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ORIGINAL PAPER

Phylogenetic analysis of rabies viruses isolated from cattle in southern Brazil

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Abstract
Bats and dogs are the main reservoirs of rabies virus (RABV) in Latin America and are responsible for the maintenance of different cycles of infection. In the two neighbour and most southern Brazilian states of Rio Grande do Sul (RS) and Santa Catarina (SC), rabies in dogs has been successfully controlled for more than 30 years. However, rabies associated to the rural cycle remains endemic, with a significant, though oscillating—annual incidence of rabies in cattle. Despite the plethora of studies on genetic analyses of Brazilian RABV, isolates from southern Brazil have only scarcely been investigated. This work was performed to identify the genetic lineages of RABVs circulating in states of RS and SC. Fifty-nine RABV cattle isolates from RS and SC were selected and submitted to reverse transcription/polymerase chain reaction (RT-PCR) followed by sequencing of the nucleoprotein gene. In RS, the circulation of two sublineages (1A and 1B) of RABV was detected, both with characteristics of lineages usually detected in vampire bats (*Desmodus rotundus*). In SC, only one sublineage of RABV (1B) was detected. Nevertheless, the findings reported here are expected to contribute to the understanding of the biology of the virus in the region and its interactions with the natural host *D. rotundus*.

Keywords Rural rabies · Genetic lineages · Rio Grande do Sul · Santa Catarina · Brazil

Introduction

Rabies is a fatal zoonosis associated with encephalitis in all mammals that is transmitted predominantly by bites from infected animals. The disease is caused by rabies virus (RABV), formally *Rabies lyssavirus*, the prototype of the genus *Lyssavirus* within the family *Rhabdoviridae* [1]. This is the only lyssavirus so far identified in Brazil [2].

Rabies virus is present on all continents except Antarctica [3]. In Brazil, the infection is endemic and is maintained in the "aerial, sylvatic and rural" cycles, where bats are the main reservoirs. In some regions of the country, such as the northeast and northern regions, the urban cycle, with domestic dogs as reservoirs, is still maintained [4]. In the states of Rio Grande do Sul (RS) and Santa Catarina (SC), the most southern Brazilian states, urban rabies has not been detected since the 1980s [5, 6]. Nevertheless, vampire bats of the species *Desmodus rotundus,* remain as the natural reservoirs of the virus. As *D. rotundus* preferably feeds on cattle, the endemic character of rabies in cattle is maintained, giving rise to sporadic outbreaks of the disease. Despite the implementation of methods of control, such as reductions in vampire bat populations, vaccination of cattle (though not compulsory) and efforts to improve public awareness on the subject, the endemic status of the infection is maintained, with the occurrence of occasional, often yearly, outbreaks of disease. Such occurrences cause significant burdens to farmers due to the loss of livestock, in addition to causing serious risks of human contamination.

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It has been possible to associate different genetic lineages of RABV with the geographic distribution of the isolates [7, 8]. Such association is important to study the routes of dissemination of the virus. Moreover, the association of particular RABV genetic lineages and the migratory profile of vampire bats may provide useful information in predicting future outbreaks of the disease. Therefore, analyses of genetic lineages of RABV could enable significant advances in the understanding of the epidemiology of infection within a particular region. Here, to investigate the genetic lineages of RABV circulating in RS and SC, phylogenetic analyses of the nucleoprotein gene (*N*) of RABV were performed on isolates recovered from infected cattle.

