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Rapid Communication

Molecular epidemiology of rabies virus isolated from different sources during a bat-transmitted human outbreak occurring in Augusto Correa municipality, Brazilian Amazon

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Abstract

We genetically characterize rabies virus (RABV) strains isolated from human cases, domestic and wild animals during a human outbreak of bat-transmitted rabies in Augusto Correa municipality, Pará state, Brazilian Amazon in 2005. Partial nucleotide sequences of the N gene (491 bp) were obtained for all strains, and phylogenetic analysis grouped these into two major clades (Pará and Central-Southeast) and identified them as bat-related viruses genotype I, *Desmodus rotundus* antigenic variant 3 (AgV3). A molecular clock was used to estimate the time of emergence for each RABV isolate. The molecular data from this study suggest the association of vampire bats with human and domestic animal cases reported in the outbreak, the circulation of at least two predominant lineages in the Pará state, and also a geographic association to lineages dispersion.

Keywords: Rabies virus; Hematophagous bat; Rabies outbreak; Molecular epidemiology; Augusto Correa municipality

Introduction

Rabies, an acute fatal encephalomyelitis that affects mammals, including humans, is a worldwide zoonotic disease caused by *Rabies virus* (RABV) (Kobayashi et al., 2006). RABV belongs to the order *Mononegavirales*, family *Rhabdoviridae*, genus *Lyssavirus*, and is a bullet-shaped virus with an approximate length of 180 nm and diameter of 75 nm. The genome consists of a single-stranded, negative-sense, non-segmented RNA, 12 kb in length. Five genes (3′-N-P-M-G-L-5′) encode for five proteins: nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and the polymerase (L) (Fauquet et al., 2005).

Currently, the genus *Lyssavirus* is phylogenetically subdivided into two major phylogroups (I and II) that include seven genotypes (genotypes 1 to 7) (Badrane et al., 2001). Phylogroup I comprises the worldwide genotype 1 (classic RABV), the *European bat lyssavirus* (EBLV) genotypes 5 (EBLV-1) and 6 (EBLV-2), the African genotype 4 (*Duvenhage virus*), and the *Australian bat lyssavirus* genotype 7. Phylogroup II is represented by the divergent African genotypes 2 (*Lagos bat virus*)

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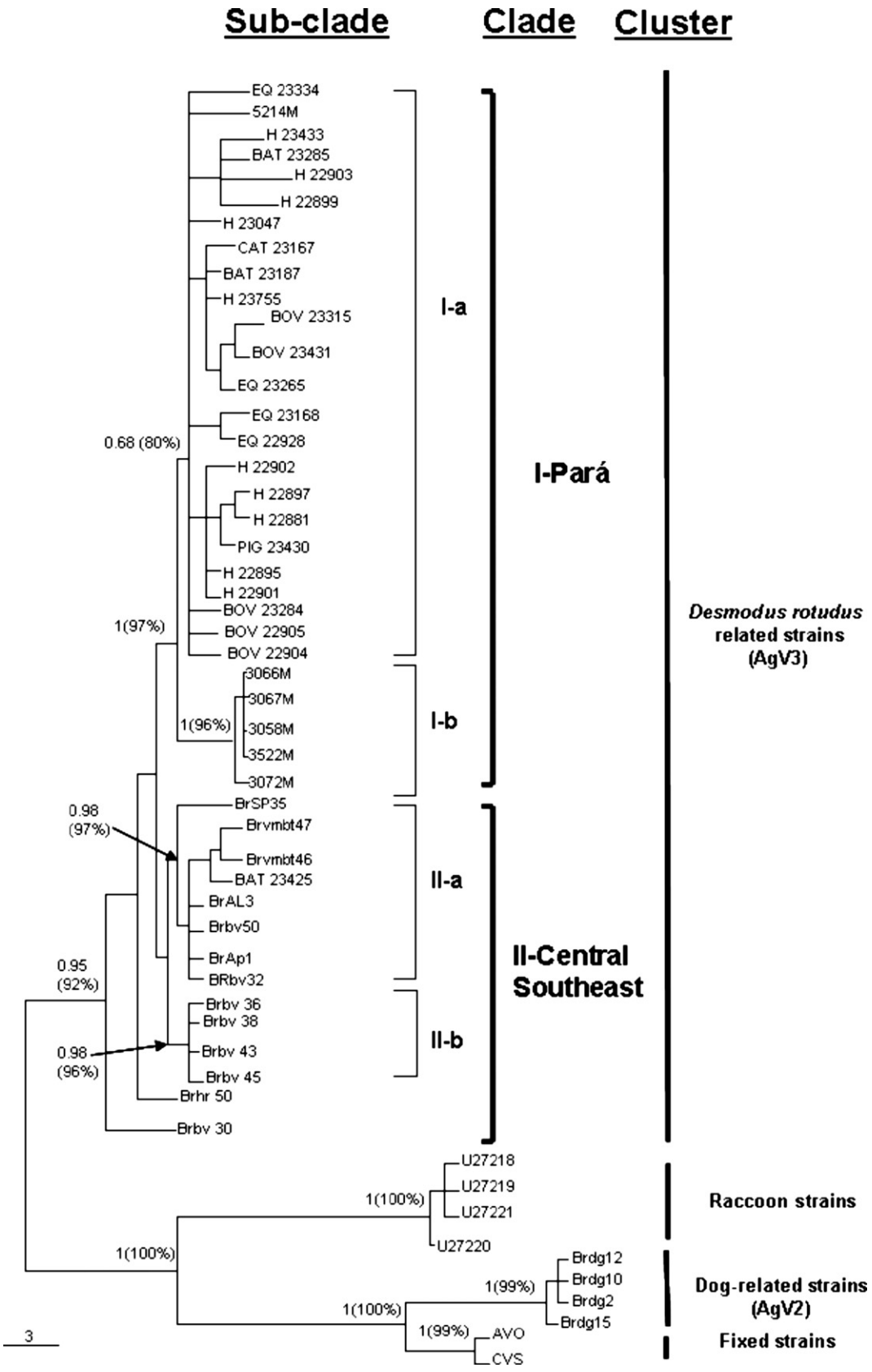

