of vampire bat-transmitted rabies by reducing the vampire bat population using warfarin and vaccinating livestock against rabies are regularly conducted in Brazil [1,2]. Knowledge of the epidemiological characteristics of vampire bat-transmitted rabies is thus important for assessing the efficacy of these control measures against rabies transmission by bats. In order to assess the epidemiological characteristics of vampire bat-transmitted rabies, the authors conducted phylogenetic and geographical analyses using sequence data of a large number of cattle rabies isolates collected over a wide geographical area in Brazil.

## Methods

The 570 rabies virus isolates genetically analyzed in this study were collected from cattle in the city of Brasília in the Federal District (DF) (n = 2), and the States of Goiás (GO) (n = 320), Maranhão (MA) (n = 18), Minas Gerais (MG) (n = 3), Mato Grosso do Sul (MS) (n = 64), Mato Grosso (MT) (n = 72), Pará (PA) (n = 1), Paraíba (PB) (n = 5), Rio de Janeiro (RJ) (n = 46), São Paulo (SP) (n = 22), and Tocantins (TO) (n = 17) between 1987 and 2006 (Accession numbers: AB307066-AB307631, AB377125-

AB377128). In addition, 96 rabies virus sequences, which were analyzed in previous studies [11,12,15], were obtained from isolates of rabid cattle in the DF (n = 1), and the States of GO (n = 32), MA (n = 1), MT (n = 26), MG (n = 4), PB (n = 14), PA (n = 1), Rondônia (RO) (n = 1), SP (n = 9), and TO (n = 7) (Accession numbers: AB083799, AB083803, AB083805, AB083809, AB083813, AB083814, AB083818, AB206423-AB206436, AB246194-AB246210, AB246213-AB246248, AB246250-AB246267, AB246268-AB246270, AB307065). Eighteen rabies virus sequences were obtained from vampire bats in the States of GO (n = 10), RJ (n = 3), and SP (n = 5) as described previously (Accession numbers: AB201803-AB201805 and AB297632-AB297646) [16,17]. The nucleotide sequences of BRdg10 and BRdg603 isolated from Brazilian dogs were obtained from GenBank (Accession numbers: AB083796 and AB263334) [18].

Viral RNA was extracted from the brains of cattle diagnosed as being rabies positive by both the direct fluorescence antibody test and the mouse inoculation test [19,20]. The mice were housed and handled with ethical principal under the committee of University of São Paulo.

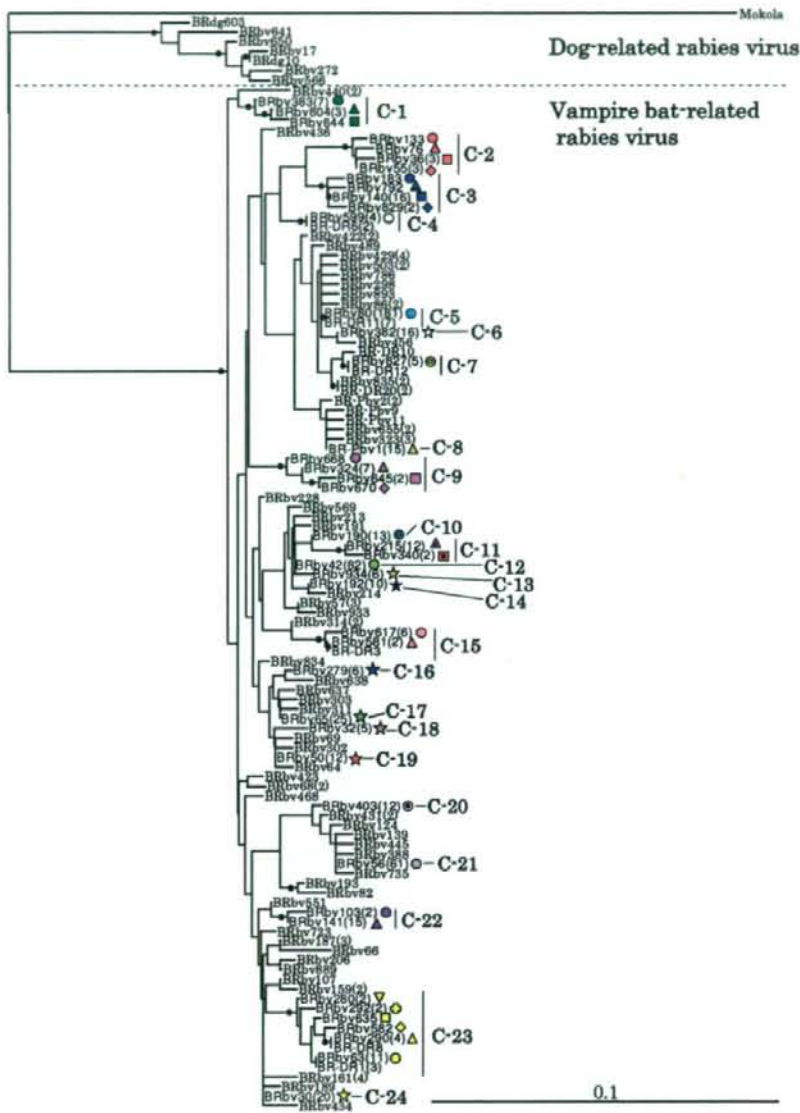
RT-PCR and sequencing methods were as described previously [12]. A 203 nt region corresponding to the nucleoprotein gene located between nucleotide 109 and 311 of the PV strain was analyzed as this locus has been employed to demonstrate association with the phylogenetic divergence of clusters in previous phylogenetic studies [11,21].

Multiple alignment and phylogenetic analysis were performed using the Clustal x program [22]. Phylogenetic trees were generated with the p-distance model using the neighbor-joining method of Saitou and Nei [23] and the Mokola virus was used as an outgroup (Accession number: Y09762). The statistical significance of the constructed phylogenies was estimated by bootstrap analysis with 1000 pseudoreplicate datasets. Bootstrap values exceeding 70% were considered to indicate phylogenetic association [24]. The TREEVIEW program was used to obtain a graphical output [25] and nucleotide sequence identities were calculated using BioEdit software [26]. The geographic origins of the sequenced Brazilian cattle and vampire bat rabies isolates were plotted at the municipal level of the respective federal states using MapInfo Professional GIS software (ver. 8.0 software MapInfo Japan K.K., Tokyo, Japan).

