Table 1. Rabies viruses samples used in this study

Sample name	Host species[d]	Isolated region	State[a]	Isolated year	Accession No.
BRdg2	Dog	Goiânia	GO	1999	AB247411
BRct3	Cat	Goiânia	GO	1999	AB110656[b]
BRct5	Cat	Goiânia	GO	1998	AB247409
BRhm6	Human	Goiânia	GO	1999	AB110657[b]
BRdg10	Dog	Pocos de caldas	MG	1987	AB110658[b]
BRdg12	Dog	Mogi Guacu	SP	1989	AB110659[b]
BRdg15	Dog	Anapolis	GO	1999	AB247410
BRhr18	Horse	Bela Vista de Goiás	GO	1999	AB110660[b]
BRhm23	Human	Goianira	GO	1999	AB247438
BRpg28	Pig	Nova Aurora	GO	1998	AB110661[b]
BRhr31	Horse	Ipora	GO	1998	AB110662[b]
BRbv32	Cattle	Sao Roque	SP	1994	AB110663[b]
BRvmbt33	*Desmodus rotundus*	Taubate	SP	No data	AB110664[b]
BRsp35	Sheep	Apiai	SP	1992	AB110665[b]
BRbv38	Cattle	No data	TO	1999	AB247393
BRbv39[c]	Cattle	Colinas do Tocantins	TO	1999	AB110666[b]
BRvmbt41	*D. rotundus*	No data	No data	No data	AB247442
BRbv43	Cattle	Alto Taquari	MT	1999	AB247394
BRbv45	Cattle	Caceres	MT	1999	AB110667[b]
BRvmbt47	*D. rotundus*	Pindamonhagaba	No data	No data	AB247443
BRbv49	Cattle	Piraju	SP	1989	AB110668[b]
BRbv50	Cattle	Corumbaiba	GO	1999	AB110669[b]
BRbv55[c]	Cattle	Montes Altos	MA	1998	AB247395
BRbv76[c]	Cattle	Xinguará	PA	2002	AB247406
BRbv87[c]	Cattle	Santa Fé do Araguaia	TO	2001	AB247407
BRbv129[c]	Cattle	Piraquê	TO	2000	AB247380
BRbv132	Cattle	Catalao	GO	2000	AB247381
BRbv136[c]	Cattle	Araguaína	TO	2000	AB247382
BRbv147	Cattle	Mundo Novo	GO	2000	AB247383
BRbv152	Cattle	Monte Alegre de Goias	GO	2000	AB247384
BRbv168[c]	Cattle	Araguaína	TO	2001	AB247385
BRfx239	Fox	Patos	PB	2002	AB247430
BRfx240	Fox	Patos	PB	2000	AB247431
BRfx241	Fox	Patos	PB	2001	AB247432
BRfx242	Fox	Patos	PB	2002	AB247433
BRfx245	Fox	Patos	PB	2002	AB247434
BRfx246	Fox	Patos	PB	2002	AB247435
BRfx247	Fox	Patos	PB	2002	AB247436
BRfx248	Fox	Patos	PB	2002	AB247437
BRbv252	Cattle	Patos	PB	2003	AB247386
BRbv259	Cattle	Patos	PB	2003	AB247387
BRbv261	Cattle	Patos	PB	2003	AB247388
BRbv270	Cattle	Patos	PB	No data	AB247389
BRdg317	Dog	Rio de Janeiro	RJ	1985	AB247412
BRdg321[c]	Dog	Bacabal	MA	No data	AB247413
BRdg322[c]	Dog	Miranda do Norte	MA	2003	AB247414
BRbv323[c]	Cattle	Presidente Dutra	MA	2004	AB247390
BRbv324[c]	Cattle	Itapecuru	MA	2004	AB247391
BRdg325[c]	Dog	Santa Inês	MA	2003	AB247415
BRhm327[c]	Human	Lago da Pedra	MA	2004	AB247439
BRhm328[c]	Human	Lago da Pedra	MA	2004	AB247448
BRbv329[c]	Cattle	Senador Alexandre Costa	MA	2004	AB247392
BRdg331[c]	Dog	Coroatá	MA	2003	AB247416

(*continued*)

Table 1 (*continued*)

Sample name	Host species[d]	Isolated region	State[a]	Isolated year	Accession No.
BRsp332[c]	Sheep	Paço do Lumiar	MA	2004	AB247441
BRct333[c]	Cat	Gonçarves Dias	MA	2003	AB247408
BRdg334[c]	Dog	Capinzal do Norte	MA	No data	AB247417
BRdg335[c]	Dog	Barra do Corda	MA	2003	AB247418
BRbv571[c]	Cattle	Nova Olinda	TO	2000	AB247396
BRbv641[c]	Cattle	Imperatriz	MA	2004	AB247444
BRbv644[c]	Cattle	Presidente Dutra	MA	2004	AB247397
BRbv645[c]	Cattle	Capinzal do Norte	MA	2004	AB247398
BRbv647[c]	Cattle	Viana	MA	2004	AB247399
BRbv654	Cattle	Pocos de Caldas	SP	2004	AB247400
BRbv655[c]	Cattle	Senador Alexandre Costa	MA	2005	AB247401
BRbv656[c]	Cattle	Codó	MA	2005	AB247402
BRdg657[c]	Dog	São Vicente Ferrer	MA	2005	AB247445
BRbv658[c]	Cattle	Fortuna	MA	2005	AB247403
BRdg659[c]	Dog	Viana	MA	2005	AB247446
BRdg661[c]	Dog	Pindaré Mirim	MA	2005	AB247447
BRdg665[c]	Dog	São João Batista	MA	2005	AB247449
BRdg666[c]	Dog	São João Batista	MA	2005	AB247419
BRpg667[c]	Pig	São Vicente Ferrer	MA	2005	AB247451
BRdg669[c]	Dog	Moncão	MA	2005	AB247420
BRbv670[c]	Cattle	Godofredo Viana	MA	2005	AB247404
BRhm673[c]	Human	Godofredo Viana	MA	2005	AB247440
BRbv674[c]	Cattle	Luis Domingues	MA	2005	AB247405
BRhm675[c]	Human	Carutapera	MA	2005	AB247450
BR-BAT5[c]	BAT[e]	Carutapera	MA	2005	AB247379
BR-DR1	*D. rotundus*	Lindóia	SP	2000	AB247421
BR-DR2	*D. rotundus*	Lindóia	SP	2000	AB247422
BR-DR3	*D. rotundus*	São José do Barreiro	SP	2001	AB247423
BR-DR4	*D. rotundus*	Taubate (Vale do Paraiba)	SP	1995	AB247424
BR-DR5	*D. rotundus*	Varewça	RJ	2002	AB247425
BR-DR6	*D. rotundus*	Laje de Muriae	RJ	1998	AB247426
BR-DR7	*D. rotundus*	Itaperuna	RJ	1997	AB247427
BR-DR8	*D. rotundus*	Tambaú	SP	2003	AB247428
BR-DR9	*D. rotundus*	Guarlhos	SP	2000	AB247429

