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The emergence of wildlife species as a source of human rabies infection in Brazil

S. R. FAVORETTO[1,2]*, C. C. DE MATTOS[3], C. A. DE MATTOS[3], A. C. A. CAMPOS[2], D. R. V. SACRAMENTO[2,4] AND E. L. DURIGON[2]

[1] *Instituto Pasteur de São Paulo, São Paulo, Brazil*
[2] *Institute of Biomedical Sciences, University of Sao Paulo, Sao Paulo, SP, Brazil*
[3] *Centers for Disease Control and Prevention, Atlanta, GA, USA*
[4] *Genomic Molecular Engineering Laboratory, São Paulo, Brazil*

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SUMMARY

Forty-five human rabies virus isolates from a wide geographical area of Brazil were characterized using an anti-nucleoprotein monoclonal antibody panel and by partial nucleotide sequencing analysis of the nucleoprotein gene. Three major antigenic groups related to the antigenic variants maintained in domestic dogs, vampire bats and marmosets were identified. Phylogenetic analyses revealed that the viruses from dog-related cases segregated into four sister clades: three associated with dog-endemic cycles in Brazil and one with the crab-eating fox cycle in the northeastern region of the country. The vampire bat- and marmoset-related viruses formed two independent groups. The topology of these clades was conserved when these samples were compared to virus representatives of the currently reported rabies endemic cycles in the Americas. These results indicated the presence of multiple endemic transmission cycles maintained in four different reservoirs, domestic dogs, crab-eating foxes, vampire bats and marmosets, which are being transmitted directly to humans and should be considered as a high-risk for rabies infection.

Key words: Host (in infections), molecular biology, rabies (human), virus infection, zoonoses.

INTRODUCTION

If the image of a snarling dog can be used to indicate the presence of rabies, then this image is the earliest record of an infectious disease. Oddly enough, in the early 21st century, 50 centuries after its discovery rabies can still be classified as an emerging infection. The epidemiology and pathogenesis of rabies are full of surprises that we have faced as long ago as the last century and as recently as a few weeks ago.

(Koprowski H, 'Raiva no Século XXI' conference, XV National Meeting of Virology, Sao Pedro-SP, Brazil, 27 September 2004)

* Address for correspondence: S. R. Favoretto, Ph.D., Instituto Pasteur of São Paulo, Brazil, Av. Paulista, 393 Paraíso, São Paulo, SP, Brasil, CEP 01311000.
(Email: srfavoretto@usp.br)

Rabies virus (RABV) has the highest fatality rate of all known human viral pathogens. With fewer than a handful of exceptions, humans who develop a symptomatic RABV infection inevitably die [1, 2]. Rabies occurs in two different cycles, the urban cycle, with the dog as the main reservoir, and the sylvatic cycle, with different wildlife species acting as reservoirs or transmitters; these cycles result in the maintenance of rabies, which varies regionally [3].

Globally, based on model predictions it is estimated that rabid dogs are the source of many thousands of deaths, about 55000 estimated each year [4], with the highest mortality in Asia and Africa [5].

In Latin America, the domestic dog acts as the main reservoir and transmitter of RABV [6].

However, a large number of other mammals can be infected and can also act as the viral transmitter. The principal epidemiological cycle of rabies in sylvatic animals throughout Brazil is maintained in the common vampire bat (*Desmodus rotundus*). Vampire bats are the source of outbreaks in cattle, but they can also infect humans, terrestrial wildlife, and other domestic animals; in addition, within non-haematophagous bats, rabies is widely distributed as well. Other wild species, such as *Cerdocyon* (*Cer.*) *thous* (crab-eating fox) and *Callithrix* (*Cal.*) *jacchus* (marmoset), play an important role in the transmission of rabies in the northeastern region of Brazil and maintain distinct cycles of transmission [7–9].

Based on antigenic and genetic analyses of the viral genome, the RABV circulating in Latin America belongs to the RABV species, phylogroup I of the genus *Lyssavirus* [10]. Techniques involving the use of monoclonal antibodies (MAbs) and molecular biology have provided a more precise understanding of rabies epidemiology, distinguished individual cases and focal outbreaks, identified new cycles, and determined the source of infection in cases of unknown exposure. These studies, conducted in the Americas and Europe, as well as in several African and Asian countries, have identified different strains that persist in certain hosts within defined geographical boundaries [3, 8, 10–15]. Awareness of the presence of the virus in different species of domestic and wild animals and of the circumstances under which humans have been exposed to the disease can clarify the reservoirs responsible for maintaining this virus in nature [16].