Fig. 1. Phylogeny of partial nucleotide sequence of RABV N gene isolated during the Augusto Correa outbreak occurred in 2005. Bayesian method and bootstrap analysis were used for three constructions. Bootstrap percentage values for ML method and Bayesian values (inside parenthesis) are placed over each main group node. Arrows are indicating the exact position of bootstrap values. Number under the scale bar corresponds to 3% nucleotide sequence divergence.

and 3 (*Mokola virus*) (Badrane et al., 2001). RT-PCR methods have been applied and widely used for molecular diagnosis (Dantas Junior et al., 2004; Heaton et al., 1997; Sacramento et al., 1991; Soares et al., 2002), and molecular epidemiologic studies of RABV have been established in order to provide a better understanding of the evolution of this virus (David et al., 2000; Ito et al., 2001; Kobayashi et al., 2005; Nadin-Davis et al., 1996, 2001; Poch et al., 1988; Sacramento et al., 1991; Tordo et al., 1986).

Four natural RABV maintenance cycles have been recognized: urban, rural, wild, and aerial cycles (Brass, 1995). The urban cycle is characterized by disease in domestic animals (dogs and cats). In the rural cycle, the rabies occurred in domestic herbivores (bovines, equines, and pigs), where hematophagous bats, mainly the vampire bat *Desmodus rotundus*, are implicated as the main species responsible for the virus transmission. The wild cycle involves RABV circulation among wild carnivores, including foxes, wolves, coatis, monkeys, and opossums. The aerial cycle includes transmission between hematophagous and non-hematophagous bats, both of which have been implicated in the maintenance and dissemination of RABV in the wild, rural, and urban environments. Humans can be accidentally infected in any of the above cycles (Kotait, 2003; Uieda et al., 1995).

Despite human cases of rabies having diminished in Latin America due to control measures in domestic animals, human rabies acquired by bats have increased in many of these countries, including Brazil, in the last 10 years (Lopez, 1991; Mayen, 2003; Travassos da Rosa et al., 2006). The first bat-transmitted human rabies case was reported in Trinidad in 1927. Retrospective studies carried out from 1931 to 1990 revealed about 330 cases of bat-transmitted rabies in Peru, Trinidad, Brazil, and Mexico (Schneider, 1995). The first bat-transmitted rabies cases reported in Brazil occurred in Juruti, Pará state in 1975, when six human fatal cases were recognized. By 1990, approximately 54 bat-transmitted human cases had been reported in the country (Schneider, 1991). In 2004, two additional vampire bat rabies outbreaks resulting in 21 human fatalities were reported in Portel and Viseu municipalities, Pará state, Brazil (Travassos da Rosa et al., 2004, 2006; Wada, 1978).

In the current study, we describe a human outbreak of bat-transmitted rabies by the vampire *D. rotundus* that occurred in June 2005 in the municipality of Augusto Correa, Pará state. We genetically characterized RABV isolates from Augusto Correa obtained from fatal human cases, domestic animals (bovine, equine, porcine, and feline), and bats (*D. rotundus*) and compared these with isolates from the 2004 outbreaks in Portel and Viseu municipalities.