[a]*MA*, Maranhão; *PA*, Pará; *MT*, Mato Grosso; *TO*, Tocantins; *GO*, Goiás; *MG*, Minas Gerais; *SP*, São Paulo and *RJ*, Rio de Janeiro. All states localized in Brazil
[b]Described in the work of Sato et al. [12]
[c]The isolates from areas pointed in Fig. 2 were shown in bold
[d]Scientific names are used for only vampire bat
[e]Species is not exactly identified

America because they play an important role in the transmission of RV to cattle. In urban areas of Brazil such as São Paulo, dog-related rabies is being controlled by an effective vaccination program [8]; however, the virus remains a problem in rural areas. Bat-related rabies also poses health threats to both livestock and humans. The cases of bat-transmitted human rabies tend to increase in areas with rapid destruction of natural ecosystems [10]. In Brazil, 703 cases of human rabies

were reported from 1986 to 2004. Most cases occurred in the northeastern and northern regions, with 84 cases reported in Maranhão alone. In 2004, 21 humans died from confirmed or suspected cases of bat-transmitted rabies in Pará (PA), and in 2005, human rabies outbreaks were reported in northwestern MA and the neighboring state of PA [1]. Although many outbreaks of rabies have occurred in northern Brazil, [2, 3, 7, 13, 14], only a few epidemiological studies of rabies have taken place there [4, 15].