A MAbs panel was utilized to characterize the antigenic variants (AgVs) and the possible reservoirs found in the Americas [17]; since then, a brief panel with eight of these MAbs has been used for antigenic characterization of samples isolated from the Americas [18]. The AgVs designated AgV1 and AgV2, maintained in dog populations, and AgV3, related to vampire bats, have been identified, as well as AgVs such as AgV4 and AgV6, related to non-haematophagous bats, and other until then unknown AgVs [7, 11, 17, 18–20].

A 93% reduction in human rabies cases has been observed in Latin American countries in the last two decades since the implementation of rabies dog control programmes, while the number of cases transmitted by bats has increased by 65% [21].

Until 2003, domestic dogs were the major disease transmitters, responsible for 72·5% of human cases, while bats were responsible for only 12·8% of cases [1]. However, human incursion into previously uninhabited environments has increased the frequency of contact between humans and bats. Of the 74 human cases reported between 2004 and 2005 in the northern region of Brazil, 64 (86·5%) were due to contact with haematophagous bats [6, 22]. In the last 5 years, during the period 2008–2011, 10 human cases were reported to the authorities, of which five (50%) were due to contact with sylvatic animals. During the same period, 696 rabies cases were reported in wildlife across the country [1, 6]. In 2006, a study performed in Ceará state, Brazil [7] reported on the rabies epidemiological situation in a small area of the country and described the main reservoirs involved in rabies cases. That study demonstrated the existence of reservoirs and transmitters in both urban and wild areas that were perpetuating the virus in this region.

Until 2005, Brazil had reported the majority of human rabies cases in Latin America. In most of these countries, socioeconomic and cultural conditions presumably play an important role in rabies epidemiology [21, 23]. In the last 10 years, 92·1% of human rabies cases in Brazil have been reported in the less developed states of northern and northeastern Brazil [1], whereas dog-related rabies is controlled in the more developed southern and southeastern states, where only sporadic cases are reported [6].

Wild carnivores and bats present a considerable risk in areas where the disease is found. Therefore, the aim of this study is to demonstrate the role of the four different reservoirs present in Brazil in the direct transmission of RABV to humans. This study will provide data on whether these species are really implicated as important sources of infection for humans in Brazil.

MATERIALS AND METHODS

Forty-five human RABV isolates obtained between 1997 and 2003 from the northern, northeastern, southeastern and middle-west regions of Brazil were antigenically and genetically characterized. Their geographical origin, year of isolation and state of origin are presented in Table 1 and Figure 1.

Antigenic characterization was performed using a MAbs panel against viral nucleoprotein (Centers for Disease Control and Prevention, USA) and was provided by the Pan American Health Organization (PAHO) against the viral nucleoprotein as described previously [18].

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Table 1. *Antigenic and genetic characterization of the 45 RABV samples isolated from humans in different states of Brazil, during 1997–2003*