Materials and methods

Viruses

Fifty-nine RABV isolates were collected between 2012 and 2015. Viruses that were recovered from fragments of tissues of the central nervous system (CNS) of cattle from the states of RS and SC were analysed in the present study (Table 1). The sequences generated in this work were deposited and inspected by GenBank staff and registered as shown in Table 1 (www.ncbi.nlm.nih.gov/genbank/). Routine identification of rabies virus antigens was performed with the direct fluorescent antibody test (DFAT), following standard diagnostic procedures [9]. The isolates included in this study

Table 1 Origin of *Rabies lyssavirus* (RABV) sequenced and examined in the present study Identification (GenBank accession numbers) and localization (Municipality and State)

Identification of samples	Localization
KX090643.1; KX090642.1; KX090641.1; KX090640.1; KX090639.1; KX090638.1; KX090637.1; KX090636.1; KX090635.1	Pinhal Grande/RS
KX090625.1; KX090629.1; KX090612.1	Camaquã/RS
KX090626.1; KX090614.1; KX090613.1	Rio Pardo/RS
KX090627.1; KX090619.1	Mariana Pimentel/RS
KX090615.1	São Jerônimo/RS
KX090628.1; KX090603.1; KX090604.1; KX090605.1; KX090631.1; KX090632.1	Gravataí/RS
KX090611.1	Porto Vera Cruz/RS
KX090608.1	Morro Redondo/RS
KX090596.1	Barra do Ribeiro/RS
KX090602.1; KX090601.1; KX090618.1	Glorinha/RS
KX090599.1	Canguçu/RS
KX090600.1	Esperança do Sul/RS
KX090594.1; KX090633.1	Arroio Grande/RS
KX090607.1	Herveiras/RS
KX090634.1	São Paulo das Missões/RS
KX090620.1	Sapiranga/RS
KX090595.1	Arroio do Tigre/RS
KX090609.1	Novo Hamburgo/RS
KX090621.1; KX090598.1; KX090597.1	Candelária/RS
KX090630.1; KX090623.1; KX090622.1	Hulha Negra/RS
KX090617.1; KX090616.1	Viamão/RS
KX090610.1	Pedro Osório/RS
KX090624.1	Bagé/RS
KX090585.1	Leoberto Leal/SC
KX090586.1	Unknown/SC
KX090589.1; KX090588.1; KX090587.1	Anitápolis/SC
KX090590.1	São Bonifácio/SC
KX090591.1	São Martinho/SC
KX090593.1; KX090592.1	Rio Fortuna/SC

All samples were isolated from Central Nervous System (CNS) of cattle

RS State of Rio Grande do Sul, *SC* State of Santa Catarina

were selected by its availability at the Reference Laboratory for diagnosis of rabies in the state of RS (Instituto de Pesquisas Veterinárias Desidério Finamor—IPVDF) and in the Laboratory of Animal Pathology of University of State of Santa Catarina. This work complies with Protocol no. 01/2015 issued by the Ethics Committee of the Pasteur Institute, São Paulo.

RT-PCR and sequencing

Total RNA was extracted from infected brain tissues with TRIzol (Thermo Fisher Scientific™), according to manufacturer's instructions. cDNA synthesis of the genomic RNA and PCR amplifications were performed with primers that target the nucleoprotein gene (*N*) of RABV: sense primer JW12 (5′-ACGCTTAACAACAARATCARAG-3′) and antisense primer 304 (5′-TTGACGAAGATCTTGCTCAT-3′) as previously described [10, 11]. Viral RNA (3–5 mg) was reverse-transcribed using 15 units of *RevertAid Premium* reverse transcriptase (Thermo Fisher Scientific™) and amplified by PCR using Taq DNA polymerase (Thermo Fisher Scientific™, 5 units/ml) in PCR buffer (1.5 mM $MgCl_2$, 50 mM KCl, 20 mM Tris–HCl, pH 8, 200 mM each dNTP) in a final volume of 50 µl. Thermal cycling conditions were as follows: 1 cycle at 94 °C for 5 min; 35 cycles at 94 °C for 45 s, 55 °C for 45 s and 72 °C for 2 min, followed by 1 final incubation at 72 °C for 10 min. The amplicons obtained were purified with *GFX™ PCR DNA and Gel Band Purification kit* (GE Healthcare™) and sequenced with 3,2 pmol same primers used for PCR and *BigDye Terminator v3.1Cycle Sequencing Kit* (Thermo Fisher Scientific™). Extracts from mice infected with the "fixed" strain Challenge Virus Standard (CVS-31) were included in all reactions as positive controls. DEPC-treated water was included as negative control throughout.