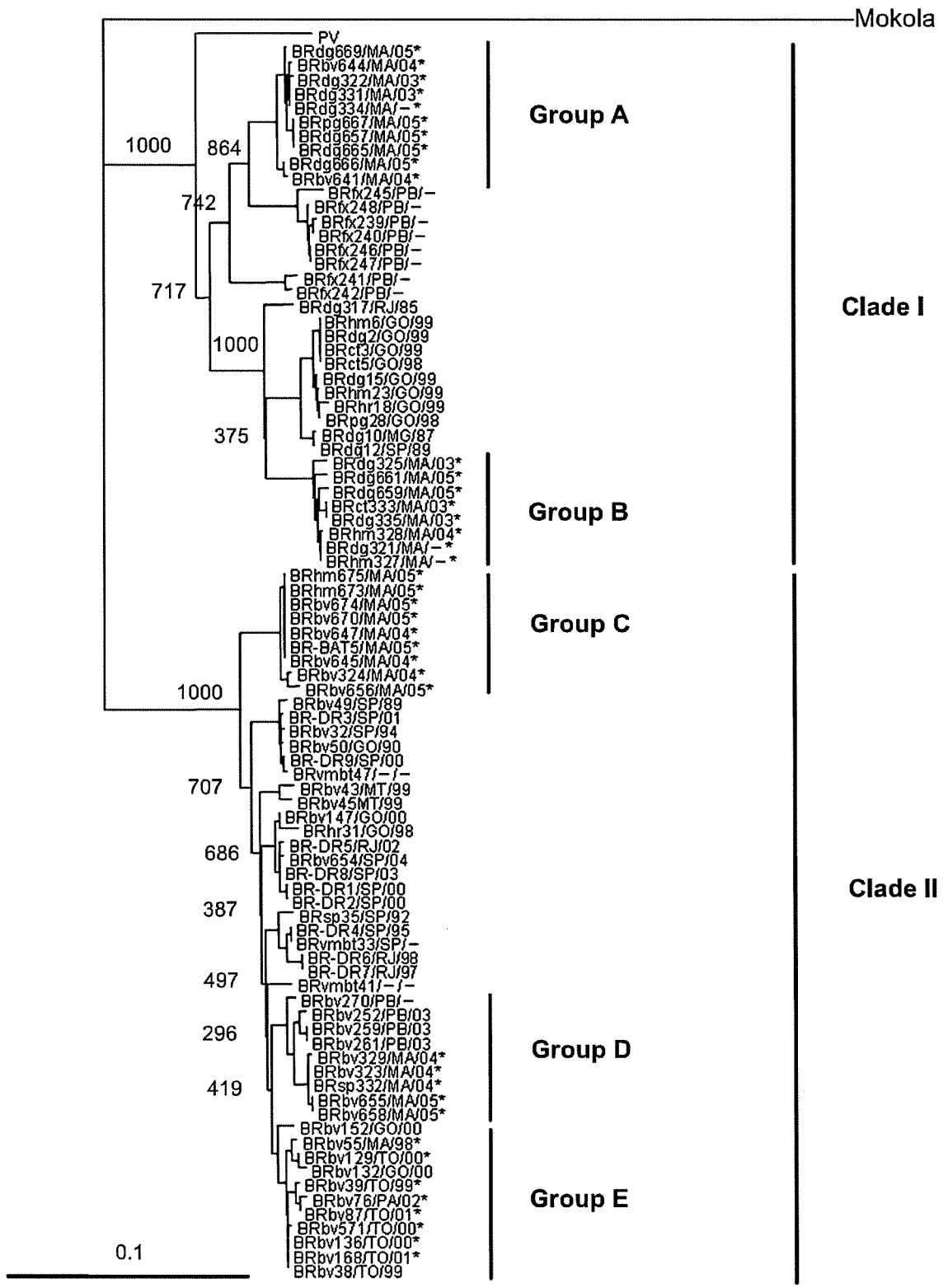
Materials and methods

In this study, RV isolates were taken from bat, dog, cat, human, and livestock populations in MA, PA, and Tocantins (TO) states. A portion of the glycoprotein gene (G) sequence from each isolate was compared with isolates from other states of Brazil. Thirty-three samples were isolated from various species in MA during 1998–2005 (the year(s) of isolation for BRdg321 and 334 are unknown), 7 samples were isolated from TO during 1999–2001, and 1 sample was isolated from PA in 2002 (Table 1). Forty-four additional livestock, human, domestic or wild species isolates from other regions of Brazil, namely Goiás (GO), Paraíba (PB), Minas Gerais (MG), Mato Grosso (MT), São Paulo (SP), and Rio de Janeiro (RJ) were also analyzed. Viral samples were extracted as a total RNA by QIAamp Viral RNA mini Kit (Qiagen) from either emulsion of the original rabid brain or from mouse brain inoculated with RV. The samples were stored at −80°C and diluted 10-fold before analysis. RV sequences were amplified using the SuperScript One-Step RT-PCR System with the Platinum Taq DNA Polymerase kit or the SuperScript III One-Step RT-PCR System with the Platinum Taq High Fidelity kit (Invitrogen) and 1 to 4 μl of RNA template. In RT-PCR, the sense primer Ga3222-40 (5′-CgCTgCATTTT(A/g)TCA(A/g)AgT-3′) and the antisense primer Gb4119-39 (5′-ggAgggCACCATTTggT(A/C)TC-3′) were used to amplify the G coding region (3221–4135 bp) from all isolates [12] . A phylogenetic tree was constructed based on a sub-region of the G gene (3318–3916; 599 bp). After the amplified RT-PCR products were confirmed by electrophoresis, direct sequencing and phylogenetic analyses were performed according to the methods described previously [12].

Results

RV isolates were divided into two clades. MA isolates from all carnivores (dogs and cat), 2 human, 2 cattle, and 1 pig belonged to Clade I (associated with dog- and fox-related isolates), while the majority of the isolates from livestock (cattle and sheep), 2 human, and 1 bat (BR-BAT5) isolates belonged to Clade II (associated with vampire bat-related isolates) (Fig. 1). Carnivore isolates were subdivided into two groups (A and B). Group A consisted of 7 dog, 2 cattle, and 1 pig isolates,

Fig. 1. A phylogenetic tree based on the G protein nucleotide sequence (3318–3916: 599 bp). Each isolate is described as follows: sample name/state/year isolated. State names are abbreviated as follow: *MA*, Maranhão; *TO*, Tocantins; *PB*, Paraíba; *PA*, Pará; *MT*, Mato Grosso; *GO*, Goiás; *MG*, Minas Gerais; *SP*, São Paulo and *RJ*, Rio de Janeiro. The bar (—) means the data is unknown. The isolates from areas pointed in Fig. 2 are indicated by an asterisk. The rabies-related virus (Mokola; Accession no. is S59447) was used as an outgroup. Numbers show bootstrap values of 1000 replicates


Mokola
PV
BRdg669/MA/05*
BRbv644/MA/04*
BRdg322/MA/03*
BRdg331/MA/03*
BRdg334/MA/-*
BRpg667/MA/05*
BRdg657/MA/05*
BRdg665/MA/05*
BRdg666/MA/05*
BRbv641/MA/04*
BRfx245/PB/-
BRfx248/PB/-
BRfx239/PB/-
BRfx240/PB/-
BRfx246/PB/-
BRfx247/PB/-
BRfx241/PB/-
BRfx242/PB/-
BRdg317/RJ/85
BRhm6/GO/99
BRdg2/GO/99
BRct3/GO/99
BRct5/GO/98
BRdg15/GO/99
BRhm23/GO/99
BRhr18/GO/99
BRpg28/GO/98
BRdg10/MG/87
BRdg12/SP/89
BRdg325/MA/03*
BRdg661/MA/05*
BRdg659/MA/05*
BRct333/MA/03*
BRdg335/MA/03*
BRhm328/MA/04*
BRdg321/MA/-*
BRhm327/MA/-*
BRhm675/MA/05*
BRhm673/MA/05*
BRbv674/MA/05*
BRbv670/MA/05*
BRbv647/MA/04*
BR-BAT5/MA/05*
BRbv645/MA/04*
BRbv324/MA/04*
BRbv656/MA/05*
BRbv49/SP/89
BR-DR3/SP/01
BRbv32/SP/94
BRbv50/GO/90
BR-DR9/SP/00
BRvmbt47/-/-
BRbv43/MT/99
BRbv45/MT/99
BRbv147/GO/00
BRhr31/GO/98
BR-DR5/RJ/02
BRbv654/SP/04
BR-DR8/SP/03
BR-DR1/SP/00
BR-DR2/SP/00
BRsp35/SP/92
BR-DR4/SP/95
BRvmbt33/SP/-
BR-DR6/RJ/98
BR-DR7/RJ/97
BRvmbt41/-/-
BRbv270/PB/-
BRbv252/PB/03
BRbv259/PB/03
BRbv261/PB/03
BRbv329/MA/04*
BRbv323/MA/04*
BRsp332/MA/04*
BRbv655/MA/05*
BRbv658/MA/05*
BRbv152/GO/00
BRbv55/MA/98*
BRbv129/TO/00*
BRbv132/GO/00
BRbv39/TO/99*
BRbv76/PA/02*
BRbv87/TO/01*
BRbv571/TO/00*
BRbv136/TO/00*
BRbv168/TO/01*
BRbv38/TO/99
1000
864
742
717
1000
375
1000
707
686
387
497
296
419
0.1
Group A
Group B
Group C
Group D
Group E
Clade I
Clade II