Sample ID	Year of isolate	Geographical region	State of origin	Antigenic variant	Genetic lineage	Group/ subgroup	Accession no. (Genbank)	Ref.
Brhm4119	1997	North	Acre	AgV2	Dog	A1	JX217782	This study
Brhm4134	1997	North	Acre	AgV2	Dog	A1	JX217784	This study
Brhm4116	1997	North	Rondônia	AgV2	Dog	A1	JX217780	This study
Brhm4118	1997	Noth	Tocantins	AgV2	Dog	A1	JX217781	This study
Brhm4137	1998	North	Pará	AgV2	Dog	A1	JX217793	This study
Brhm4107	1998	North	Rondônia	AgV2	Dog	A1	JX217787	This study
Brhm4117	1998	North	Rondônia	AgV2	Dog	A1	JX217788	This study
Brhm4128	1998	North	Rondônia	AgV2	Dog	A1	JX217791	This study
Brhm4126	1998	North	Tocantins	AgV2	Dog	A1	JX217790	This study
Brhm4099	1998	North	Tocantins	AgV3	Vampire bat	B	JX217785	This study
Brhm5385	1999	North	Rondônia	AgVLab	Vampire bat	B	JX217798	This study
Brhm5308	2000	North	Acre	AgV2	Dog	A1	JX217799	This study
Brhm5363	2000	North	Acre	AgV2	Dog	A1	JX217802	This study
Brhm5393	2000	North	Pará	AgVLab	Vampire bat	B	JX217805	This study
Brhm5375	2000	North	Rondônia	AgV2	Dog	A1	JX217803	This study
Brhm5376	2000	North	Rondônia	AgV2	Dog	A1	JX217804	This study
Brhm5313	2000	North	Rondônia	AgVLab	Vampire bat	B	JX217800	This study
Brhm4093	1997	Northeast	Bahia	AgVLab	Dog	A1	JX217779	This study
Brhm4531	1997	Northeast	Ceará	AgV2	Crab-eating fox	A4	DQ447947	[7]
Brhm4138	1998	Northeast	Ceará	Nova*	Marmoset	C	AY654587	[8]
Brhm4097	1998	Northeast	Ceará	Nova*	Marmoset	C	AY654585	[8]
Brhm4121	1998	Northeast	Paraíba	AgVLab	Dog	A3	JX217789	This study
Brhm4543	1998	Northeast	Pernambuco	AgVLab	Dog	A3	JX217794	This study
Brhm4130	1998	Northeast	Pernambuco	AgV2	Dog	A1	JX217792	This study
Brhm4558	1999	Northeast	Pernambuco	AgV2	Dog	A1	JX217797	This study
Brhm5325	2000	Northeast	Ceará	AgV2	Dog	A1	DQ447949	[7]
Brhm5348	2001	Northeast	Bahia	AgVLab	Dog	A1	JX217806	This study
Brhm5691	2001	Northeast	Ceará	AgV2	Dog	A1	DQ447954	[7]
Brhm5700	2001	Northeast	Piauí	New*	Marmoset	C	JX217808	This study
Brhmu1403	2003	Northeast	Bahia	AgV3	Vampire bat	B	JX217813	This study
Brhmu142	2003	Northeast	Ceará	AgV2	Dog	A1	DQ447963	[7]
Brhmu130	2003	Northeast	Ceará	AgV2	Dog	A1	DQ447964	[7]
Brhmu146	2003	Northeast	Ceará	AgV2	Dog	A1	DQ447965	[7]
Brhmu145	2003	Northeast	Ceará	AgV2	Dog	A1	DQ447966	[7]
Brhmu129	2003	Northeast	Ceará	AgV2	Dog	A1	DQ447967	[7]
Brhmu131	2003	Northeast	Ceará	AgV2	Dog	A1	DQ447968	[7]
Brhmu138	2002	Northeast	Ceará	AgV2	Dog	A1	DQ447962	[7]
Brhmu1303	2003	Northeast	Maranhão	AgV2	Dog	A1	JX217812	This study
Brhm4103	1998	Middle-west	Goiás	AgV3	Vampire bat	B	JX217786	This study
Brhm4548	1999	Middle-west	Goiás	AgV2	Dog	A2	JX217795	This study
Brhm5322	2000	Middle-west	Goiás	AgV2	Dog	A2	JX217801	This study
Brhm4555	1999	Southeast	Minas Gerais	AgV3	Vampire bat	B	JX217796	This study
Brhm5704	2001	Southeast	Minas Gerais	AgV2	Dog	A1	JX217809	This study
Brhm5699	2001	Southeast	São Paulo	AgV3	Vampire bat	B	JX217810	This study
Brhmu1203	2003	Southeast	Espírito Santo	AgV3	Vampire bat	B	JX217811	This study

AgVLab, Reagent to all monoclonal antibodies; AgV2, antigenic profile related to RABV maintained by the dog population; AgV3, antigenic profile related to RABV maintained by the haematophagous bat (*Desmodus rotundus*) population.
* Antigenic profile related to marmosets (*Callithrix jacchus*).