Phylogenetic analysis

The RABV sequences obtained were edited with the CHROMAS software (version 2.24 Copyright© 1998–2004 Technelysium Pty Ltd.). Homology analyses were performed with basis on RABV sequences obtained at this work (59 sequences) and sequences available at the GenBank database as shown in Table 2 (64 sequences, including 56 sequences with genetic lineages from vampire bat *D. rotundus*, isolated from different regions of Brazil and other countries from Latin America, and 4 sequences corresponding to genetic lineages of RABV of nonhematophagous bats and 4 sequences of the genetic lineage of domestic dogs). Fig. 1 shows the geographical distribution of samples of RABV included in this study. A total of 123 nucleotide sequences of the RABV *N* gene were included in the phylogenetic analyses. Sequences were aligned with CLUSTAL/W using the BioEdit software version 7.1.3.0 [12] and the phylogenetic tree was performed by the Maximum Likelihood method based on the Tamura-Nei model [13], with MEGA 7 software [14]. A total of 1.218 nucleotides of the coding region of the RABV *N* gene, nucleotide positions 202–1420 (amino acids 45–450 in the PV strain of RABV-GenBank accession number: GB M13215.1) were analysed.

Results

Sequencing and phylogenetic analysis

A phylogenetic tree was constructed utilizing the 59 RABV isolates sequenced in this work. Another 64 RABV sequences recovered from GenBank were also included in the constructed tree (Fig. 2). Three clusters were formed: cluster 1 corresponded to sequences of viruses recovered from vampire bats; cluster 2 corresponded to sequences originating from nonhaematophagous bats and cluster 3 comprised sequences recovered from canids. Moreover, cluster 1 (vampire bats) could be further split into two subclusters: 1A, which included 37 isolates from RS, and 1B, which included 13 isolates from RS plus the 9 isolates from SC examined in this study.

Identification of sublineages of RABV

Subcluster 1A included, in addition to isolates from RS, a number of isolates from other regions of Brazil (the Southeast and North) and from Ecuador. In subcluster 1B along with isolates from RS and SC, sequences corresponding to viruses recovered from GenBank isolated from Uruguay and Argentina were grouped together.

These findings reveal at least two sublineages (1A and 1B) of RABV circulating in RS, for which the main reservoir is the vampire bat *D. rotundus*. Sublineage 1A seems to have a wider geographic distribution than sublineage 1B in RS, as 1A was identified in the state's northern, eastern, southern and central regions. Sublineage 1B appears to have a more restricted geographic distribution, since representatives of this sublineage were recovered only in the northern and eastern regions of the state. Cocirculation of the two sublineages in the same region was detected in two municipalities (Gravataí and Glorinha, Eastern RS; Fig. 3a).

In the state of SC, only sublineage 1B was identified; however, the number of municipalities from which samples were available for this study were somewhat limited, including three in the southern area of the state (the municipalities of Rio Fortuna, São Martinho and São Bonifácio) and two in the eastern area of the state (the municipalities of Anitápolis and Leoberto Leal) (Fig. 3b).

Table 2 Description of sequences of *Rabies lyssavirus* (RABV) recovered from GenBank (GB) and used in phylogenetic analysis (GenBank accession numbers, animal origin, localization, year, genetic lineage identified and reference)