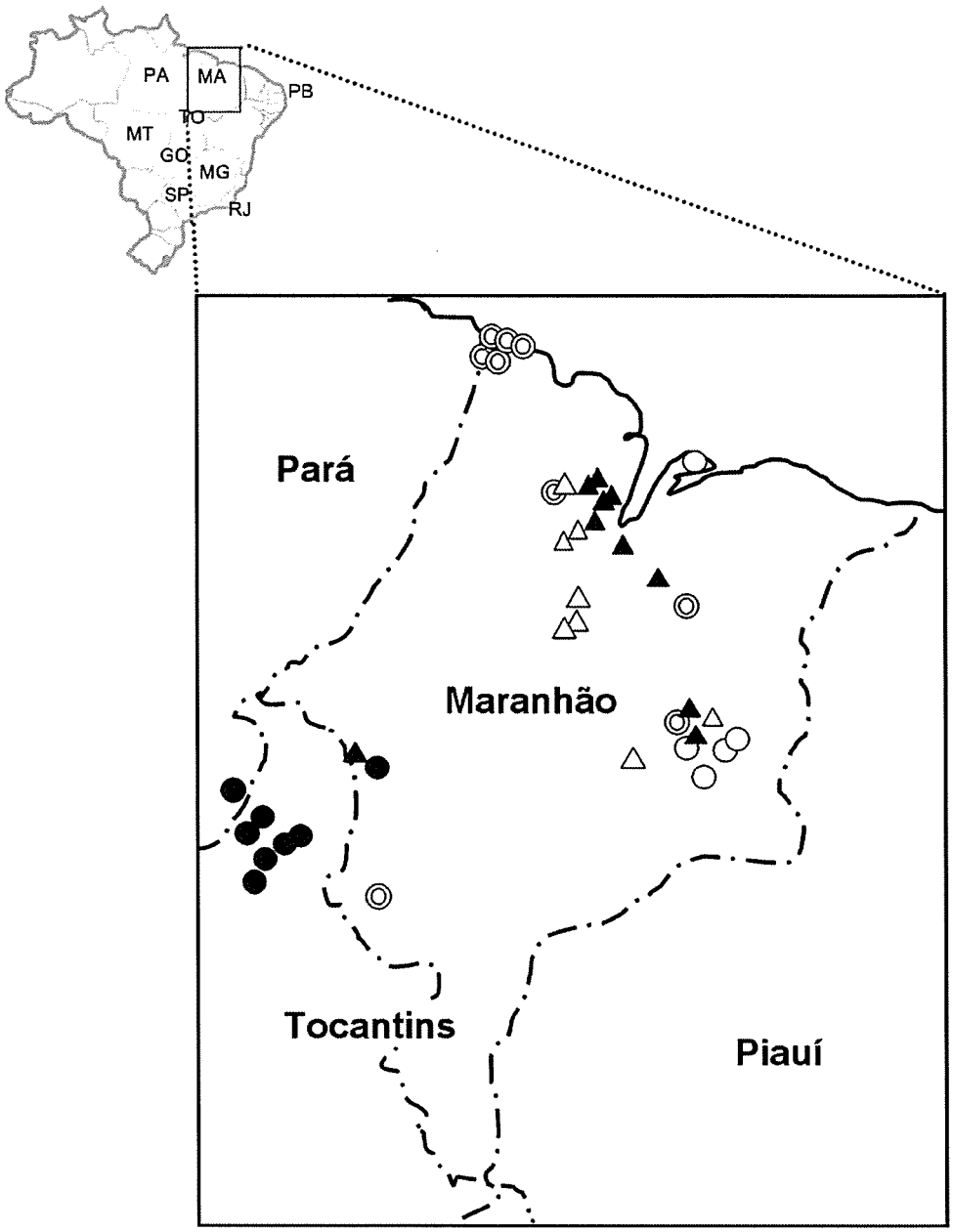


Fig. 2. Geographic origins of each isolate shown in bold in Table 1. The symbols indicate geographic origins of samples. Each symbol corresponds to a group shown in Fig. 2, namely, group A (▲), B (△), C (◎), D (○), and E (●). The two overlapping symbols indicate that they were isolated from the same place

and group B included 5 dog, 1 cat, and 2 human isolates. Although the geographic origins of groups A and B were close to each other (Fig. 2), these and other groups of this clade clearly divided in the tree, as indicated by high bootstrap values.

Sequences from group A were close to those isolated from foxes (*Pseudalopex vetulus* or *Dusicyon vetulus*) in Patos, a central city of PB (these samples were obtained from fox road-kills). Sequences from group B were close to the group isolated from central - southeastern areas in Brazil (GO, MG and SP).

MA isolates belonging to Clade II were divided into 3 groups (C, D and E). Group C included 6 cattle, 2 human, and 1 bat isolates, group D included 4 cattle and 1 sheep isolates, and group E consisted of 8 cattle isolates. Group C consisted of only MA isolates, and it was divided from other groups in Clade II by a high bootstrap value. However, Clade II generally showed very low bootstrap values. The geographic origins of these isolates were mapped out with carnivore isolates (Fig. 2).

The Clades I and II isolates shared nucleotide homology greater than 90.2% and 94.9%, respectively. Except for fox isolates, nucleotide homology of Clade I was greater than 92.6%.

Discussion

In this study, we conducted phylogenetic analyses of RV isolates from the states of MA, PA, and TO in northeastern Brazil. Our results are consistent with reports of rabies transmission by carnivorous mammals and vampire bats in northern and northeastern regions of Brazil [2, 3, 7, 13, 14].

In a previous study that analyzed a 203-bp sequence in the nucleoprotein gene, it was concluded that dog-related RV isolated from central to southern areas (GO, MG and SP) of Brazil were more closely related to one another than to vampire bat-related RVs [9]. Therefore, it was speculated that a relatively uniform population of dog-related RV was spread widely in Brazil. Similar results were obtained by using the G protein coding gene [12]; however, these results indicate that dog-related RV isolates display greater diversity than vampire bat-related RV isolates. These results suggest that carnivoran-related RVs display more genetic diversity than previously suspected.

Interestingly, the isolates derived from carnivores in MA fell into two distinct phylogenetic groups despite their proximate geographic origins. Moreover, group A was closer to the group consisting of wild fox isolates. The geographic origins of these carnivoran strains did not show geographical correlation. This result indicates that RV can be transmitted between domestic animals and wild foxes, and it supports the report that hoary foxes play a role in maintenance and dissemination of rabies in Brazil, as described by Bernardi et al. [4]. Wild carnivoran rabies cases have been reported all over Latin America. For example, the incidence of rabies associated with wild fox and coyote was demonstrated in Mexico [6], Argentina [5], and Colombia [11]. In addition, isolates from wild dog (*Cerdocyon-thous*) were also reported in Brazil [15]. Therefore, it is not surprising that the rabies cycle is maintained by wild carnivores (such as the fox) in northeastern Brazil. Unfortunately, PB, where these fox rabies isolates were obtained, is geographically distant from MA, and no isolates exist to bridge the gap between the two areas. Thus, to demonstrate the phylogeny of carnivoran rabies viruses in northern Brazil, epidemiological studies of carnivoran rabies in these gap areas are necessary.

Groups D and E showed distinct geographical distribution. However, as indicated by its high bootstrap value, group C localized independently from not only other MA isolates but also from any Brazilian vampire bat-related rabies. Moreover, when a phylogenetic tree was created with non-haematophagous bat isolates, group C clearly belonged to the clade of vampire bat-related viruses (data not shown). Thus, this group is likely associated with vampire bats but not with non-haematophagous bats such as insect bats. Therefore, it is possible that distinct bat-transmitted rabies strains may exist in MA or in northern Brazil.