Results

RT-PCR and sequence analysis

RT-PCR products were obtained for all 24 RABV strains used in this study, and their nucleotide sequences from positions 868 to 1359 (491 nt) were determined. Initial analysis using the BLAST search toll (NCBI, http://www.ncbi.nlm.nih.gov/) showed high similarity among the studied strains and different bat-related RABV from distinct geographic areas. Multiple sequencing analyses revealed nucleotide and deduced amino acid sequence homologies ranging from 98 to 99% and from 99 to 100%, respectively.

Phylogeny

Phylogenetic analysis of the RABV partial N gene nucleotide sequences using MP, NJ, and ML methods (model selected by Modeltest was SYM+G; Base=equal Nst=6 Rmat=[1.7919 5.5880 1.2398 1.4125 10.9630], Rates=gamma, Shape=0.6073, Pinvar=0), as well as the Bayesian method resulted in trees with similar topology. Although all methods generated trees with similar topology, the ML and Bayesian reconstructions showed more reasonable topologies. In this case, the ML tree is presented, however the bootstrap values for ML method and the Bayesian values are represented for the main groups. The phylogeny depicted all studied RABV isolates as members of the genotype I antigenic variant III of RABV, strains originally related to the vampire bat *D. rotundus* (AgV3). Two major clades were determined for this group hereafter denominated clade I (Pará state) and clade II (Central/Southeast). The mean divergence was estimated in 0 and 0.27% for clade II and clade I, respectively. However, between these clades the mean divergence was 3.55%. The clade I (Pará) which included strains isolated in Pará state was subdivided into two sub-clades denominated Ia and Ib. With exception of one strain (BAT 23425), all isolates of Augusto Correa were grouped into the sub-clade Ia together with strains isolated in Viseu in the years of 2004 (5214M) and 2005 (H23755, H23433, and BOV 23188). The sub-clade Ib grouped strains exclusively in Portel municipality in the Marajo region. For the clade II (Central/Southeast), the sub-clade IIa grouped the strain BAT 23425 together with strains isolated in São Paulo (BRAL3, BRAP1, Brbv 32, BrSP 35) and Goias states (BrBv50). Strains isolated in Tocantins (Brbv36, Brbv38) and Mato Grosso (Brbv 43, Brbv 45) states were included in the sub-clade IIb. Furthermore, the strains Brh50 and Brbv30 isolated in Goias state did not group in any sub-clade of the described clades (Fig. 1).

Molecular clock and divergence time

In order to evaluate the molecular clock hypothesis for rabies virus isolated in Pará state, a separate tree was constructed including all rabies isolates circulating among humans and domestic (pig, bovine, horse, and cat) and wild (bat) animals,

Table 1
Analysis of maximum-likelihood ratio for the molecular clock hypothesis

Data set	No. of taxa	Log likelihood		2(ln ML − ln MLK)	*df*	*p*	Rejection of the molecular clock
		Clock −ln	Nonclock −ln				
All strains	53	2266.761	2,213.807	105.90	51	<0.05	Yes
Human	17	1052.891	1064.594	23.40	15	>0.05	No

ML=maximum-likelihood method; MLK=maximum-likelihood method enforcing a molecular clock.