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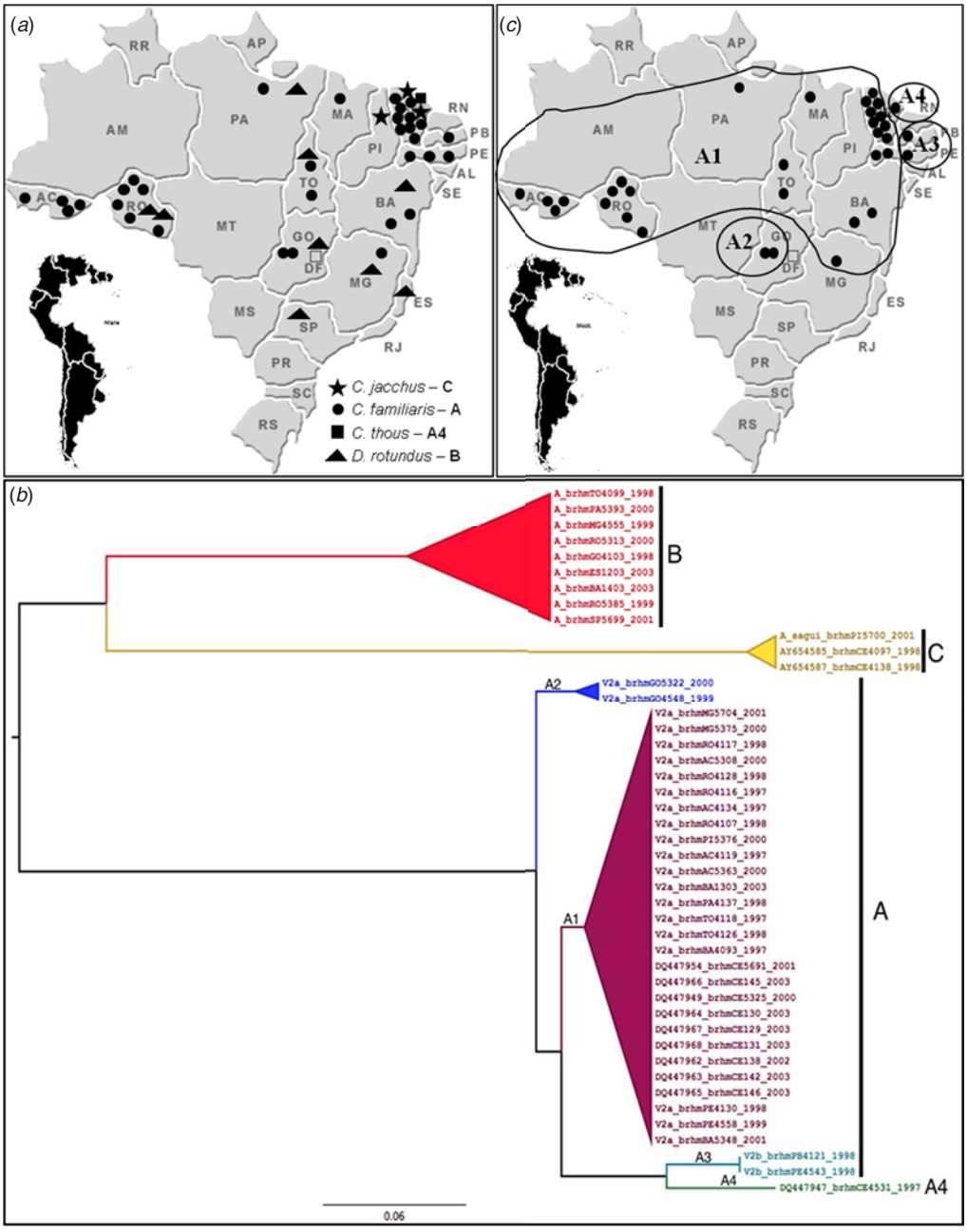

Fig. 1 [*colour online*]. Maps and phylogenetic tree of rabies virus (RABV) distribution of 45 Brazilian human samples studied; samples were collected during 1997–2003. (*a*) Map of RABV distribution in the geographical study region according to genetic lineage representative of four major reservoirs: A, B, C and A4. (*b*) Phylogenetic tree of 45 RABV samples related to different cycles of transmission. (*c*) Map showing distribution of Brazilian human samples studied; samples were collected during 1997–2003 in the geographical study region according to genetic lineage related to antigenic variant 2 (AgV2) maintained in dogs with four major subclades: A1, A2, A3 and A4.

Partial gene sequence data of the viral nucleoprotein gene (positions 1157–1476) were analysed after a reverse transcriptase–polymerase chain reaction procedure using primers 21 g/304, as described previously [13, 24]. Sequencing of these PCR products was performed with Big Dye Terminator Cycle Sequencing Ready Reaction (Applied Biosystems, USA) and processed on the ABI Prism™ 377 DNA Sequencer (Applied Biosystems) [13, 24]. The sequence alignments were performed using ClustalW in BioEdit (available at http://www.mbio.ncsu.edu/bioedit/bioedit.html). The average intrinsic distance (AID) was

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determined using molecular evolutionary genetics analysis (MEGA) 4 software (available at http://www.megasoftware.net/mega4/mega.html). The phylogenetic analyses were performed using the genetic algorithm for rapid likelihood inference (GARLi) program (available at http://www.bio.utexas.edu/faculty/antisense/garli/Garli.html). Phylogenetic trees were reconstructed using the maximum-likelihood (ML) method with Kimura-2 evolutionary distance correction statistics and the branching pattern was statistically evaluated by bootstrap analysis of 1000 replicates with the GARLi program to determine the confidence value of each node. Trees were converted to a graphics format using the FigTree program (http://tree.bio.ed.ac.uk) (Fig. 2).