This is the first epidemiological investigation of rabies in MA and surrounding states. In this paper, we showed that bat- and dog-transmitted rabies occur in northwestern Brazil, and implied that the diversity of rabies virus associated with wildlife in Brazil differs from conventional views. However, this study only covered isolates from the eastern end of an extensive forest in northern Brazil. Vaccination campaigns and education about rabies have been got behind in northern Brazil, and dog- and bat-transmitted rabies threaten not only livestock but also humans. Thus, more extensive epidemiological studies and further countermeasures are necessary in these regions.

Acknowledgement

This work was supported in part by the Academic Frontier Project for Private Universities from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan.

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NOTE *Virology*

Geographical Distribution of Vampire Bat-related Cattle Rabies in Brazil

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(Received 7 February 2006/Accepted 1 June 2006)

ABSTRACT. Seventy-seven rabies virus (RV) isolates originating from Brazilian cattle were genetically characterized. Partial nucleoprotein gene sequences of these isolates were phylogenetically and geographically analyzed. Cattle isolates, which clustered with the vampire bat-related RV group, were further subdivided into nine genetic subgroups. These subgroups were distributed widely in lowland regions, with some subgroups separated from each other by mountain ranges. In addition, separation of the groups in mountainous regions was correlated with altitude. These results indicate that cattle rabies is derived from several regionally-defined variants, which suggests that its geographical distribution is related to that of the vampire bat population.

KEY WORDS: cattle, geographical analysis, rabies.

J. Vet. Med. Sci. 68(10): 1097–1100, 2006

The rabies virus (RV), which belongs to genotype 1 of the *Lyssavirus* genus within the *Rhabdoviridae* family, has an almost global distribution throughout the world. The principal RV reservoirs are known to differ between regions in the areas where the virus occurs, and dogs and vampire bats (*Desmodus rotundus*) are the major known RV vectors in Brazil. Vampire bats, which are widely distributed in Latin America, transmit RV to herbivore species and are recognized as an important RV vector in herbivore rabies [2, 3, 10]. Vampire bats live in small colonies of 10 to 300 animals and range within an area of activity of between 10 and 20 km^2 [2, 14]. During the period of 1993–2002, 31,187 cases of cattle rabies were reported in Latin America, and vampire bat-transmitted RVs have had an economic impact in the livestock industry [4, 10]. In this study, to define the epidemiological characteristics of vampire bat-transmitted rabies, we performed a phylogenetic analysis and related the findings to the geographical distribution of 86 vampire bat-transmitted RV isolates from cattle in several states in Brazil.

Seventy-seven of the RV isolates were collected from cattle in the city of Brasília, the Federal District, and the states of Goiás, Maranhão, Minas Gerais, Mato Grosso, Rondônia, Pará, São Paulo, and Tocantins between 1998 and 2003 (Table 1). Additionally, nine isolates were collected from cattle (BRbv30, 32, 36, 38, 39, 43, 45, 49, and 50), eight isolates were collected from vampire bats (BRvmbt33, 34, 41, 46 and 47, and BR-DR1–3), and two isolates were collected from dogs (BRdg10 and 12) as described previously [7, 8]. All cattle isolates were identified as vampire bat-related RV by sequencing and phylogenetic analysis. Viral RNA was extracted from the brains of cattle diagnosed as RV positive by both the direct fluorescence antibody test [5] and the mouse inoculation test [9]. RT-PCR was used to amplify nucleoprotein (N) gene sequences of the Brazilian RV strain as described previously [8] using P1/P2 primer pairs. Sequencing was performed using the methods described in our previous study [8]. The 203 nucleotide sequences corresponding to positions 109–311 of the N gene sequence of the PV strain were determined using the P1 and BRABN-C3 primers. This locus has been reported to be associated with the phylogenetic divergence of clusters and has been used in other phylogenetic studies [1, 7]. The nucleotide sequences were aligned using the Clustal X program [13], and a neighbor-joining tree was constructed using the method of Saitou and Nei [12]. Bootstrap values were calculated using 1,000 replicates, and homologies between nucleotide sequences were identified using the BioEdit software [6].

The nucleotide sequences of the 203 RT-PCR products corresponding to the RV N gene were determined for all 77 RV isolates and were deposited in GenBank (Accession numbers AB246194–246270). The cattle isolates were clustered in nine major subgroups characterized by high nucleotide identities (above 93%) except for a small number of the cattle isolates and these subgroups were distributed in limited areas associated with geomorphology (Fig. 1). The geographic locations of the isolates belonging to the nine subgroups are shown in Fig. 2. Subgroup A consisted of 9 samples from the lowland regions of northern Tocantins and its neighboring states. These samples were distributed among different hydrographic basins separated by several rivers. Subgroup B consisted of three samples from low-lying mountainous regions approximately 300 km to the south of subgroup A. Subgroup C consisted of 13 samples from Brasília and eastern Goiás. These isolates were distributed along low altitude areas in mountainous ranges. Subgroup D consisted of eight cattle and three vampire bat

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Table 1. List of Brazilian cattle isolates used for the phylogenetic analysis

Place of isolation	Identification number
Brasília city	BRbv296
Goiás state	BRbv30[a)],50[a)],56,80,82,86,89,93,94,95,103,106,119, 124,132,138,141,145,147,150,152,153,157,160,161, 162,169,170,181,182,185,187
Maranhão state	BRbv55
Mato Grosso state	BRbv43[a)],45[a)],57,186,192,193,194,195,197,200,202, 206,207,208,210,214,216,217,221,225,229,230,232, 234,235,238
Minas Gerais state	BRbv63,74,282,287
Rondônia state	BRbv68
Pará state	BRbv76
São Paulo state	BRbv32[a)],49[a)],62,64,65,66,69,71,75,118,
Tocantins state	BRbv36[a)],38[a)],39[a)],87,129,133,140,168,178,183,

a) The following data were obtained from GenBank: BRbv30 (AB083803), BRbv32 (AB083805), BRbv36 (AB083809), BRbv38 (AB083810), BRbv39 (AB083811), BRbv43 (AB083813), BRbv45 (AB083814), BRbv49 (AB083817), and BRbv50 (AB083818).

isolates from mountainous regions at altitudes below 800 m in the states of Goiás and São Paulo, but the strength of the node in the phylogenetic tree was low (bootstrap values of 307). Subgroup E consisted of 18 samples that were distributed over an extensive area that included both the mountainous and lowland regions of the states of Goiás and Mato Grosso. Subgroup F consisted of nine samples from the mountainous regions of central Goiás. These isolates were distributed in low altitude regions and were separated from the isolates in subgroup C and E by high mountains. Subgroup G consisted of four samples from lowland regions surrounding mountainous areas between the state of Goiás and Mato Grosso from which subgroups E and F were isolated. Subgroup H consisted of isolates from six cattle and two vampire bats from mountainous regions between the state of Minas Gerais and São Paulo. These samples were isolated in areas that were higher in altitude compared to subgroup D isolated in São Paulo state. Interestingly, although subgroup I consisted of 8 widely distributed isolates from a large lowland region between the state of Goiás and Mato Grosso, the nucleotide identities were 100%.