## Results

Brazilian rabies viruses could be divided into two phylogenetic groups, dog- and vampire bat-related rabies virus variants (Fig. 1). Almost all (99.2%) of cattle isolates ana-





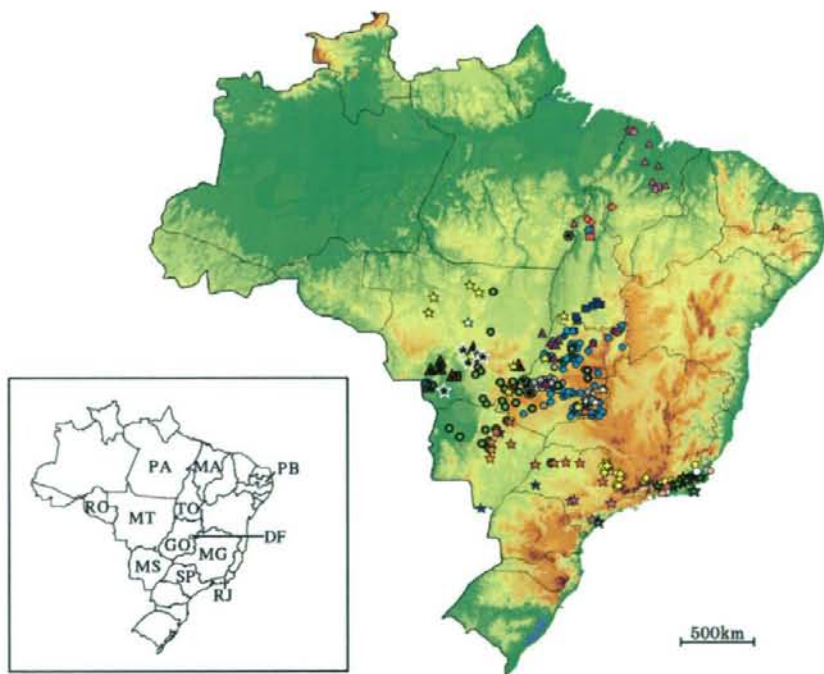
**Figure 1**  
**Distance tree generated by the neighbor-joining method.** Mokola virus was used as an outgroup. Bootstrap values were calculated with 1,000 iterations, and black circles indicate internal branches with bootstrap values of  $\geq 70\%$ . Letters of BRbv, BRdg, and BR-DR indicate samples from Brazilian cattle, dogs and vampire bats, respectively. The number of samples exhibiting 100% nucleotide identity is shown in parenthesis. The symbols corresponding to the phylogenetic clusters with a  $\geq 70\%$  bootstrap value on internal branches, and the clusters consisting of more than five samples, C-1 – C-24, are shown in this figure.

lyzed in this study were identified as being the vampire bat-related rabies virus variant, which consisted of a large number of phylogenetic lineages, the other five cattle samples collected from the States of GO, MA, MT, and SP during 1995–2004 were characterized as being dog-related rabies virus variants.

To assess geographical distribution pattern of the virus, vampire bat-related cattle isolates belonging to clusters with bootstrap values at the shared internal branches of lineages exceeding 70% (Fig. 1), and clusters consisting of more than five samples with 100% nucleotide similarity were plotted on topographical Brazilian maps. Consequently, 593 samples belonging to 24 clusters, C-1 – C-24, were mainly analyzed and plotted in maps (Fig. 2, 3,

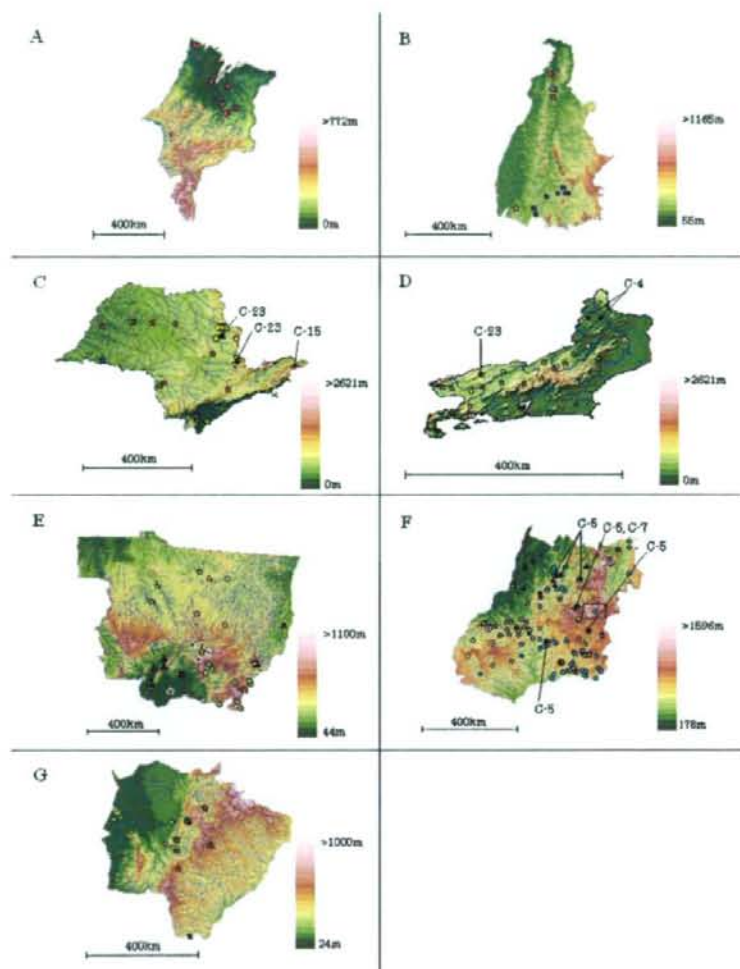
4). The nucleotide sequence identities among 24 clusters ranged between 93.1 – 99.5%. C-1 – C-3, C-9, C-11, C-15, C-22 and C-23 consisted of several lineages with bootstrap values exceeding 70%. The nucleotide sequence identities within these clusters ranged between 97.5 – 99.5%, and the distributions of the samples belonging to these clusters were observed to overlap (Fig. 2).