RESULTS

The antigenic characterization of the RABV isolates that were studied revealed four different antigenic patterns. AgV2 was identified in 29 (64·4%) samples. The variant maintained in vampire bats (*D. rotundus*), AgV3, was found in six (13·3%) isolates, and three (6·6%) samples showed an antigenic profile characteristic of a previously reported variant that circulates in marmosets (*Cal. jacchus*) in northeastern Brazil [8]. Seven (15·5%) samples were positive for all MAbs in the panel (Table 1).

The geographical distribution of the genetic lineage is shown in Figure 1. All samples belonged to RABV species of the *Lyssavirus* genus [25]. The phylogenetic analyses revealed three main lineages, related to clades termed A, B, and C, a result that was supported by high bootstrap values. Clade A included viruses circulating in dogs, clade B included isolates from cases involving human exposure to vampire bats, and clade C included isolates from cases related to human exposure to marmosets in northeastern Brazil (Fig. 1*b*).

Clade A contained 29 AgV2 isolates from 1997 to 2003 and four isolates that were reactive with all MAbs in the panel. Clade A was formed by four subclades, designated A1, A2, A3 and A4 (Fig. 1*b, c*). The genetic distances between these subgroups ranged from 5·8% to 11%. Subclade A1 was formed by 26 isolates from the northern, northeastern and southeastern regions of Brazil, collected between 1997 and 2001, as well as two samples with a positive reaction to all MAbs in the panel. The within-group AID was 1·2%. Subclade A2 included two viruses from the middle-west region, collected in 1999 and 2000, with a within-group AID of 1·3%. Subclade A3 contained two isolates from a restricted geographical area in the northeastern region of Brazil that were obtained in 1998 and showed a positive reaction to all MAbs in the panel. Compared to rabies isolates from dogs and wildlife from the rest of Brazil and the Americas, these isolates segregated with a sample isolated from a dog in the state of Ceará (Brdg5693) in 2001 (Fig. 1*b, c*) showing a within-group AID of 2·2%. Subclade A4 was formed by the isolate BRhmCE4531_1997 from the northeastern region. The between-group AID ranged from 6·9% to 10·3% compared to the other subclades and was grouped with viruses obtained from *Procyon cancrivorous* and *Cer. thous*, by comparison with RABVs from Brazilian wildlife species (Fig 2).

Clade B was formed by nine (20%) isolates from rural and urban areas in a wide geographical region (north, northeast, middle-west, southeast) obtained during 1998–2003. Six were antigenically characterized as AgV3, and three reacted with all the MAbs in the panel. When compared to viruses from the Americas' *Chiroptera* species, they clustered with isolates from *D. rotundus* and *D. rotundus*-related cases from Latin America that were isolated from frugivorous bats, dogs, humans and livestock (bootstrap value 94), and with a within-group AID of 3·8% (Fig. 2).

Clade C comprised three isolates from two different states from the northeast (Fig. 2). These sequences showed a within-group AID of 1·0%, but showed low similarity when compared to other lineages circulating in Brazil and worldwide. The nucleotide divergence of this lineage with clades A and B was 29·2% and 26·3%, respectively.

A similar topology was observed when additional comparisons were conducted with isolates maintained in marmosets, other terrestrial mammals, and bat species in the Americas (Fig. 2). The isolates belonging to lineages A, B and C continued to segregate with high statistical support (bootstrap value 100), revealing a distant genetic relationship, and when the between-group AID was observed, these groups were maintained. The within-group AID remained when the samples were analysed individually (A1 = 2·5%, A2 = 1·0%, A3 = 2·2%, A4 = 2·7%, B = 3·1%, C = 1·0%).