Phylogenetic analysis of the N gene showed that the cattle isolates were genetically related to the vampire bat RV group and that they formed a clade consisting of nine subgroups. The geographical distributions of each subgroup were separated by mountains, with additional differences observed between the lowland and mountainous regions. The separation attributed to mountain ranges was particularly apparent in subgroups C, E, and F. Although the samples of these subgroups were isolated in areas neighboring each other, the samples could be divided into three phylogenetic groups by the distribution of mountains in the regions where the samples were isolated. In the lowland regions, cattle isolates were widely distributed and separated from the other subgroups by mountains. Epidemiological evidence of herbivore rabies suggests that the clusters originated from the same hydrographic basin in the state of Rio de Janeiro [11]. Furthermore, it has been suggested that bats need to fly longer distances in order to feed in large regions, and that spread of the virus may thus occur in a shorter period of time [11]. In this study, the flatland regions from which subgroups A, E, and I were isolated are characterized as having numerous rivers. However these groups were not separated by rivers and had widespread distributions. These findings, corroborated by Romijn *et al.* [11], suggest that vampire bat-related RV tends to spread in lowland regions. The formation of each subgroup observed in the mountainous regions was correlated with altitude. Subgroup H, which was distributed between 800 and 1,600 m, was separated from subgroup D, which was located at lower altitudes. Furthermore, two vampire-bat isolates, BR-DR1 and 2, were distributed in this area. Vampire bats are known to inhabit areas at altitudes below 2,000 m [3]. In high altitude areas, vampire bat populations may retain specific RV variants.

The present study indicates that cattle rabies is derived from several variants that are regionally well-defined. This finding implies that the geographical distribution of cattle rabies is dependent on that of the vampire bat populations because the major transmitter of cattle rabies is vampire bats [3] and vampire bats live in colonies with a limited migratory range [2, 14]. Moreover, the transmission of vampire bat-related RV appears more prevalent in flatland regions and is limited by the distribution of mountains. Similarly, RV circulation was observed to be related to altitude. This therefore reveals that the geographic isolation of bat populations, especially in mountainous and lowland areas, is an important factor in the development of the epidemiological characteristics of vampire bat-transmitted RV.

ACKNOWLEDGMENTS. This work was partly supported by the Academic Frontier Project for Private Universities from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan, the Grant-in Aid for Sci-