Most of the samples analyzed in this study were found along rivers, and a large number of samples characterized as the same variant were observed to be distributed along the same river (Fig. 3). Although the distributions of several clusters overlapped, regional variations in the distributions of these clusters were observed in Brazil (Fig. 2). C-2, C-8 and C-9 were mainly distributed in the northern



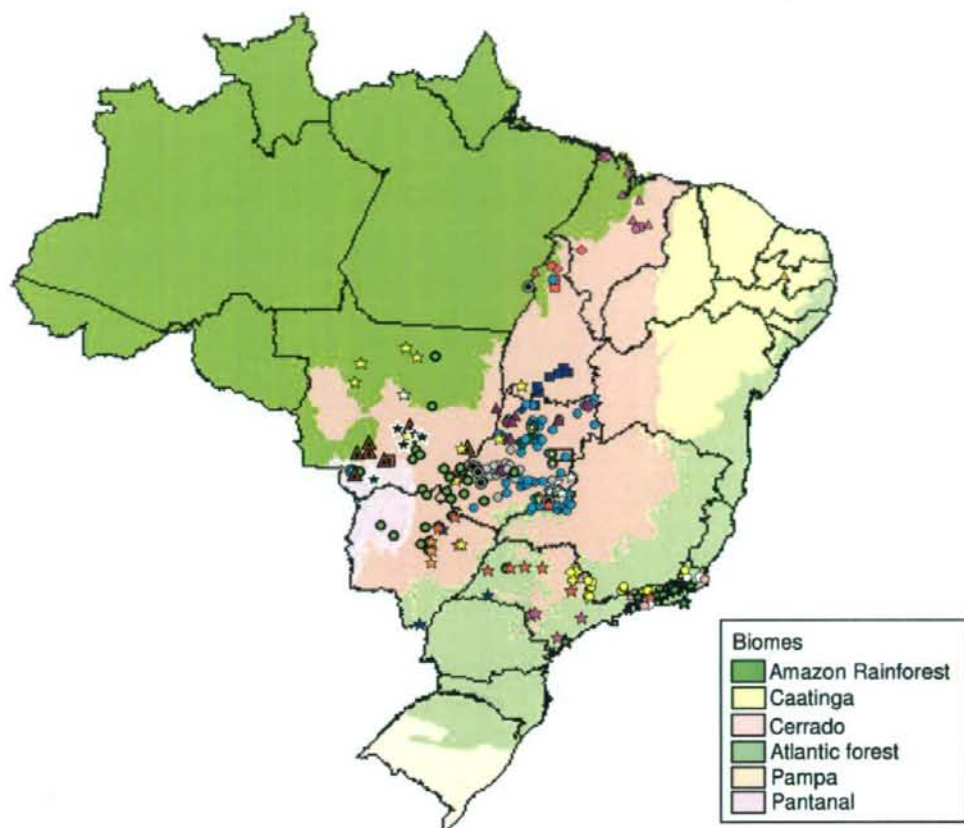
**Figure 2**  
**Geographic distribution of cattle isolates classified as genetic variants in this study.** City and state abbreviations are as follows: DF, Brasília City in the Federal District; GO, Goiás State; MA, Maranhão State; MG, Minas Gerais State; MT, Mato Grosso State; MS, Mato Grosso do Sul State; RJ, Rio de Janeiro State; RO, Rondônia State; PA, Pará State; PB, Paraíba State; SP, São Paulo State; TO, Tocantins State. The symbols for the cattle isolates correspond to those used in figure 1. Samples for which the geographic origin and the genetic variant are identical are illustrated using the same symbol.





**Figure 3**

**Detailed geographic distribution of cattle and vampire bat isolates classified as genetic variants.** (A) Maranhão State, (B) Tocantins State, (C) São Paulo State, (D) Rio de Janeiro State, (E) Mato Grosso State, (F) Goiás State and Brasília City of the Federal District, and (G) Mato Grosso do Sul State. Closed circles indicate the geographic origins of vampire bat isolates classified as genetic variants. Other symbols correspond to those of the cattle isolates used in figure 1. Rivers appear as blue lines. Samples for which the geographic origin and the genetic variant are identical are illustrated using the same symbol. Brazilian maps were obtained from Brasil em Relevo – Embrapa Monitoramento por Satélite <http://www.relevobr.cnpm.embrapa.br/>.



**Figure 4**  
**Geographic distribution of cattle isolates classified as genetic variants in Brazilian biomes.** Symbols correspond to those used in figure 1. The Brazilian biome map was obtained from IBGE Mapas Interativos <http://mapas.ibge.gov.br/>.

regions, C-1, C-3, C-5 – C-7, C-10 – C-14, C-20 – C-22 and C-24 in the central regions, and C-4, C-15 – C-19 and C-23 in the southern regions of Brazil. In other 86 cattle samples which were not categorized by the above-mentioned method, the distributions of the samples with 100% similarity were also observed in adjacent geographic areas (data not shown).

The distributions of several clusters were separated by geographic boundaries such as mountain ranges. For example, mountain ranges in the States of GO, MT and MS were observed to separate C-24 in the north and northwest, C-10 – C-12 and C-14 in the southwest areas, C-5 and C-20 – C-22 in the northeast areas, and C-13, C-16 and C-19 in

the southeast (Fig. 2). In addition, the distributions of C-1, C-3, C-5 and C-22 were affected by the mountain ranges in the eastern areas of the State of GO (Fig. 3-F). C-21 localized in a region between two mountain ranges in the State of GO. C-4 and C-17 were mainly distributed in the State of RJ, and were surrounded by the mountain ranges in the States of MG, SP and RJ with altitudes of 800 m above (Fig. 3-D).