GB accession numbers	Animal origin	Localization	Year	Genetic lineage identified	Reference
GU552789	*Molossus molossus*	Brazil	2006	Lineage 2	Oliveira et al. [15]
GU552796	*Molossus sp.*	Brazil	2007	Lineage 2	Oliveira et al. [15]
AB201815	*Molossus molossus*	Brazil	2005	Lineage 2	Kobayashi et al. [7]
AB201818	*Molossus sp.*	Brazil	2005	Lineage 2	Kobayashi et al. [7]
AB685222	Cattle	RS/Brazil	2016	Lineage 1 sublineage 1A	Itou et al. [16]
AB685226	Cattle	RS/Brazil	2016	Lineage 1 sublineage 1A	Itou et al. [16]
EF152264	Canids	Brazil	2005	Lineage 3	Carnieli et al. [17]
EF152275	Canids	Brazil	2005	Lineage 3	Carnieli et al. [17]
EF152279	Canids	Brazil	2005	Lineage 3	Carnieli et al. [17]
EF152280	Canids	Brazil	2005	Lineage 3	Carnieli et al. [17]
EF363727	Human	Ecuador	2005	Lineage 1 sublineage 1A	Castilho et al. [18]
EF363728	Human	Ecuador	2005	Lineage 1 sublineage 1A	Castilho et al. [18]
EF363745	Human	PA/Brazil	2004	Lineage 1 sublineage 1B	Castilho et al. [18]
EF363748	Human	PA/Brazil	2004	Lineage 1 sublineage 1B	Castilho et al. [18]
EF363751	Human	PA/Brazil	2004	Lineage 1 sublineage 1A	Castilho et al. [18]
EF363755	Human	PA/Brazil	2005	Lineage 1 sublineage 1A	Castilho et al. [18]
EF363756	Human	PA/Brazil	2005	Lineage 1 sublineage 1A	Castilho et al. [18]
EF363757	Human	PA/Brazil	2005	Lineage 1 sublineage 1A	Castilho et al. [18]
EF428576	*D. rotundus*	RJ/Brazil	2010	Lineage 1 sublineage 1A	Vieira et al. [19]
EF428577	*D. rotundus*	RJ/Brazil	2010	Lineage 1 sublineage 1A	Vieira et al. [19]
EF428578	*D. rotundus*	RJ/Brazil	2010	Lineage 1 sublineage 1A	Vieira et al. [19]
EF428579	*D. rotundus*	RJ/Brazil	2010	Lineage 1 sublineage 1A	Vieira et al. [19]
EF428580	*D. rotundus*	RJ/Brazil	2010	Lineage 1 sublineage 1A	Vieira et al. [19]
EU981923	Horse	Uruguay	2008	Lineage 1 sublineage 1B	Guarino et al. [20]
EU981925	Cattle	Uruguay	2008	Lineage 1 sublineage 1B	Guarino et al. [20]
EU981926	Cattle	Uruguay	2008	Lineage 1 sublineage 1B	Guarino et al. [20]
EU981927	Cattle	Uruguay	2008	Lineage 1 sublineage 1B	Guarino et al. [20]
EU981928	Cattle	Uruguay	2008	Lineage 1 sublineage 1B	Guarino et al. [20]
EU981929	Cattle	Uruguay	2008	Lineage 1 sublineage 1B	Guarino et al. [20]
EU981930	*D. rotundus*	Uruguay	2007	Lineage 1 sublineage 1B	Guarino et al. [20]
EU981931	*D. rotundus*	Uruguay	2007	Lineage 1 sublineage 1B	Guarino et al. [20]
GQ160921	Cattle	MG/Brazil	2007	Lineage 1 sublineage 1A	Macedo et al. [21]
GQ160956	Cattle	MG/Brazil	2007	Lineage 1 sublineage 1A	Macedo et al. [21]
GQ160917	Cattle	MG/Brazil	2007	Lineage 1 sublineage 1A	Macedo et al. [21]
GQ160910	Cattle	MG/Brazil	2007	Lineage 1 sublineage 1A	Macedo et al. [21]
GQ160918	Cattle	MG/Brazil	2007	Lineage 1 sublineage 1A	Macedo et al. [21]
GQ160927	Cattle	MG/Brazil	2008	Lineage 1 sublineage 1A	Macedo et al. [21]
GQ160928	Cattle	MG/Brazil	2008	Lineage 1 sublineage 1A	Macedo et al. [21]
GQ160936	Cattle	MG/Brazil	2008	Lineage 1 sublineage 1A	Macedo et al. [21]
GQ160946	Cattle	SP/Brazil	2008	Lineage 1 sublineage 1A	Macedo et al. [21]
GQ160923	Cattle	SP/Brazil	2008	Lineage 1 sublineage 1A	Macedo et al. [21]
GQ160924	Horse	SP/Brazil	2008	Lineage 1 sublineage 1A	Macedo et al. [21]
GQ160944	Cattle	SP/Brazil	2008	Lineage 1 sublineage 1A	Macedo et al. [21]
GQ160931	Horse	SP/Brazil	2008	Lineage 1 sublineage 1A	Macedo et al. [21]
GQ160941	Cattle	SP/Brazil	2008	Lineage 1 sublineage 1A	Macedo et al. [21]
GQ160916	Cattle	SP/Brazil	2008	Lineage 1 sublineage 1A	Macedo et al. [21]
GQ160922	Horse	SP/Brazil	2008	Lineage 1 sublineage 1A	Macedo et al. [21]
GQ160935	Horse	SP/Brazil	2008	Lineage 1 sublineage 1A	Macedo et al. [21]
GQ160912	Cattle	SP/Brazil	2008	Lineage 1 sublineage 1A	Macedo et al. [21]