At the antigenic site of the nucleoprotein, at residues 377–379 [based on the Pasteur virus (PV) strain], the presence of amino-acid residues Thr-Asp-Val (TDV) was observed in the PV strain of the dog and

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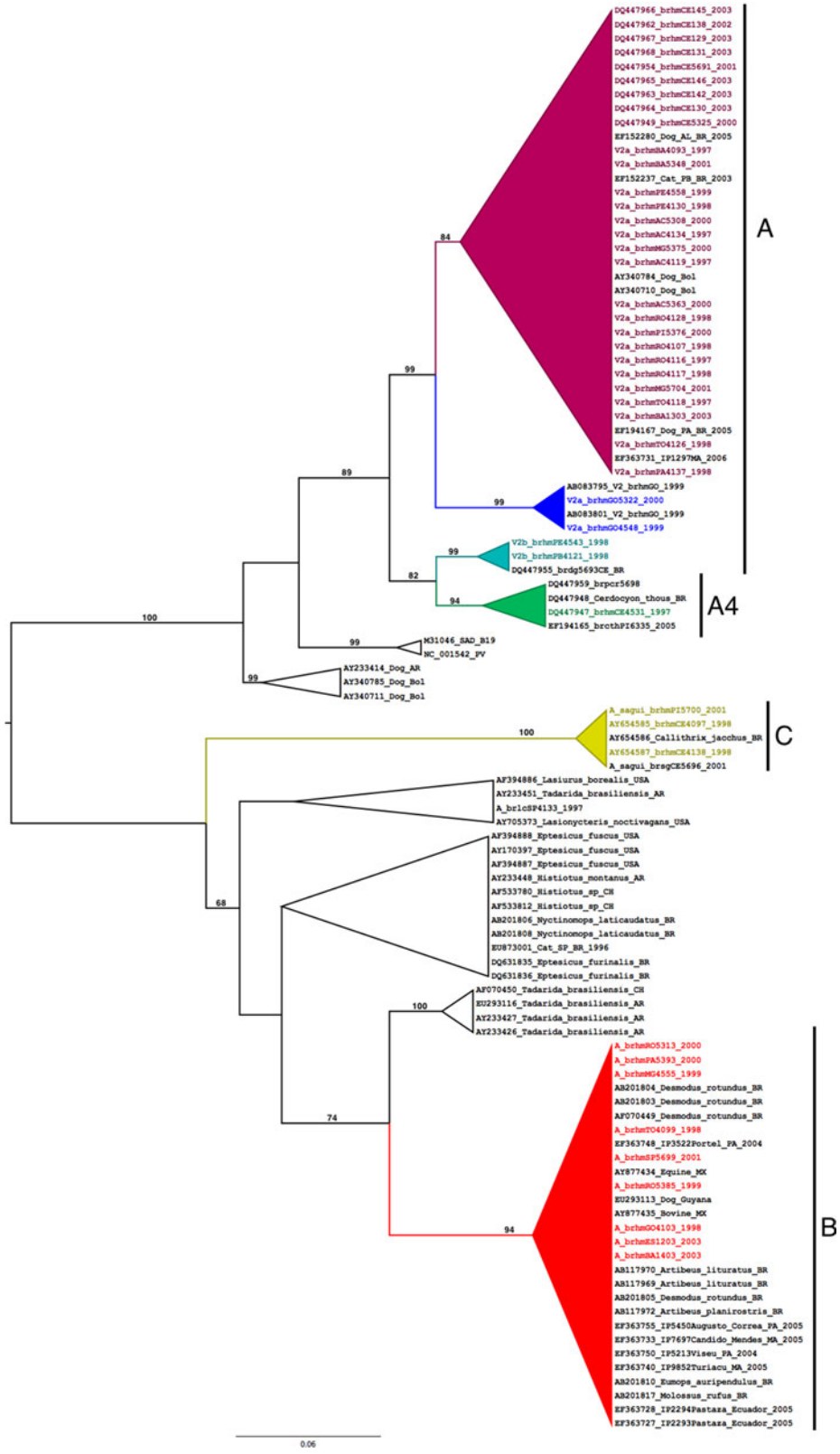

Fig. 2 [*colour online*]. Phylogenetic tree with samples from Brazil and the Americas reconstructed by maximum likelihood using the GARLi program. Bootstrap values were obtained from 1000 resamplings. Only bootstrap values >60% are shown at the branching points. The phylogenetic groups A, B, C and A4 previously observed are maintained after this analysis.