Elevations in distribution regions of clusters defined in this study were various heights. For example, C-5, C-7, C-11, C-12, C-14, C-19 and C-23 were dispersed in mountain regions at altitudes 1000 m above to its surrounding lowland regions at altitudes 100 m below (Fig. 3). C-2, C-



4, C-9 and C-22 were distributed in lowland regions at altitudes less than 300 m, and C-6 was primarily distributed at an altitude of 700 m in mountainous area of the State of GO (Fig. 3-F).

The effect of the different Brazilian biomes on the distribution patterns of bat rabies isolates was also assessed (Fig. 4). C-2, C-9, C-11, C-12, C-14, C-19 and C-24 were found to be distributed across several regions with different biomes, while other clusters tended to be restricted to regions with a particular biome.

Using the above-mentioned method of categorization, the 10 vampire bat isolates in 18 categorized were genetically classified as falling into C-4, C-5, C-7, C-15 and C-23. The distributions of the vampire bat isolates belonging to C-5, C-7 and C-23 overlapped with cattle isolates of the same lineage (Fig. 3-C, D, F). Although vampire bat isolates belonging to C-4 were isolated in remote areas several tens-of kilometers from the geographic origins of cattle isolates belonging to the same lineage, several vampire bat and cattle isolates were distributed along the same river (Fig. 3-D). A vampire bat isolate belonging to C-15 was isolated in adjacent geographic areas where the cattle isolates were classified as the same variant (Fig. 3-C).

The number of cattle isolates of C-2, C-6, C-17 and C-20 in remote areas was small compared to the size of the groups from the principal distribution regions (Fig. 2). While the majority of the samples belonging to C-6 and C-20 were mainly distributed in the mountain regions of the State of GO, one sample belonging to C-6 originated from the central lowland region in the State of MT and one sample in C-20 from the southeastern region of the State of PA were also observed. While C-17 consisted mainly of samples from the State of RJ, one sample was derived from the southern area of the State of SP. Similarly, while C-2 primarily contained samples from the northern lowland areas of TO, one sample was derived from the southeastern mountainous region of MT.

## Discussion

The previous research reported the existence of nine regionally-defined rabies virus variants in Brazilian vampire bats [12]. The distribution patterns of these variants were observed to differ with respect to the geographic origins of the viruses and suggested that the epidemiological characteristics of the rabies virus variants were associated with the ecology of the bat populations.

Vampire bats usually select the shorter routes between the roost and their preferred prey, often traveling several kilometers one-way to find prey [7]. In addition, although vampire bat population in a colony remains relatively stable, they regularly visit neighboring roosts within activity

area of ranges of 10 to 20 km<sup>2</sup> [7,9,27]. This tendency to visit neighboring colonies promotes indirect communication between almost all of the colonies within an area forming a loosely defined assemblage or network [28]. Given these aspect of bat ecology, the spread of rabies in this species is mainly due to contact between the infected individuals of one colony and the susceptible individuals of another [8,28]. Rabies outbreaks in vampire bats have been observed to move slowly at an average rate of 40 km per year [3].

Although the categorization of a genetic group employed in this study differed from that employed previously [12], a large number of regionally-differentiated rabies virus variants were identified, and were widely distributed in both previously-studied and new areas of Brazil. The distributions of the variants identified in this study were observed to differ between regions in Brazil and tended to be separated by mountain ranges, which corroborated the observations of Kobayashi *et al.* and Velasco-Villa *et al.* [12,13]. In addition, since the distributions of the classified genetic variants from both vampire-bat and cattle isolates were found to overlap relatively closely, the epidemiological characteristics of cattle rabies are markedly similar to those of vampire bat rabies, with the genetic diversity between these variants attributed to aspects related to bat ecology and the topographical features of the habitat. Consequently, it appears that the rabies viruses might be circulated among bat populations inhabiting in contiguous areas, and although the variant distributions may change slowly over time, natural barriers such as mountain ranges may play an important role in delimiting rabies foci, resulting in regionally-differentiated rabies virus variants. A similar geographic orientation has been observed in rabies virus variants transmitted within populations of other non-migratory bat species, i.e. *Eptesicus fuscus* in North America [29].

The authors previously reported that genetic variants of vampire bat-related rabies in mountainous regions were correlated with altitude [12]. However, in this study, several variants were observed to disperse across of the potential altitude barriers defined previously, moving from high altitude mountainous regions to lowland. In addition, the distributions across several distinct biomes were also observed. Vampire bats utilize rivers to move between areas, and paths of outbreaks have been observed to follow tributaries and splits in the river due to the abundance of suitable roosts and caves along their banks [28]. Consequently, the presence of rivers and adequate shelter has been recognized as important considerations underlying the incidence and spread of vampire bat-transmitted cattle rabies. Most of the samples analyzed in this study were isolated from adjacent areas linked by rivers, which agreed with epizootic observations in cattle rabies. In addition, a



large number of samples characterized as the same variant that was distributed along the same river. These findings suggest that the distribution patterns of variants may correlate with the behavior of vampire bats, and that the spread of the virus is associated with bat migration.

In this study, a small number of variants were isolated in areas that were relatively remote and removed from the principal areas. Similarly, distribution patterns of the virus have been also observed that appear to indicate the movement of either vampire bats or infected animals across several Brazilian regions [14]. 86 of the samples in this study could not be categorized as genetic variants, and surveillance of cattle rabies has not yet been performed in several Brazilian regions. Subsequent epidemiological studies are, therefore, necessary in order to assess the temporal and spatial characteristics of the bat rabies distribution and also to obtain additional detailed epidemiological information related to bat rabies.

Since the epidemiological characteristics of vampire bat-transmitted rabies in cattle may be associated with topographical and geographical features of the areas where cattle are maintained as well as aspects of bat ecology, it is important that these factors are considered in plans directed at mitigating the spread of bat-transmitted cattle rabies.