Table 2 (continued)

GB accession numbers	Animal origin	Localization	Year	Genetic lineage identified	Reference
GQ160919	Cattle	SP/Brazil	2008	Lineage 1 sublineage 1A	Macedo et al. [21]
GQ160952	Cattle	SP/Brazil	2008	Lineage 1 sublineage 1A	Macedo et al. [21]
GQ160954	Cattle	SP/Brazil	2008	Lineage 1 sublineage 1A	Macedo et al. [21]
GQ160957	Cattle	SP/Brazil	2008	Lineage 1 sublineage 1A	Macedo et al. [21]
GQ160958	Cattle	SP/Brazil	2008	Lineage 1 sublineage 1A	Macedo et al. [21]
KF864327	Cattle	Argentina	2006	Lineage 1 sublineage 1B	Macedo et al. [21]
KF864328	Cattle	Argentina	2006	Lineage 1 sublineage 1B	Torres et al. [22]
KF864329	Cattle	Argentina	2006	Lineage 1 sublineage 1B	Torres et al. [22]
KF864330	Cattle	Argentina	2006	Lineage 1 sublineage 1B	Torres et al. [22]
KF864331	Cattle	Argentina	2006	Lineage 1 sublineage 1B	Torres et al. [22]
KF864332	Cattle	Argentina	2006	Lineage 1 sublineage 1B	Torres et al. [22]
KF864379	Cattle	Argentina	2006	Lineage 1 sublineage 1A	Torres et al. [22]
KF864386	Cattle	Argentina	2006	Lineage 1 sublineage 1A	Torres et al. [22]
KF864397	Cattle	Argentina	2006	Lineage 1 sublineage 1A	Torres et al. [22]
KF864407	Cattle	Argentina	2006	Lineage 1 sublineage 1A	Torres et al. [22]

RS State of Rio Grande do Sul, *PA* State of Pará, *RJ* State of Rio de Janeiro, *MG* State of Minas Gerais, *SP* State of São Paulo