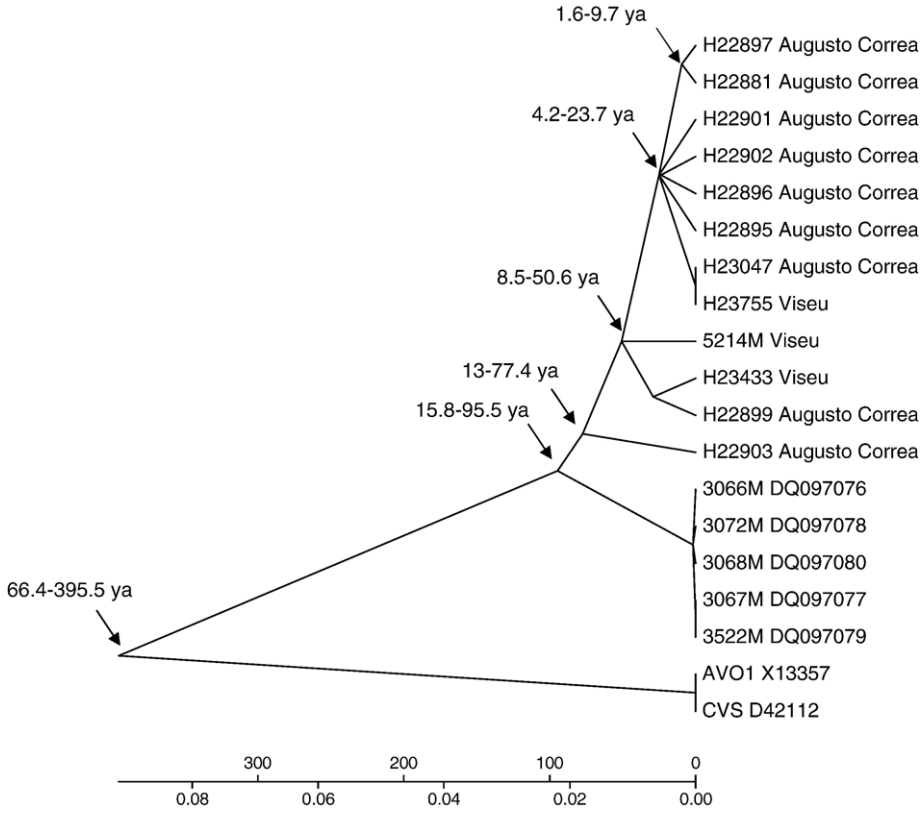

Fig. 2. Schematic rabies virus tree rooted with AVO1 and CVS strains indicating the starlike evolution. Divergence times (upper and lower values) are shown over all nodes of the tree.

for which partial nucleoprotein (N) gene sequences were available. The clock hypothesis was rejected when rabies strains isolated from different species were used and accepted when tested with rabies strains isolated only from humans (Table 1) (model selected by Modeltest was TrNef+G; Base=equal Nst=6 Rmat=(1.0000 1.6975 1.0000 1.0000 5.3994) Rates=gamma Shape=0.9267 Pinvar=0). Furthermore, estimation of the mean of divergence time at the node of rabies strains isolated from human inhabitants of Viseu and Augusto Correa municipalities suggests that these strains diverged from each other about 77 years ago (range of 13–77.4 years) (Fig. 2).

Discussion

Rabies is one of the oldest documented diseases of humankind. Ancient writers in Mesopotamia, China, Greece, Rome, and India described classic symptoms that have both intrigued physicians and terrified the public for millennia (Patterson, 1993). Rabies has affected several thousand domestic animals and people worldwide, causing an impressive, though often neglected, health impact (Rupprecht et al., 1995).

Bats are responsible for the aerial cycle of RABV, maintained and transmitted among vampire, insectivorous, and frugivorous bats, and from bats to wild animals (Uieda et al., 1995). Vampire bats, in turn, have been incriminated as the main host in the transmission of RABV to wild and domestic animals, with dogs representing the primary link in virus transmission to humans in urban and rural areas (Ito et al., 2001). For decades, the forest environment in Brazil has been the focus of intense changes due to deforestation, urbanization, and development (e.g. construction of highways and hydroelectric dams). These environmental changes have had an important impact on the ecology of forested regions and a powerful influence on the movement of bats to rural and urban areas where domestic animals and human populations are established (Constantine, 2003).

Our findings reveal the association of bats with human, wild, and domestic animal deaths and incriminate the vampire bat *D. rotundus* as the species responsible for this occurrence. The phylogenetic analysis suggests that the strains BRbv 30 (accession number AB083803) and BRhr 50 (accession number AB083804) isolated in Goias state between 1998 and 1999, represents a possible ancestral strains to those isolated during the outbreak occurring in Portel, Viseu, and Augusto Correa municipalities in 2004–2005. The phylogram also revealed the segregation of two independent groups geographically distinct hereafter namely clade I (Pará) and II (Central–Southeast) (Fig. 1). The viruses belonging to the clade I clearly segregated from those included in the clade II and showed different ancestral strains suggesting a distinct evolutionary origin. We also observed that at least two distinct lineages have been circulated predominantly in the Pará state (lineages Ia and Ib; clade I) in which strains from sub-clade Ib probably represent the evolutionary origin to strains from the sub-clade Ib. These two clade I lineages are phylogenetically distinguishable from two other clade II lineages (lineages IIa and IIb) represented by strains

isolated in the south of Amazon region (Tocantis state), as well as by strains isolated in the Central (Mato Grosso and Goias states) and Southeast Brazilian regions (São Paulo state) (Fig. 1). Of interest, the genetic relationship observed between the strain BAT 23425 and those belonging to lineage IIb suggests that the Amazonian strains have its evolutionary origin related to strains that circulate in Central and Southeast regions of Brazil. Thus, based on our molecular data we assume that the rabies virus dispersion has been occurred from Central–Southeast toward the Amazon region. These results may reflect the vampire bat or infected animals (wild or domestic animals) movement across the Brazilian regions and also suggest an important correlation between geographic distribution and lineages dispersion in Brazil. Although reasonable, other strains isolated in Brazil and in the Amazon region must be sequenced in order to test this hypothesis.