### Conclusion

The present study revealed that several dozen of regionally-defined rabies virus variants associated with vampire bats exist in Brazil, and that areas where rabies occurs were affected by mountain ranges and rivers. These findings suggest that epidemiological characteristics of vampire bat rabies may be influenced by the topographical and geographical features of the areas in which cattle are maintained as well as aspects related to bat ecology. Consequently, the authors propose that these factors should be considered in measures directed to control the bat-transmitted cattle rabies.

### Competing interests

The authors declare that they have no competing interests.

### Authors' contributions

YK, carried out the molecular genetic studies, and edited the manuscript. GS, NM and SH, conducted the RT-PCR and the sequencing reactions. TI, AABC, AA, HPS, FHI and TS, participated in the elaboration of the study design, management, coordination, and assisted drafting the manuscript. All of the authors have read and approved the final manuscript.

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## A unique substitution at position 333 on the glycoprotein of rabies virus street strains isolated from non-hematophagous bats in Brazil

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**Abstract** The amino acid R or K at position 333 on the glycoprotein of the rabies virus is considered necessary for virulence in adult mice. Although some exceptions exist, substitution at this position causes expression of a phenotype that is either less pathogenic or non-virulent. To date, such substitutions have only been found in fixed strains of rabies virus. In this study, the authors found 333H, 333N, and 333Q substitutions at this position in rabies virus street strains isolated from non-hematophagous bats in Brazil. These strains showed pathogenicity and lethality on passage using adult mice with the intracerebral route and were confirmed rabies-positive by immunofluorescent assay. This suggests that these strains maintain virulence. Our findings indicate that rabies virus street strains with these substitutions exist in the field and may result in infection cycles.

**Keywords** Rabies virus · Glycoprotein · Non-hematophagous bat · Brazil

### Introduction

Rabies is a zoonotic viral disease of the central nervous system that is caused by rabies virus (RV), which belongs to the genus *Lyssavirus*. The five coding regions of the RV genome (3'N-P-M-G-L5'), encode nucleoprotein, phosphoprotein, matrix protein, glycoprotein, and RNA polymerase protein, and the G protein within this region is strongly linked to RV pathogenicity. Since the G protein is the only surface-exposed viral coat protein, it is capable of eliciting the production of neutralizing antibodies [26] and is responsible for host cell receptor recognition [23, 25] and membrane fusion [7]. Therefore, mutations in the G protein often change the viral antigenicity and pathogenicity. The amino acids 333R and 333 K are responsible for the virulence phenotype of RV. RV variants that have a Q, I, G, M, or S substituted at the position 333 in the G protein express a phenotype that is either less pathogenic or non-virulent compared to the parental wild-type RV when inoculated intracerebrally into adult immunocompetent mice [3, 18, 24]. However, pathogenicity of RV strains is not entirely explained by substitution at this position, since exceptional variants have been reported. For example, there are some recombinant RVs with neuroinvasive capacity or pathogenicity for adult mice that have a 333Q substitution [14, 28]. Moreover, RC-HL, a non-pathogenic RV fixed strain has conserved an R at this position [8]. Recently, it was reported that the SPBNGA-K RV recombinant strain, which has 194K and 333E substitutions, has increased pathogenicity for adult mice, but that parental SPBNGA, which has 194N and 333E substitutions, was non-pathogenic [6]. Therefore,

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it is apparent that pathogenicity of RV strains is not entirely dependent on substitution at this position. Until now, these unique substitutions have only been found in RV fixed strains. In this study, we report cases in which a unique substitution occurred at position 333 in the G protein of RV street strains isolated from non-hematophagous bats. We also show the results of comparison between deduced amino acid sequences of the full-length G protein of these strains and those of other RV street strains.

## Materials and methods

All RV street strains were isolated from non-hematophagous bats in Brazil (Table 1). We previously conducted phylogenetic analysis based on the N gene, and this demonstrated eight variants (I–VIII) of bat-related RV [9]. The

samples used in this study were variants VI, V, and VII. Only BR-EA1 was not used in this research, but it was confirmed that this sample belonged to the VII variant by phylogenetic analysis based on the N and G genes (data not shown). All samples were diagnosed as lethal and rabies-positive by the direct intracerebral mouse inoculation test and immunofluorescent assay, respectively, at some laboratories in São Paulo state (data not shown). The mice were housed and handled with ethical principles under the regulations of these laboratories. The viral RNA samples were extracted from emulsions of the brains of dead mice using the QIAamp Viral RNA Mini Kit (QIAGEN). RT-PCR and sequencing were performed for viral RNA samples. RV sequences were amplified using the SuperScript One-Step RT-PCR System with the Platinum Taq DNA Polymerase kit and 1 to 4  $\mu$ l of RNA template. For RT-PCR, two sense primers and two antisense primers (Table 2) were used to amplify the full-length G protein coding region (3,318–4,892; 1,575 bp) from all strains. After the amplified RT-PCR products were confirmed by electrophoresis, direct sequencing was performed according to methods described previously [17]. The primers used in direct sequencing are shown in Table 2. Amino acid sequences were deduced using computer software (GENETYX Ver. 6.1.0.) and compared with other G sequence data for RV street and fixed strains as follows: full-length G protein of Brazilian bat-related RVs (*Artibeus* sp.  $n = 8$ , *Eumops* sp.  $n = 1$ , *Molossus* sp.  $n = 13$ , *Nyctinomops* sp.  $n = 3$ , *Tadalida* sp.  $n = 2$ , Vampire bat  $n = 16$ , and Bat  $n = 8$ ); full-length G protein of fixed RVs (Nishigahara, RC-HL, CVS, Flury-HEP) and street RVs obtained from published data (Bat  $n = 5$ , Cattle  $n = 3$ , Buck  $n = 1$ , Camel  $n = 1$ , Coyote  $n = 1$ , Dog  $n = 12$ , Fox  $n = 2$ , Human  $n = 9$ , Mongoose  $n = 1$ , Raccoon  $n = 3$ , Sheep  $n = 1$ , Skunk  $n = 3$ , and Unknown  $n = 2$ ) [1]; fragments of G protein (position 145–241) (*Eptesicus fuscus*  $n = 15$ , *Myotis* sp.  $n = 6$ , *Lasiurus* sp.  $n = 7$ , *Lasionycteris*