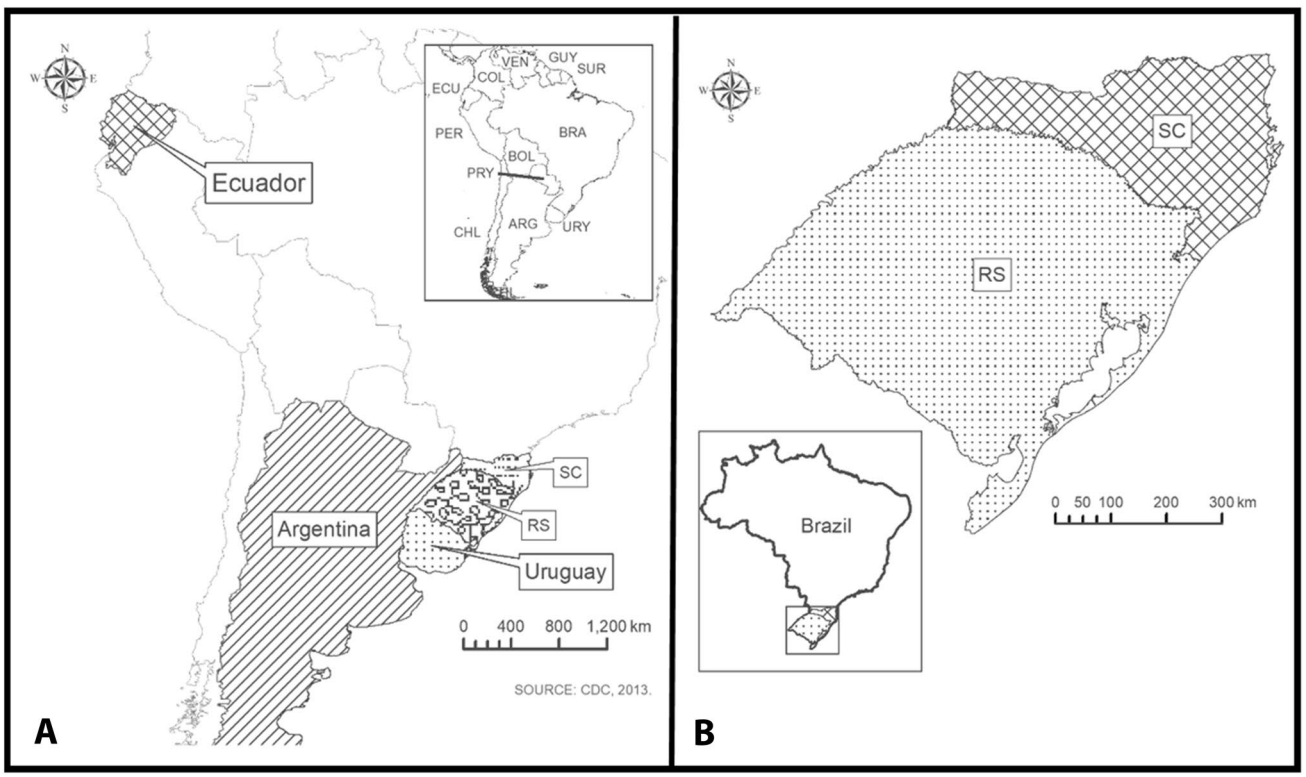

Fig. 1 Geographical distribution of samples of rabies virus (RABV) included in this study, **a** Map of Latin America showing countries where sequences of RABV were collected, **b** Map of Brazil showing each state where RABV isolates were collected and sequenced in this study

Discussion

Vampire bats of the species *D. rotundus* are distributed throughout Latin America [2]. However, the introduction and dissemination of cattle in the subcontinent have provided a widely available source of food for the species. The states of RS and SC, the two most southern states in Brazil,