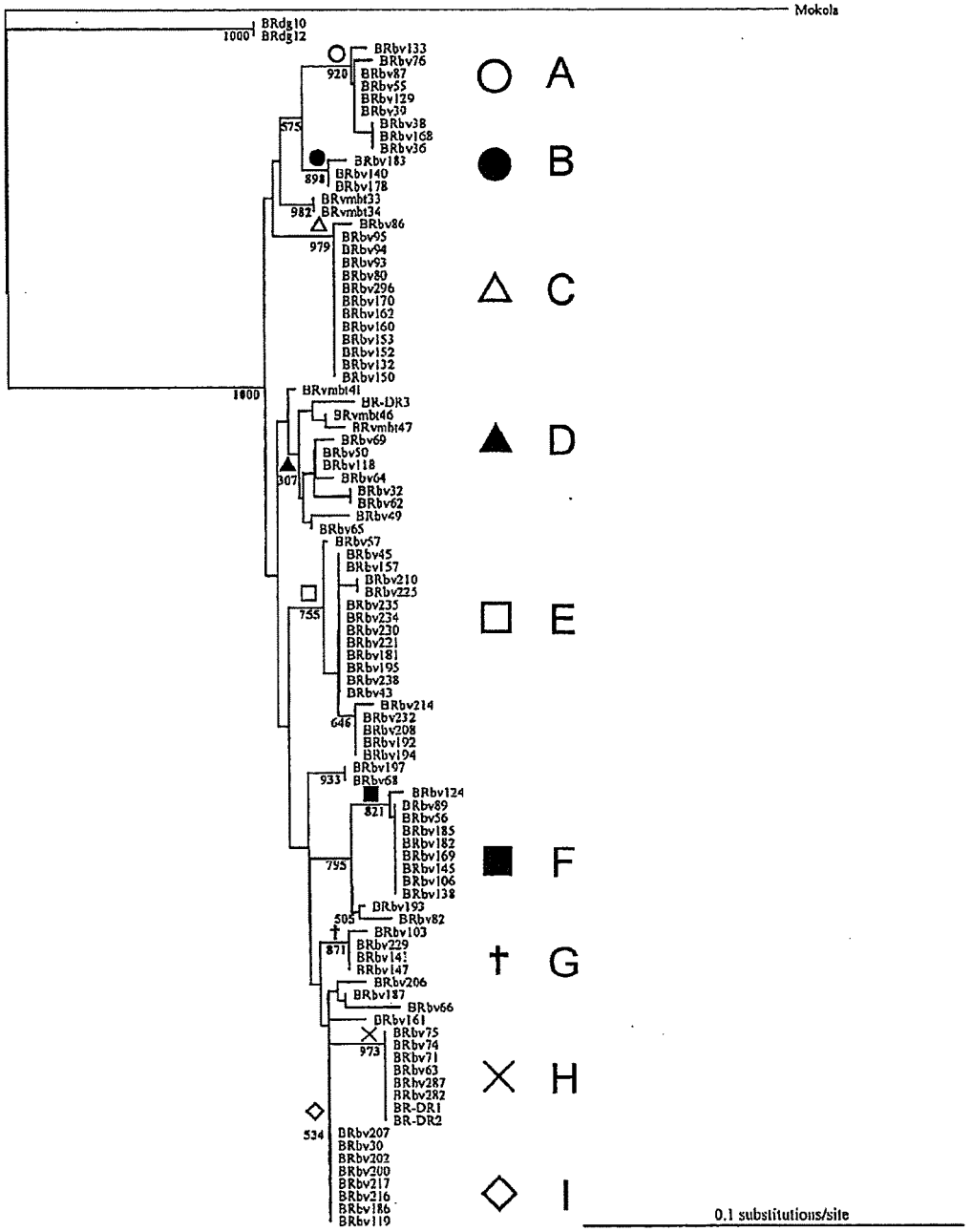

Fig. 1. Phylogenetic tree based on a partial N gene sequences. Mokola virus was used as outgroup. The Brazilian dog isolates are as follows: BRdg10 (AB083796) and BRdg12 (AB083797). The vampire bat isolates are as follows: BRvmbt33 (AB083806), BRvmbt34 (AB083807), BRvmbt41 (AB083812), BRvmbt46 (AB083815), BRvmbt47 (AB083816), BR-DR1 (AB201803), BR-DR2 (AB201804), and BR-DR3 (AB201805). These isolates were employed in previous reports (Ito *et al.* 2001 and Kobayashi *et al.* 2005). The symbols correspond to the nine subgroups (groups A to I). Bootstrap values, determined from 1,000 replicates of the data, are shown to the left of all major branches.

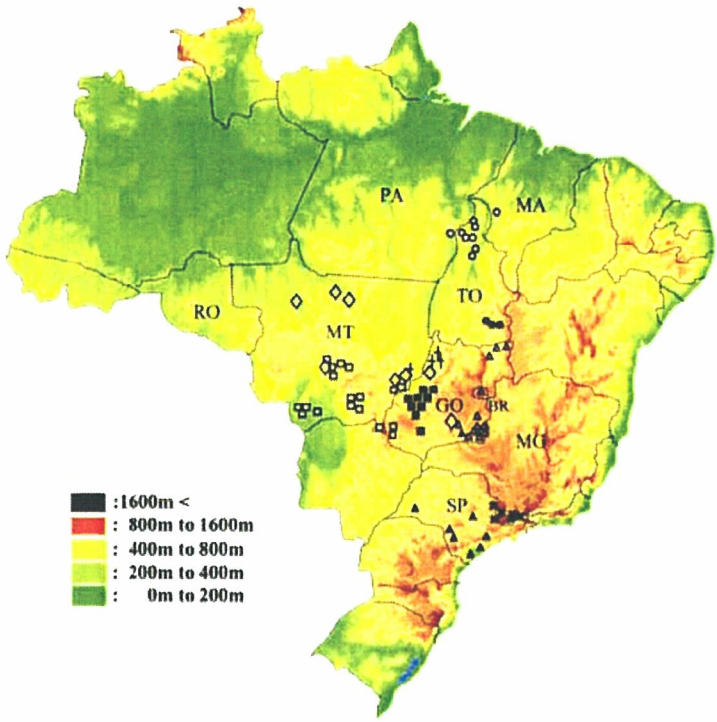

Fig. 2. Geographical distribution of cattle isolates clustered into nine subgroups in the phylogenetic analysis. The symbols correspond to Fig. 1. Subgroup A, ○; subgroup B, ●; subgroup C, △; subgroup D, ▲; subgroup E, □; subgroup F, ■; subgroup G, +; subgroup H, ×; subgroup I, ◇. City and state abbreviations are as follows: BR, Brasília city; GO, Goiás State; MA, Maranhão State; MG, Minas Gerais State; MT, Mato Grosso State; RO, Rondônia State; PA, Pará State; SP, São Paulo State; TO, Tocantins State.

entific Research B from Japan Society for the promotion Science, and the grant for the Research on Emerging and Re-emerging Infectious Diseases, Ministry of Health, Labour and Welfare, Japan.

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希望審査分野：ウイルス学

分類：原著

表題：Phylogenetic Characterization of Rabies Virus Isolates from Carnivora in Brazil

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RUNNING HEAD: ANALYSIS OF CARNIVORA RABIES IN BRAZIL

ABSTRACT. The incidence of canine rabies has been widely reported in Brazil, and new rabies virus (RV) variants, genetically similar to canine RV, have recently been isolated from foxes. In order to derive the epidemiological characteristics of Brazilian Carnivora RV, Brazilian RVs isolated from dogs, cats, and foxes were genetically analyzed. Brazilian Carnivora RV isolates were divided into two main lineages. The predominant lineage was found in dogs and cats, which included the Argentinean and Bolivian Carnivora RV isolates, and was extensively distributed throughout Brazil and surrounding countries. The other lineage consisted of three sublineages containing Brazilian dog and fox RV isolates, with the dog sublineages located on an internal branch of two fox sublineages, suggesting that RV transmission events might have occurred between foxes and dogs in the past. These results suggest that contact between dogs and wildlife has the potential to generate new rabies variants and that it is important to control RV infection cycles in both dogs and wildlife to prevent spread of rabies infection.

KEY WORDS: Brazil, Carnivora rabies, phylogenetic analysis, rabies virus

INTRODUCTION

Rabies virus (RV) belongs to genotype 1 of the Lyssavirus genus of the Rhabdoviridae family and is capable of infecting all warm-blooded animals to cause a lethal form of encephalitis [15]. The orders of Carnivora and Chiroptera are known to be the principal RV reservoirs of the virus. The epidemiological cycle is divided into two different forms: urban rabies, in which the primary transmitter to human is dogs, and sylvatic rabies, in which the transmitter and reservoir is one of several wildlife species.

Rabies is an endemic disease in Brazil, and dogs and vampire bats are known to transmit RV to both humans and livestock. Although the incidence of canine rabies has tended to decrease in response to effective vaccination programs undertaken in urban areas [1], during the period of 1993 to 2002, the highest numbers of canine and human rabies cases transmitted by dogs in the Pan-American region were recorded in Brazil [1]. Recently, new RV variants exhibiting genetic characteristics similar to canine rabies have been isolated from foxes in northeastern Brazil [2, 4, 23]. Additionally, RVs isolated from dogs in the state of Maranhão were found to consist of two distinct phylogenetic groups, with one group being genetically similar to the fox RV variant [22]. However, the phylogenetic relationship among RVs isolated from foxes and dogs is uncertain. Since dogs are primary transmitters of RV and therefore pose unique problems within the context of public health, epidemiological study of dissemination of rabies among wildlife and dogs is important.

Phylogenetic analysis and surveillance data increase our understanding of the epidemiological characteristics of RVs and contribute to development of prophylaxes against viral infection. In this study, to derive the epidemiological characteristics of Brazilian Carnivora RVs, we analyzed RVs isolated from dogs, cats, and foxes from Brazil.