**Table 1** The rabies virus strains analyzed in this study

Sample	Country	Origin/host species	Accession no.
Nishigahara	Japan		AB044824
RC-HL	Japan		D16330
CVS			AF406694
FluryHEP			AB085828
SHBRV-18	USA	Silver-haired bat	U52946
BR-BAT3	Brazil	Bat	AB383162
BR-BAT28	Brazil	Non-hematophagous bat	AB383165
BR-BAT29	Brazil	Non-hematophagous bat	AB383166
BR-EA1	Brazil	<i>Eumops auripendulus</i>	AB383167
BR-EF1	Brazil	<i>Eptesicus furinalis</i>	AB383168
BR-EF2	Brazil	<i>Eptesicus furinalis</i>	AB383169
BR-EF3	Brazil	<i>Eptesicus furinalis</i>	AB383170
BR-EF4	Brazil	<i>Eptesicus furinalis</i>	AB383171
BR-NL2	Brazil	<i>Nyctinomops laticaudatus</i>	AB383172

**Table 2** Primers used in this study

Primer	Nucleotide sequence(5'–3')	Position <sup>a</sup>	Sense	Use
Ga3222-40	CGCTGCATTTTRTCARAGT	3,221–3,239	+	RT-PCR/Direct sequencing
GS3994	CGGMTTGTGGATGAAAGRGGC	3,995–4,016	+	RT-PCR/Direct sequencing
Gb4119-39	GGAGGGCACCAATTTGGTMTTC	4,116–4,135	–	RT-PCR/Direct sequencing
G-antiBR2072-1	CATGAAGTATGTGAAGGGC	4,989–4,971	–	RT-PCR/Direct sequencing
Ga3222-40-1	GGGATACATCTCTGCCATA	3,518–3,536	+	Direct sequencing
Gb4119-39-1	GGGATTTGTCGTATGGGTC	3,801–3,819	–	Direct sequencing
IBM2-1004	AGCTTTCCCAAATCCTGGA	4,316–4,298	–	Direct sequencing
GS3994-1	GACTTGGAAACGAGATCATCC	4,373–4,392	+	Direct sequencing
GSI-1	CTGTAATCCCYCTAATGC	4,546–4,564	+	Direct sequencing
IBM2-1342	GGAGACCTGTTGTGAATATCTGGCAA	4,658–4,632	–	Direct sequencing

<sup>a</sup> Nucleotide positions are numbered according to the PV sequence (GenBank; accession no. M13215)

*noctivagans*  $n = 5$ , *Tadalida brasiliensis*  $n = 1$ , *Desmodus rotundus*  $n = 2$ ) [15]; and fragments of G protein (positions 19–284) (*Eptesicus fuscus*  $n = 9$ , *Lasiurus ega*  $n = 1$ , *Myotis* sp.  $n = 4$ ) (Accession No. AY170418-20, 27, 28, 30, 31, 33-38) from published data.

## Results

Open reading frames of the gene for the G protein of all Brazilian RV street strains were composed of 1,575 nucleotides and encoded 524 amino acids, as previously found on other RV strains (Fig. 1). Amino acid sequence similarity between open reading frame of the Brazilian RV street strains and those of other fixed strains ranged from 83.5% to 88.1%, and that among Brazilian RV street strains ranged from 88.7% to 100%.

**Fig. 1** Multiple alignments of G protein amino acid sequences. Boxes with discontinuous lines (–19 to 0 and 440–461) indicate signal peptide and transmembrane domains, respectively. Boxes with continuous lines indicate antigenic sites in the following order: antigenic site II (34–42 and 198–200), antigenic site I (231), VI (264), III (330–338), and “a” (342). N-linked glycosylation sites are underlined. Position 333 is indicated in bold. The closed triangle indicates 168F substitution. The open triangle indicates 194T substitution. Crosses indicate residues similar to the acetylcholine receptor binding site of snake venom curaremimetic neurotoxins. Open arrows indicate positions 242, 255, and 268, which are considered important for pathogenicity of the Nishigahara RV strain. Closed arrows indicate 318F and 352H, which are reported to be important for binding to the p75NTR neurotrophin receptor