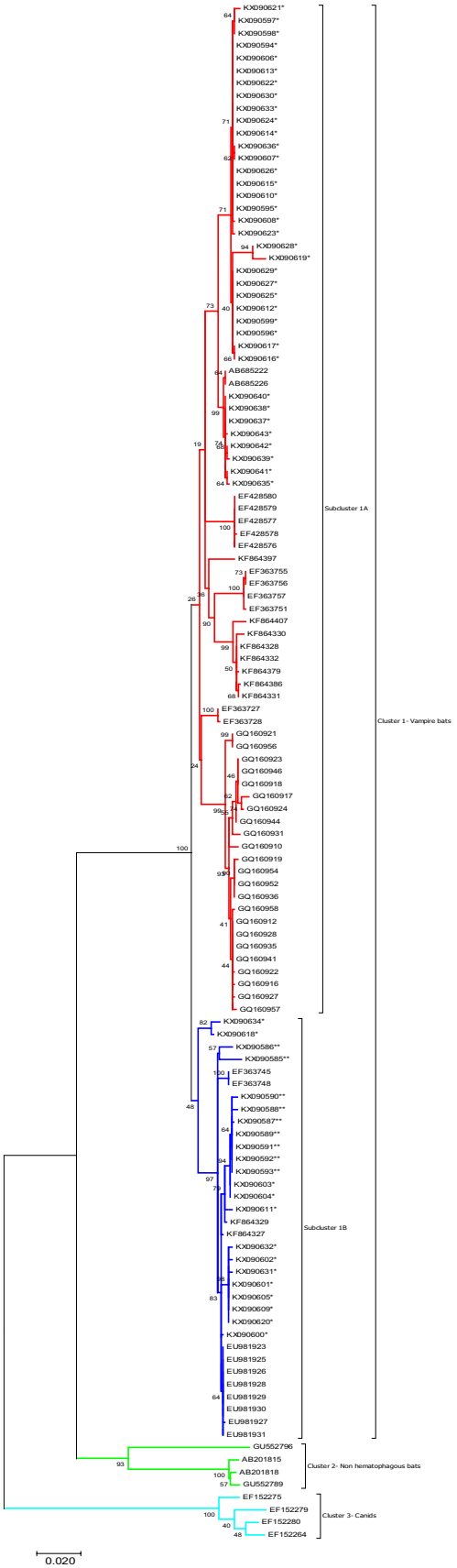

Fig. 2 Phylogenetic tree based on the partial 1.218-nt region within the N gene of RABV (nucleotide position 202–1420 in the standard sample of RABV PV-Pasteur Virus, GenBank accession number: GB M13215.1). Tree was generated by using Maximum Likelihood method based on the Tamura-Nei model (Tamura et al. 1993), with MEGA 7 software (Kumar et al. 2001). Cluster 1 (divided in subcluster 1A, marked in red and 1B, marked in blue), cluster 2 marked in green and cluster 3 marked in light blue. Samples sequenced in this study are marked with *RS and **SC

have been successful in controlling urban rabies transmitted by dogs. However, rural rabies remains endemic, mostly affecting cattle, since the species is the preferred food source for *D. rotundus*. Vaccination of cattle against rabies is not compulsory as the disease often occurs in outbreaks; farmers sometimes prefer not to vaccinate with the expectation that "there will be no rabies" on that particular season or year. Consequently, new outbreaks of rabies in cattle can be expected to occur and are invariably associated with the presence of infected vampire bats in the region. However, more detailed studies aiming to investigate the genetic lineages of RABV that may be circulating in southern Brazil are scarce [16, 23–25]. In view of this scarcity, in the present study, the coding region sequences of the RABV *N* gene from isolates recovered from infected cattle in the states of RS and SC were sequenced and compared to equivalent sequences available in GenBank. Such an approach has been used in a number of previous studies [7, 8, 21, 23, 24]. Through examination of the topology of the phylogenetic tree generated by the analyses, two genetic sublineages of RABV (1A and 1B) were identified to be circulating in southern Brazil, both having the vampire bat *D. rotundus* as their natural reservoir.

Indeed, the distributions of the isolates examined here do not reflect statistically representative samplings of all geographic areas of these two states. However, the sampling covered regions with high incidences of rabies in cattle. A few other studies have addressed the same issue; however, in those studies, sampling was performed in quite limited regions [16, 21, 25]. Therefore, available data on RABV sequences circulating in those regions are still quite scarce.

The current study identified RABV isolates of two sublineages (1A and 1B) that have been invariably associated with transmission by the vampire bat *D. rotundus*. Sublineage 1A was more widely distributed than 1B and was detected in all RS regions sampled. This sublineage clustered with RABV isolates from regions in northeast and central RS obtained in previous studies [16, 24]; the same sublineage has also been detected in southeastern Brazil (in the states of São Paulo, Rio de Janeiro and Minas Gerais), northern Brazil (in the State of Pará), Argentina and Ecuador, evidencing its wide geographic distribution. Sublineage 1B, in contrast, seemed to have a more restricted geographic distribution, since it was detected