The molecular clock hypothesis was rejected when viruses isolated from different species were included in the analysis. However, in the analysis including only strains isolated from humans the clock hypothesis was accepted, indicating a non-constant rate of evolution for RABV among distinct hosts. This could be explained in part due to different microenviromental factors such as immunologic pressure and virus selection, as well as other evolutionary factors such as bottleneck (Novella et al., 1999; Wittkea et al., 2002). Consequently, such factors could result in very different branch lengths in the tree including with different hosts (Fig. 1), when compared to the human samples tree (Fig. 2), which presents more regular branch lengths. For viruses isolated from humans, the molecular clock method depicted a clear picture regarding the time when each strain emerged. Based on the results, the strains from Portel (3522M, 3066M, 3067M, 3068M, and 3072M) were estimated to originate approximately in 1911 (approximately 95 years before), giving rise to strains isolated in Augusto Correa (H22903) that emerged in the region around 18 years later, in 1929. The molecular clock phylogeny also indicated that the strains isolated in Viseu (H23433) and Augusto Correa (H22899) probably diverged one from another during the 1950s (1956) and the 1980s (1983). These data suggest an interesting movement of RABV through distinct and distant geographic areas of the Amazon region (Viseu and Augusto Correa: Bragantina region; Portel: Marajo region) and also indicate that strains from Portel, Augusto Correa, and Viseu have been circulating in the area for at least 100, 75, and 50 years, respectively, before these outbreaks.

Furthermore, 100 years ago the Augusto Correa, Viseu, and Portel municipalities probably were completely covered by tropical rainforest, where viruses, including RABV, established their harmonic wild cycles. Since human populations have invaded the forest and changed the environment dramatically in the last four decades, new and enforced host associations have been established between vampire bats and domestic animals such as bovines, horses, porcines, and canines, as well as with humans. In this way, it is possible that RABV began its route of evolution and adaptation to new hosts, resulting in the emergence of rabies with a more eclectic animal range. Recently in the Amazon region, several cases of human rabies outbreaks have been related to bat attacks, indicating an intense alteration of the natural environment that has forced bats to migrate to urban areas in search of new food sources (Travassos da Rosa et al., 2006). This has resulted in transmission of RABV to humans and domestic animals found in abundance in urban and suburban areas.

Finally, the association of genetic and ecologic data can provide a better understanding of RABV evolution and may explain the generation of RABV diversity associated with host-selection factors, as well as should provide new, more efficient control measures to prevent rabies outbreaks.

Materials and methods

Area of occurrence

The municipality of Augusto Correa (46°39′W, 01°01′S) has 34,695 inhabitants (39.19 inhabitants/km^2) and is located in the northeastern region of Pará state, 256 km distant from the state capital of Belém. The geographic location of Augusto Correa municipality and other places where the rabies virus strains used in the phylogenetic analysis where isolated is shown in Fig. 3.

The outbreak

In June 2005, 15 human rabies cases were reported in the Arai, Piçarreira, and Cachoeira villages in Augusto Correa municipality. Ten patients were diagnosed as rabies cases based on clinical, epidemiological, and laboratorial data (immunofluorescence assay of and virus isolation from brain tissue obtained at autopsy), while the five other diagnoses were made only based on clinical and epidemiological information.

Among the 10 clinic and laboratory confirmed rabies cases, 50% were male, with patients' ages ranging from 6 to 50 years old. All patients showed fever, flaccid paralysis, disphagy, and intestinal constipation. Paresis in the place of animal bite was observed in 90% of cases, while hypersalivation was present in 80% of cases followed by headache (70%) and hydrophobia (50%). The mean time from disease onset to death was estimated at 7.3 days, and the mean incubation period was approximately 2.5 months.

Laboratory diagnostics

The laboratory diagnosis of rabies required the detection of viral antigen by direct immunofluorescence assay (DIFA) and viral isolation by intracranial inoculation of central nervous system samples obtained at autopsy into newborn mice (2–3 days old). Positive samples were identified antigenically using a panel constituted by eight monoclonal antibodies (MAbs) produced by the Centers for Disease Control and Prevention (CDC), Atlanta, USA.