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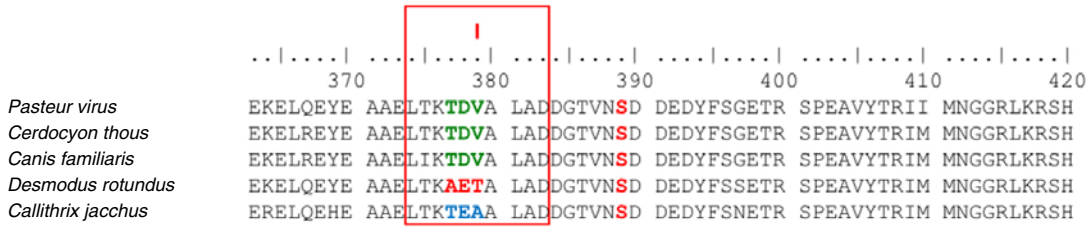

Fig. 3 [*colour online*]. Gene partial alignment of nucleoprotein antigenic site with Pasteur virus, *Cerdocyon thous, Desmodus rotundus*, and *Callithrix jacchus* samples. Amino-acid substitutions are related to different endemic cycles.

crab-eating fox (*Cer. thous*) samples; however, a change, to Ala-Glu-Thr (AET), was observed in the *D. rotundus* isolates, and another change, to Thr-Glu-Ala (TEA), was observed in the *Cal. jacchus* isolates (Fig. 3).

DISCUSSION

A thorough description of rabies epidemiology depends on a comprehensive surveillance programme and the application of accurate molecular methods to discriminate the presence of different variants and the emergence of new foci [13]. Antigenic and sequencing analyses were used in this study to better understand the epidemiological events in human rabies in Brazil between 1997 and 2003.

The reactions observed in the MAbs panel against the viral nucleoprotein identified the presence of three AgVs previously related to hosts and reservoirs described in the Americas and particularly in Brazil [7, 9, 13, 20, 26–30]. AgV2 was observed in all regions of the country where human rabies cases were reported, confirming that in this period, the dog was the most important reservoir of rabies transmission to humans. Additionally, AgV3 and the AgV previously observed to be marmoset-related also transmitted rabies to humans [8, 9, 20]. The reports related to these variants, similar to the case of rabies transmitted by a raccoon, occurred mostly in urban areas, although some studies have described the transmission locations as wild or rural areas.

The genetic analyses of the human rabies isolates and their comparison with RABVs from dogs and wildlife from Latin America corroborated the dog as the main source of infection for humans during this period and revealed the presence of multiple cycles of RABV transmission maintained in the Brazilian dog population.

This study also highlights the increasing importance of terrestrial and arboreal wildlife species as a source of infection for humans. Due to the success of the dog rabies control programmes, it is probable that human rabies exposure in Brazil has been moving towards to a level that cannot be further reduced without targeting wildlife. Most rabies cases occurring in terrestrial mammals are the result of infection with the virus variant that circulates in the dominant wildlife reservoir host [7, 9].

A genetic lineage previously described in cases of rabies in the crab-eating fox (*Cer. thous*) and in the raccoon (*P. cancrivorous*) was found in a human case from the state of Ceará. *Cer. thous* has been reported as the species responsible for RABV maintenance and transmission of this genetic lineage to humans and wildlife in the states of northeastern of Brazil [1, 7, 9]. This genetic lineage could have been established in this canid population after a spillover event from dogs [7, 9].

The crab-eating fox is a medium-sized neotropical canid with generalist habits and a wide distribution in South America. The species is relatively common and omnivorous, and given the disturbance of its natural habitat by human activities, its diet may have switched to cultivated fruits, domestic fowl and refuse. This change in its behaviour may increase the frequency of contact with humans and their animals and, consequently, the risk of rabies transmission.

An epidemiological investigation of an isolate (DQ447947) that segregated in the crab-eating fox subgroup revealed that it was obtained from a child attacked by a raccoon (*P. cancrivorous*) [7]. This suggests that this procyonid could also be a reservoir for this variant or that this particular child was probably infected by a crab-eating fox. These two species are sympatric and have similar feeding behaviours that could facilitate rabies transmission between them.

The common vampire bat is an outstanding sylvatic rabies reservoir in rural areas of Latin America [31]. However, lately, its presence has been expanding to urban centres in Brazil [32–34]. It has been reported

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in cities such as Belo Horizonte city, the third largest Brazilian city, and in Sao Paulo state [35, 36].