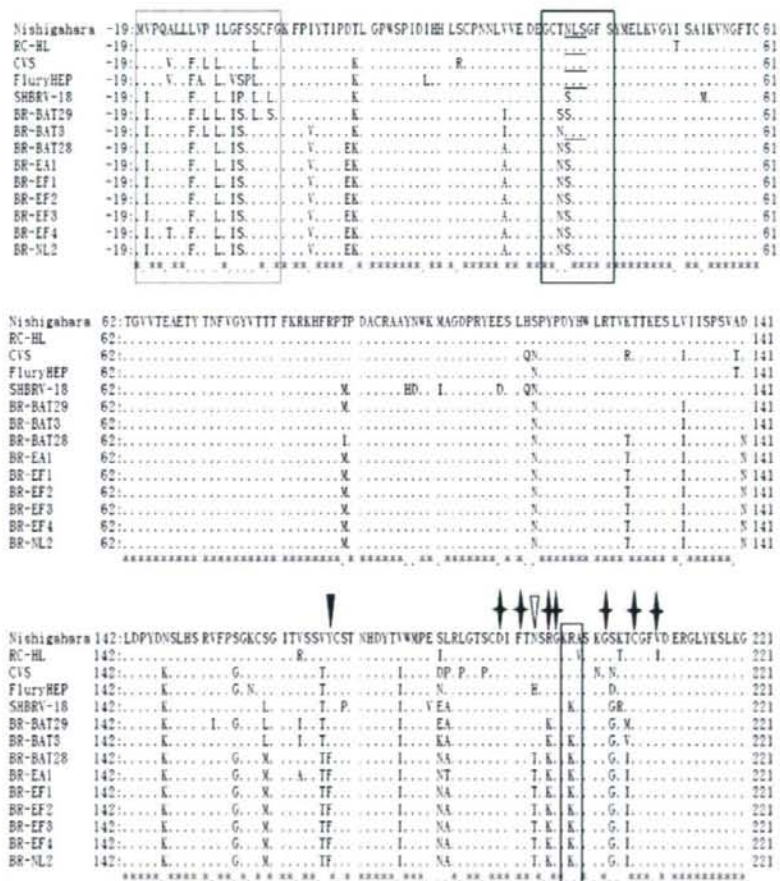




Fig. 1 continued

Nishigahara	222:	ACKLKLQGLV	GLRLMDGIV	ANQTSN	ETAW	CPPTQLVNLH	DURSDREIHL	VIEELVKARE	ECLDALES	IITKSYSFRL	301								
RC-HL	222:	.....	.....	S.....	N.....	.....	L.....	.....	.....	.....	301								
CVS	222:	R.....	.....	D.....	S.....	H.....	V.....	D.....	T.....	M.....	301								
FluryHEP	222:	.....	.....	.....	.....	G.....	.....	E.....	.....	M.....	301								
SHBRV-18	222:	P.....	N.....	SI.....	DDI.....	.....	.....	V.....	I.....	G.....	301								
BR-BAT29	222:	P.....	.....	SV.....	PD.....	S.....	.....	V.....	.....	T.....	301								
BR-BAT3	222:	P.....	T.....	SV.....	PD.....	A.....	.....	V.....	.....	T.....	301								
BR-BAT28	222:	S.....	L.....	TI.....	D.....	I.....	K.....	.....	V.....	R.....	301								
BR-EA1	222:	S.....	L.....	TI.....	D.....	I.....	K.....	.....	V.....	R.....	301								
BR-EF1	222:	S.....	L.....	TI.....	D.....	I.....	K.....	.....	V.....	R.....	301								
BR-EF2	222:	S.....	L.....	TI.....	D.....	I.....	K.....	.....	V.....	R.....	301								
BR-EF3	222:	S.....	L.....	TI.....	D.....	I.....	K.....	.....	V.....	R.....	301								
BR-EF4	222:	S.....	L.....	TI.....	D.....	I.....	K.....	.....	V.....	R.....	301								
BR-NL2	222:	S.....	L.....	TI.....	D.....	I.....	K.....	.....	V.....	R.....	301								
<div style="text-align: center;"> </div>																			
Nishigahara	302:	SYLRALVPGF	GKATYF	SKT	LNEAEARHS	VRTWNEI	PSK	DCLRGGRC	HPHYNGVFN	GIILPGDGHV	LIPENQSSLL	381							
RC-HL	302:	H.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	381							
CVS	302:	H.....	.....	DV.....	.....	.....	.....	K.....	.....	.....	DR.....	381							
FluryHEP	302:	H.....	.....	.....	.....	Q.....	.....	E.....	.....	S.....	.....	381							
SHBRV-18	302:	H.....	.....	N.....	D.....	.....	V.....	K.....	F.....	.....	N.....	381							
BR-BAT29	302:	H.....	.....	D.....	.....	IQ.....	.....	K.....	.....	L.....	N.....	381							
BR-BAT3	302:	H.....	.....	D.....	.....	N.....	V.....	K.....	.....	L.....	N.....	381							
BR-BAT28	302:	H.....	.....	D.....	.....	N.....	.....	T.....	.....	.....	.....	381							
BR-EA1	302:	H.....	.....	D.....	.....	N.....	.....	T.....	.....	.....	.....	381							
BR-EF1	302:	H.....	.....	D.....	.....	N.....	.....	T.....	.....	.....	.....	381							
BR-EF2	302:	H.....	.....	D.....	.....	N.....	.....	T.....	.....	.....	.....	381							
BR-EF3	302:	H.....	.....	D.....	.....	N.....	.....	T.....	.....	.....	.....	381							
BR-EF4	302:	H.....	.....	D.....	.....	N.....	.....	T.....	.....	.....	.....	381							
BR-NL2	302:	H.....	.....	D.....	.....	N.....	.....	T.....	.....	.....	.....	381							
<div style="text-align: center;"> </div>																			
Nishigahara	382:	QRIELLESS	VIPLNHPLAD	PFTVFEDGGE	TEDF	IEVHLP	DVHEQNSVD	LGLPNQGV	LLSAGTLIAL	NLIIFLMTCC	461								
RC-HL	382:	.....	.....	.....	.....	.....	.....	.....	.....	.....	461								
CVS	382:	R.....	M.....	S.....	E.....	A.....	V.....	YRKI.....	K.....	MT.....	AM.....	G.....	V.....	FS.....	V.....	461			
FluryHEP	382:	M.....	.....	S.....	.....	V.....	.....	V.....	.....	K.....	MI.....	A.....	.....	.....	.....	461			
SHBRV-18	382:	M.....	.....	S.....	.....	A.....	V.....	E.....	D.....	S.....	K.....	L.....	M.....	A.....	AT.....	I.....	A.....	I.....	461
BR-BAT29	382:	M.....	.....	S.....	.....	A.....	V.....	E.....	.....	F.....	R.....	M.....	A.....	AI.....	T.....	.....	.....	.....	461
BR-BAT3	382:	M.....	.....	S.....	.....	A.....	V.....	E.....	.....	F.....	R.....	M.....	A.....	AI.....	T.....	.....	.....	.....	461
BR-BAT28	382:	M.....	.....	S.....	.....	A.....	V.....	E.....	.....	I.....	K.....	I.....	F.....	VT.....	L.....	.....	.....	.....	461
BR-EA1	382:	M.....	.....	S.....	.....	A.....	V.....	E.....	.....	I.....	K.....	I.....	F.....	VT.....	L.....	.....	.....	.....	461
BR-EF1	382:	M.....	.....	S.....	.....	A.....	V.....	E.....	.....	I.....	K.....	I.....	F.....	VT.....	L.....	.....	.....	.....	461
BR-EF2	382:	M.....	.....	S.....	.....	A.....	V.....	E.....	.....	I.....	K.....	I.....	F.....	VT.....	L.....	.....	.....	.....	461
BR-EF3	382:	M.....	.....	S.....	.....	A.....	V.....	E.....	.....	I.....	K.....	I.....	F.....	VT.....	L.....	.....	.....	.....	461
BR-EF4	382:	M.....	.....	S.....	.....	A.....	V.....	E.....	.....	I.....	K.....	I.....	F.....	VT.....	L.....	.....	.....	.....	461
BR-NL2	382:	M.....	.....	S.....	.....	A.....	V.....	E.....	.....	I.....	K.....	I.....	F.....	VT.....	L.....	.....	.....	.....	461
<div style="text-align: center;"> </div>																			
Nishigahara	462:	RKYDRPESTQ	RSLRGTGRNV	SVTSQSGKFI	PSWESYKSGG	EITGL	505												
RC-HL	462:	.....	.....	.....	.....	N.....	505												
CVS	462:	RAN.....	K.....	FG.....	G.....	V.....	IR.....												
FluryHEP	462:	RAN.....	SN.....	G.....	P.....	V.....	S.....	R.....											
SHBRV-18	462:	RAN.....	TE.....	GH.....	ES.....	GA.....	AP.....	N.....	I.....	S.....	L.....	ES.....	M.....	505					
BR-BAT29	462:	.....	K.....	TD.....	S.....	GEL.....	K.....	P.....	V.....	S.....	E.....	R.....	505						
BR-BAT3	462:	.....	R.....	N.....	KDLSR.....	S.....	GEL.....	K.....	P.....	N.....	V.....	S.....	E.....	R.....					
BR-BAT28	462:	.....	RAN.....	RV.....	Q.....	GES.....	KK.....	F.....	IP.....	N.....	V.....	S.....	N.....	A.....	R.....				
BR-EA1	462:	.....	RAN.....	RV.....	Q.....	GES.....	KK.....	F.....	IP.....	N.....	V.....	S.....	N.....	A.....	R.....				
BR-EF1	462:	.....	RAN.....	RV.....	Q.....	GES.....	KK.....	F.....	IP.....	N.....	V.....	S.....	N.....	A.....	R.....				
BR-EF2	462:	.....	RAN.....	RV.....	Q.....	GES.....	KK.....	F.....	IP.....	N.....	V.....	S.....	N.....	A.....	R.....				
BR-EF3	462:	.....	RAN.....	RV.....	Q.....	GES.....	KK.....	F.....	IP.....	N.....	V.....	S.....	N.....	A.....	R.....				
BR-EF4	462:	.....	RAN.....	RV.....	Q.....	GES.....	KK.....	F.....	IP.....	N.....	V.....	S.....	N.....	A.....	R.....				
BR-NL2	462:	.....	RAN.....	RV.....	Q.....	GES.....	KK.....	F.....	IP.....	N.....	V.....	S.....	N.....	A.....	R.....				