Genetic studies

Twenty-four RABV strains were genetically characterized, 21 (9 humans, 5 bovines, 3 bats, 2 equines, 1 feline, and 1 porcine)

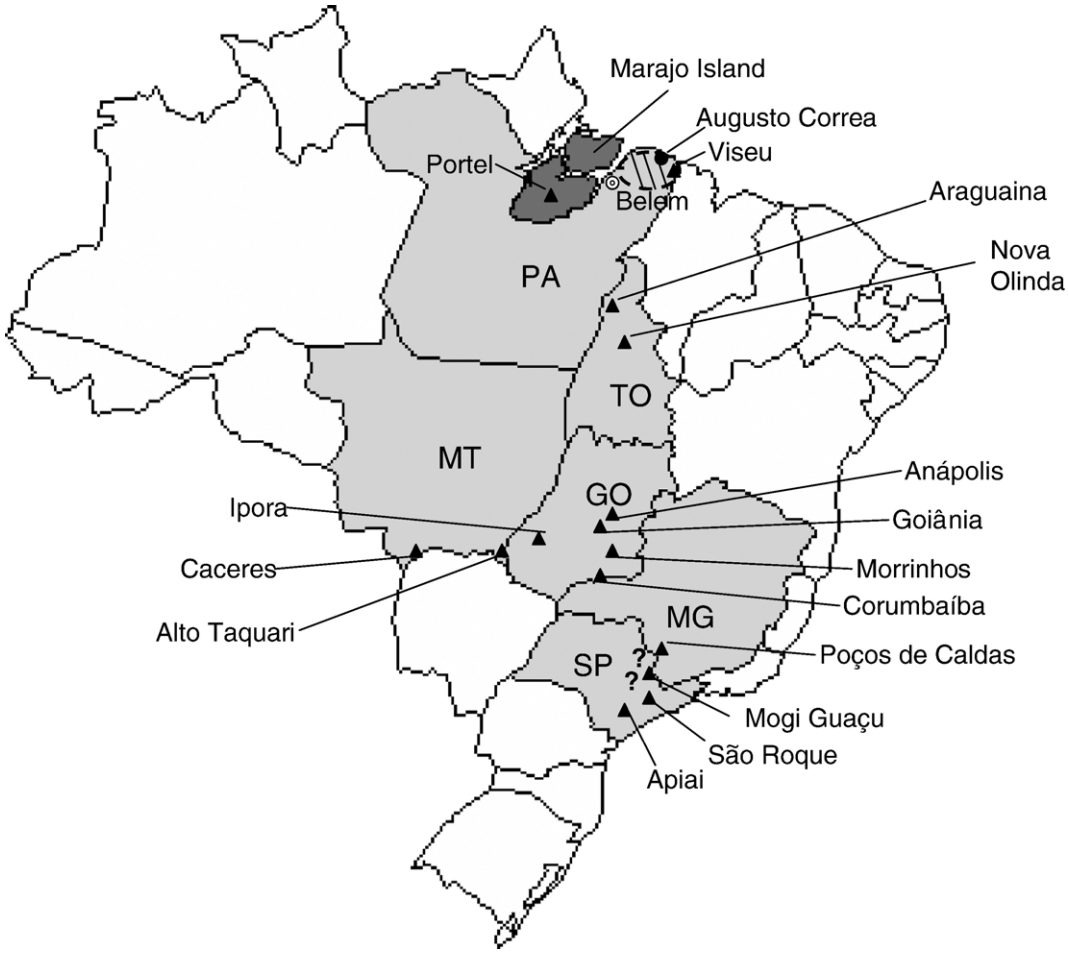

Fig. 3. Geographic position of the places where the RABV strains used for phylogenetic analysis was isolated. Black dot corresponds to Augusto Correa Municipality, Pará state [PA]. Triangles represent other municipalities of different Brazilian states which are highlighted in light gray (TO: Tocantins; MT: Mato Grosso; GO: Goias; MG: Minas Gerais; SP: São Paulo). Areas highlighted in dark gray represent the Marajo region in which Portel municipality is included. Dashed areas correspond to the northeast region of Pará state including Augusto Correa and Viseu municipalities. ?: unknown municipalities.

from Augusto Correa municipality and three (2 human and 1 bovine) from Viseu municipality. Samples from Augusto Correa were collected during and after the outbreak and samples from Viseu were isolated in the same period as the Augusto Correa outbreak. Nucleotide sequences from RABV isolated from distinct geographic regions, time periods, and host association were used for genetic studies (Table 2).

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

Viral RNA from all 24 samples was extracted directly from human and animal central nervous system tissue by using the TRIZOL reagent (Invitrogen, San Diego, CA) following the manufacturer's instructions. For partial amplification of the N gene, a two step RT-PCR standard protocol was used. Highly conserved and phylogenetic informative region of the RABV N gene was identified applying the parsimony-informative site toll available in the Mega 3.0 software (Kumar et al., 2004) and used to design specific primer pair namely RABV NF (G G A A G A G A T A A G A A G A A T G T T T G) and RABV NR (T T G G A G C T G A C T G A G A C A T A) hybridizing to nucleotide positions 868 and 1359 for an estimated product size of 491 bp.