During the last decade, human rabies cases related to the *D. rotundus* variant have also been reported in urban areas in Brazil. The Brhm5699 isolate was obtained from a woman admitted to a health service centre in São Paulo state exhibiting clinical symptoms of rabies; she died a few days later. The laboratory results confirmed the presence of RABV in the analysed tissues and identified AgV3 as the cause of disease. This patient resided in an urban area, and her own cat had bitten her at home; the patient sought medical attention for the bite wound, but she was not instructed about the need for rabies post-exposure treatment [37]. Earlier, the cat had captured an unidentified dying bat in the backyard that was not referred for diagnosis. The possible scenarios are that the bat was a *D. rotundus* or a non-haematophagous bat infected by a *D. rotundus* bat, an epidemiological situation that has been reported previously [29]. These events stress the complexity of bats' inter-species interactions and their impact on disease transmission, the rabies-related epidemiological significance of the movement of bat populations over a wide geographical area as a consequence of habitat modification and the need to strengthen public awareness about bat behaviour in urban settings.

Human cases of rabies related to the common vampire bat are more commonly identified and reported in sylvatic areas, e.g. cases that occur in the northern Amazon region in the indigenous population. Although indigenous peoples have suffered from common vampire bat bites for generations, they are not aware of the risk of contracting rabies from these animals; previously, they have attributed the death to a curse in the area and moved the village to a new place. Education on the role of vampire bats in rabies transmission and bite prevention will be a pivotal component of a successful prevention programme [23, 38]. Another important issue to consider in this programme is the rabies pre-exposure immunization of indigenous groups living in remote areas without rapid access to proper medical care and in constant danger of attacks by vampire bats [22, 39].

Interestingly, although rabies has been diagnosed in numerous non-haematophagous bat species, no human case has been described due to lineages maintained in these bats in Brazil. This is in contrast to the epidemiological situation in the USA and Chile, where insectivorous bats are the main source of human rabies infection [27, 40].

A unique antigenic and genetic variant associated with sagui (*Cal. jacchus*) was identified in three human cases in the northeastern region of Brazil. This variant has been previously described in cases of rabies in sagui in Ceará state [8]. The sagui is a small marmoset endemic to Brazil; its habitats include a variety of forests, and although it is arboreal, it can run/walk along the ground in order to move from one group of trees to another [41].

The proximity of this marmoset to urban settlements and the common practice of capturing and keeping them as pets are two contributing factors to a higher risk of rabies transmission. Common marmosets have been introduced to areas outside of their natural geographical range in Brazil and can be found living within cities such as Rio de Janeiro, Bahia, etc. and even Buenos Aires, Argentina [42]. The transmission of this particular rabies variant and the continuous geographical dispersion and introduction of the species into urban centres could increase the importance of this animal as a public health risk and should prompt further studies of this distinctive rabies transmission cycle [8].

When the nucleotide substitutions at residues 377–379 were analysed, they showed distinct amino-acid changes in the antigenic site of the nucleoprotein that were representative of *D. rotundus* and *Cal. jacchus* reservoirs. These changes were not observed for dogs and *Cer. thous* reservoirs for the terrestrial cycle of transmission, which is to be expected because the PV strain (NC_001542) has a canine origin. The lineage maintained in *Cer. thous* is probably a spillover of the lineage related to dogs.

The complexity of rabies epidemiology in Brazil is characterized by its wide geographical range, coupled with its multiplicity of ecological niches and its high socioeconomic and cultural diversity, as well as the intense intra- and inter-regional migration activities of its human population. Other key factors in this complexity are the high diversity of mammalian species and the loss of their habitat.

This study is the only report on RABV reservoirs and genetic lineages involving humans that shows the antigenic and genetic characteristics of rabies isolates obtained from humans in a wide geographical area of Brazil. Our results confirmed the presence of at least four main reservoirs as the source of rabies infections in humans involving dogs, haematophagous bats, wild carnivores and marmosets. Recently, human rabies cases have been diagnosed that were related to lineages maintained in dogs, vampire bats

and marmosets. No human cases related to the *Cer. thous* lineage have been diagnosed, although samples of this lineage were isolated from *Cer. thous* [1]. As control programmes succeed in diminishing the importance of dogs as rabies reservoirs, continuous epidemiological surveillance is required both to monitor the changes in the epidemiology of this disease and to discover new cycles in this highly biodiverse environment.

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DECLARATION OF INTEREST

None.

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