MATERIALS AND METHODS

Viruses: Thirty-seven brain samples (32 dogs and 5 cats) were collected in the states of Goiás, Maranhão, Minas Gerais, São Paulo, and Rio de Janeiro between 1985 and 2005 (Table 1). These samples were diagnosed as rabies positive using the immunofluorescence antibody assay and mouse inoculation test [7, 18]. In addition, 12 isolates were collected from dogs, 8 from foxes, 11 from cats, 2 from insectivorous bats, and 2 from vampire bats as reported previously [12, 17, 23].

RT-PCR and sequencing: Extraction and purification of viral RNA, RT-PCR, and sequencing were performed as described previously [17]. The primers for RT-PCR and sequencing are shown in Table 2. The RHN1/RHNS3 primer set was used for amplification of the nucleoprotein (N) coding region of dog-related RV [14]. For the BRct319 and BRdg339 isolates, which did not yield detectable products, RT-PCR was performed using the P1/N8 primer set, which was designed previously for detection of the N gene of vampire bat-related RV [13]. Similarly, direct sequencing was performed using specific primers based on the nucleotide sequences of the dog- and vampire bat-related RVs [13].

Phylogenetic analysis: Phylogenetic trees were generated using the neighbour-joining method of Saitou and Nei [21, 24]. Bootstrap values were calculated using 1,000 replicates, and homologies and multiple alignments between nucleotide sequences were identified using the BioEdit software [11].

RESULTS

Phylogenetic analysis revealed two major genetic clusters corresponding to the Carnivora and Chiroptera RV variants (Fig. 1). The Brazilian Carnivora RV isolates from dogs, cats and foxes were grouped within the Carnivora RV cluster of variants with a high bootstrap value

(bootstrap value: 919), with the exception of BRct319 and BRdg339. BRct319 and BRdg339 belonged to the Chiroptera RV variant cluster and were closely related to BR-DR1 and BR-DR2, which are vampire bat isolates. The Brazilian Carnivora RV isolates formed a monophyletic group and were divided into two major lineages identified as lineage A, containing domestic dogs and foxes, and lineage B, containing domestic dogs and cats. RV isolates from domestic dogs and foxes in Argentina and Bolivia were observed to cluster into two major groups, with one group, which was typed as antigenic variant (AgV) 2 [5, 9], belonging to lineage B (data not shown). Another group, which was typed as AgV1 [5, 9], was located at a remote position from the cluster containing the Brazilian Carnivora RV variants. Lineage A was further divided into three sublineages (A-1, A-2, and A-3). Sublineage A-1 consisted of RV isolates from dogs in the state of Maranhão, and sublineages A-2 and A-3 consisted of fox RV isolates from the state of Paraíba (Fig. 2). Lineage B was further divided into two sublineages (B-1 and B-2). Sublineage B-1 consisted of RV isolates from dogs and a cat from the states of Maranhão, Rio de Janeiro, and Minas Gerais in Brazil and Bolivian dog RV isolates. Sublineage B-2 consisted of RV isolates from dogs and cats from the states of São Paulo, Minas Gerais, and Goiás.

DISCUSSION

RV transmissions among dogs and wild canids has been reported in Africa, Europe, Colombia, Mexico, and Turkey, and infection of wildlife species contributes to the difficulties associated with controlling and eradicating rabies in wildlife [3, 16, 19, 20, 25]. Fox RV isolates from northeastern Brazil have been reported to form specific clades that group independently of dog RV isolates despite evidence suggesting that dog and fox rabies are derived from a common ancestor [2, 4, 22, 23]. In this study, although lineage A consisted primarily fox RV isolates, several RV isolates from sublineage A1, which consisted of dog RV isolates, were placed within

sublineages of A2 and A3, which consisted of fox RV isolates. One dog infected with fox-typed RV was found in the state of Pernambuco in northeastern Brazil [4]. While not conclusively demonstrated, it is postulated that the dog RV isolates of sublineage A-1 may have been directly or indirectly transmitted by rabid foxes, and the virus might have been spread in the dog population of northeastern regions in the past. RV transmission events among wildlife and dogs have the potential to generate new RV variants, which complicates control of viral infectious disease, and RV transmission from wildlife to dogs especially increases public health problems. Therefore, further epidemiological analysis of these RV variants is needed. Additionally, dog and cat RV isolates that were genetically very similar to vampire bat RV isolates were found in this study, suggesting that these Carnivora RV isolates were transmitted by vampire bats.

Lineage B, which consisted of dog and cat RV isolates, contained RV isolates from Brazilian, Argentinean, and Bolivian Carnivora with a wide geographical distribution that were collected along the borders of Bolivia, Paraguay, and Brazil and typed as AgV2 [5, 9]. Analyses using monoclonal antibody panels revealed that RVs isolated from dogs in Latin America can be characterized as AgV 1 or 2 and that these AgVs correspond to the two phylogenetic groups [5, 9]. AgV1 was found in dogs throughout Latin America, while AgV2 was only isolated in dogs from Argentina, Bolivia, Brazil, and Paraguay [8, 10]. Since the Carnivora-related RVs isolated in Brazil consisted only of the AgV2 type, the AgV2 type is referred to as the Brazilian-type RV [4, 8, 10]. Furthermore, given that dogs migrate with humans along transport routes, such as roads and borders [5, 6, 9], it seems likely that lineage B would be maintained within the dog reservoir in extensive regions of Brazil and that it might spread from Brazil into neighboring countries with human migration. Rabies incidences associated with dogs and coyotes have been reported at the border between the United States and Mexico, suggesting that the coyote RV variant may have emerged

via dog translocation at some time in the past [25]. These findings suggest that controlling both canine rabies and dog translocation is essential for preventing the spread of rabies infection.

Contact between dogs and wildlife has the potential to generate new rabies variants. To prevent future spread of rabies infection and the incidence of new rabies variants, it is important to control RV infection cycles in both dogs and wildlife. In addition, we believe that both continuation of vaccination programs for dogs and surveillance of wildlife rabies are necessary.

ACKNOWLEDGMENTS. This work was supported in part by the Academic Frontier Project for Private Universities from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan, a Grant-in Aid for Scientific Research B from the Japan Society for the Promotion Science, and a grant for Research on Emerging and Re-emerging Infectious Diseases from the Ministry of Health, Labour and Welfare of Japan.

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