For the first strand synthesis a 20 μL reaction mixture was used, consisting of 5 μL viral RNA (1 ng to 5 μL) and 15 μL of the RT master mix including 1× first-strand buffer (250 mM Tris–HCl pH 8.3, 100 mM NaCl, 15 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT), 0.5 mM dNTP, 10 mM DTT, 20 U RNaseOUT (Invitrogen), 2.5 μM of the forward primer RABV NF, and 10 U of RT enzyme (Superscript II Reverse Transcriptase, Invitrogen). Viral RNA was reverse transcribed for 90 min at 42 °C to generate the complimentary DNA (cDNA).

The PCR was performed using 5 μL (~2 ng) of cDNA and a PCR mixture containing 1× PCR buffer (250 mM Tris–HCl pH 8.3, 100 mM NaCl, 0.1 mM EDTA), 1.5 mM $MgCl_2$, 200 μM of dNTPs, 1 μM of each forward RABV NF and reverse RABVNR primers, and 0.05 U/μL of DNA polymerase (Platinum Taq DNA Polymerase, Invitrogen) adjusted for a final volume of 25 μL. PCR reactions were carried out for 35 cycles of 94 °C/40 s, 55 °C/40 s, and 74 °C/1 min. Amplicons were visualized on a 1.2% agarose gel, purified using the Quiack gel extraction kit (Qiagen, Valencia, CA), following the manufacturer's instructions, and then sequenced.

Direct sequencing

Nucleotide sequences of the RABV strains were determined using the ABI PRISM Dye Terminator kit (Applied Biosystems, Foster City, CA) in an ABI 377 DNA sequencer. Direct sequencing was carried out using the primer pair described above.

Alignment and phylogenetic analyses

Nucleotide sequences for all 24 RABV isolates were aligned with homologous sequences available from GenBank (Table 2) using Clustal X software. Phylogenetic trees were constructed by neighbor joining (NJ) (Saitou and Nei, 1987), maximum likelihood (ML), and maximum parsimony (MP) methods in the

Table 2
Rabies virus strains used for genetic characterization according with its host association, geographic origin, and year of isolation

Strain	Host association	Geographic origin			Year of isolation	Access number
		City	State	Country		
H22881	Human	Augusto Correa	Pará	Brazil	2005	–
H22895	Human	Augusto Correa	Pará	Brazil	2005	–
H22896	Human	Augusto Correa	Pará	Brazil	2005	–
H22897	Human	Augusto Correa	Pará	Brazil	2005	–
H22899	Human	Augusto Correa	Pará	Brazil	2005	–
H22901	Human	Augusto Correa	Pará	Brazil	2005	–
H22902	Human	Augusto Correa	Pará	Brazil	2005	–
H22903	Human	Augusto Correa	Pará	Brazil	2005	–
H23047	Human	Augusto Correa	Pará	Brazil	2005	–
BOV 22904	Bovine	Augusto Correa	Pará	Brazil	2005	–
BOV 22928	Bovine	Augusto Correa	Pará	Brazil	2005	–
CAT 23167	Feline	Augusto Correa	Pará	Brazil	2005	–
BAT 23187	*Hematophagous bat* (*Desmodus rotundus*)	Augusto Correa	Pará	Brazil	2005	–
BOV 23284	Bovine	Augusto Correa	Pará	Brazil	2005	–
EQ 23285	Equine	Augusto Correa	Pará	Brazil	2005	–
BAT 23286	*Hematophagous bat* (*Desmodus rotundus*)	Augusto Correa	Pará	Brazil	2005	–
BOV 23315	Bovine	Augusto Correa	Pará	Brazil	2005	–
EQ 23334	Equine	Augusto Correa	Pará	Brazil	2005	–
BAT 23425	Hematophagous bat (*Desmodus rotundus*)	Augusto Correa	Pará	Brazil	2005	–
PIG 23430	Porcine	Augusto Correa	Pará	Brazil	2005	–
BOV 23431	Bovine	Augusto Correa	Pará	Brazil	2005	–
BOV 23188	Bovine	Viseu	Pará	Brazil	2005	–
H23433	Human	Viseu	Pará	Brazil	2005	–
H23755	Human	Viseu	Pará	Brazil	2005	–
5214M	Human	Viseu	Pará	Brazil	2004	DQ097075
3066M	Human	Portel	Pará	Brazil	2004	DQ097076
3067M	Human	Portel	Pará	Brazil	2004	DQ097077
3072M	Human	Portel	Pará	Brazil	2004	DQ097078
3522M	Human	Portel	Pará	Brazil	2004	DQ097079
3068M	Human	Portel	Pará	Brazil	2004	DQ097080
BR-AL3	*Frugivorous bat* (*Artibeus literatus*)	Unknown	São Paulo	Brazil	1998	AB117971
BR-AP1	*Frugivorous bat* (*Artibeus literatus*)	Unknown	São Paulo	Brazil	1998	AB117972
BRbv32	Bovine	São Roque	São Paulo	Brazil	1994	AB083805
BRsp35	Dog	Apiai	São Paulo	Brazil	1992	AB083808
BRdg12	Dog	Mogi Guaçu	São Paulo	Brazil	1989	AB083797
BRbv30	Bovine	Morrinhos	Goiás	Brazil	1999	AB083803
BRbv50	Bovine	Corumbaiba	Goiás	Brazil	1999	AB083818
BRhr50	Equine	Ipora	Goiás	Brazil	1998	AB083804
Brdg2	Canine (dog)	Goiânia	Goiás	Brazil	1999	AB083792
Brdg15	Canine (dog)	Anápolis	Goiás	Brazil	1999	AB083798
BRbv36	Bovine	Nova Olinda	Tocantins	Brazil	1998	AB083809
BRbv38	Bovine	Araguaxna	Tocantins	Brazil	1998	AB083810
BRbv43	Bovine	Alto Taquari	Mato Grosso	Brazil	1999	AB083811
BRbv45	Bovine	Caceres	Mato Grosso	Brazil	1999	AB083813
Brdg10	Canine (dog)	Poço de Caldas	Minas Gerais	Brazil	1987	AB083796
PA R89	Canine (Racon)	Unknown	Ontario	Canada	Unknown	U27221
CAN-RC	Canine (Racon)	Unknown	Ontario	Canada	Unknown	U27218
NY 771	Canine (Racon)	Unknown	Ontario	USA	Unknown	U27219
USA-RC	Canine (Racon)	Unknown	New York	USA	Unknown	U27220
BRvmbt46	Hematophagous bat (*Desmodus rotundus*)	Unknown	Unknown	Brazil	Unknown	AB083816
BRvmbt47	Hematophagous bat (*Desmodus rotundus*)	Unknown	Unknown	Brazil	Unknown	AB083815
CVS	Fixed strain	–	–	–	–	D42112
AVO1	Fixed strain	–	–	–	–	X13357