antigenic site II that are involved with reduction of pathogenicity for adult mice, at positions 34, 42, and 198 [16], were conserved. Fragment 189–214 that is proposed to be important for RV G-binding to the nicotinic acetylcholine receptor (nAChR) [12, 13] was conserved, but R196K

substitution occurred in the dataset of critical residues (190D, 192F, 196R, 197G and 203G, 207C and 210V) which are similar to the acetylcholine receptor binding site of snake venom curaremimetic neurotoxins. However, 318F and 352H, reported to be important for binding to the



p75 neurotrophin receptor [11], were conserved. Among Asn37, Asn247, and Asn319, the three sequons known as the N-linked glycosylation site on the RV G gene [19, 20], substitutions were found on Asn37 and Asn247, but Asn319 was conserved. As an exception, Asn37 was conserved in BR-BAT3. In the three residues shown to be important for pathogenicity of Nishigahara RV strain [22], 255N and 268I were conserved in all street isolates, but A242S substitutions were found in BR-BAT3 and BR-BAT29. Moreover, A242T substitution was found in the other seven RV strains with 333N. These substitutions on the antigenic sites, those on RV G-binding site to the nAchR, those on N-linked glycosylation sites, and those on position 242 were found not only in RV strains with substitution at position 333 but also in some other RV street strains, particularly bat-related RVs. However, in the region between amino acids 164 and 210 that may be related in part to the pathogenicity of the Nishigahara RV strain [22], I68F substitution was found only in the seven RV strains with 333N. Moreover, at position 194, at which K to N substitution reportedly increases pathogenicity of recombinant RV strain with 333Q substitution in adult mice [6], K to T substitutions were also found in these seven RV strains with 333N.

## Discussion

The substitutions found in the present study at position 333 do not seem to have occurred incidentally in the mouse brain because the pattern of these substitutions corresponded with the lineage(s) classified according to the neighbor-joining tree based on the N gene [9]. These findings indicate that RV street strains with these substitutions exist in the field and may result in infection cycles. RV street strains with 333H, 333Q, and 333N substitutions have not been previously reported. Although viral titrations were not calculated for each RV sample, these RV strains with position 333 substitution showed pathogenicity and lethality for adult mice via the intracerebral route and were confirmed rabies-positive by immunofluorescent assay. This suggests that these strains maintain virulence, at least for adult mice. Interestingly, despite reports that 333Q causes an avirulent mutation of the CVS strain [18], a similar mutation in the RV strain BR-BAT29 showed lethality to adult mice in this study. It is likely that the neighboring 332I substitution found on this RV sample is involved in reverse pathogenicity. Unfortunately, the present study could not determine whether virulence for adult mice is related only to 333Q, 333H, and 333N substitution. However, these results supported that amino acids R and K at 333 of the G gene are not the absolutely requirement for RV pathogenicity [6, 14, 28] and that other

gene regions in addition to the G gene are involved in pathogenicity of RVs [21, 27]. In addition, it was known that sequences of RVs maintained in bats have some differences from those of RVs maintained in carnivores, recombinant and vaccine strains. However, the effect of these differences on bat RVs function has not been clarified. To clarify whether these substitutions at position 333 are related to virulence of the non-hematophagous bat Brazilian RV street strains, further studies are needed. These should include not only inoculation assays with virus titration but also determination of the full length of the genome, and experiments using recombinant RVs.

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