PAUP 4.0b10 (Swofford, 2002). The program Modeltest version 3.6 (Posada and Crandall, 1998) was used to determine which model of nucleotide substitution best explained the data using Akaike information criteria (AIC). For the NJ analysis, a distance matrix was calculated from the aligned sequences using the Tamura-3 parameter model with a different gamma distribution parameter (gamma parameter = 1.0). For the MP analysis, a transition/transversion rate of 4:1 was used. Bootstrap analyses (1000 replicates) were implemented to place confidence values on phylogenetic groupings (Felsenstein, 1985) in the MP and NJ methods using PAUP, however, for the ML method we used the PHYML program (Guindon and Gascuel, 2003). This was used instead of PAUP due to its greater speed. Bayesian analysis with Markov chain Monte Carlo (MCMC) sampling was performed with MrBayes3.0b4 run for 2 million generations and sampling once every 1000 trees. Bayesian posterior probabilities were estimated on a 50% majority rule consensus after burn-in (Huelsenbeck and Ronquist, 2001). The TRACER program (evolve.zoo.ox.ac.uk) was used to verify if MrBayes runs reached the appropriate convergence.

Molecular clock and divergence time analysis

Using the partial nucleoprotein gene sequence alignments, a molecular clock hypothesis that assumed a constant rate of evolution was tested using the best-fit model of sequence evolution selected by the Modeltest 3.6 (Posada and Crandall, 1998) in PAUP, version 4.0b10 (Swofford, 2002). To establish statistical significance, twice the difference in likelihood was assumed to be distributed as a χ^2 with $n-2$ degrees of freedom, where n is the number of sequences (Felsenstein, 1993).

Aiming to estimate divergence times for the rabies strains isolated from Augusto Correa and Viseu municipalities, we determined the genetic distance among viruses using the F84 model/DNAML implemented in the DNADIST software in PHYLIP 3.5v (Felsenstein, 1993). The distances were corrected for gamma-distributed and gamma-plus-invariant-sites-distributed rates of change in different sites. The time of divergence (T) was obtained as upper and lower values for different divergence times by calculating $r = k/(2T)$, where k is the mean of genetic distance and r is the nucleotide substitution ratio (Kimura, 1980). Divergence times were expressed as a range of dates for each node in the tree. We assumed an evolutionary rate (r) for the N gene ranging from 1.38×10^{-3} to 2.32×10^{-4} as reported by Hughes and colleagues for North American bats (Davis et al., 2006, Hughes et al., 2005).

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