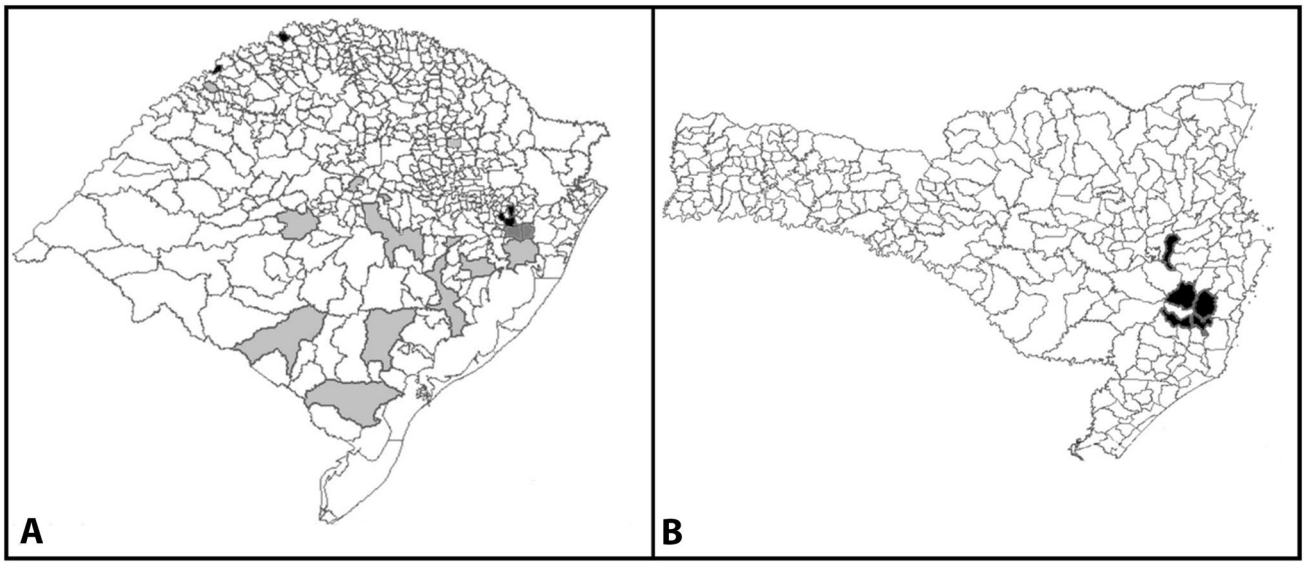

Fig. 3 Map of State of Rio Grande do Sul (**a**) and State of Santa Catarina (**b**) from southern Brazil showing locality of municipalities according to the sublineage of *Rabies lyssavirus* (RABV) identified. Light grey indicates RABV sublineage 1A, black indicates sublineage 1B and dark grey indicates municipalities where both sublineages 1A and 1B are identified

only in the states of RS and SC, the two most southern states in Brazil, and in Argentina and Uruguay, countries bordering southern Brazil. This is the only sublineage identified in the state of SC.

The sublineages identified in the study could have originated from two different colonies of *D rotundus* if independent coevolution occurred between each specific colony of bats and RABV. The geographic distribution of each viral sublineage may be related to the ecology of *D. rotundus*, which is able to move over long distances; such ecology is hypothesized to have contributed to the formation of the two sublineages of RABV.

In the present study, the analyses were focused on the *N* gene due to the large number of sequences of such genes available for comparisons in databanks; however, sequences corresponding to RABVs from southern Brazil are not readily available in databanks. The current study contributes to the availability of sequences in the public databanks, providing additional resources for a better understanding of the occurrence of rabies in the region.

The findings reported here are expected to contribute to the understanding of the biology of the virus in the region and its interactions with the natural host *D. rotundus*. Eventually, such studies may be expected to contribute to decreases in the numbers of cattle lost due to rabies. It is likely that different *D. rotundus* colonies or clones segregated over time within the sampled region; this might have led to adaptation of the best suited viral sublineages.

Conclusion

Two genetic sublineages of rabies virus (RABV), 1A and 1B, both associated with the vampire bat *D. rotundus* as their natural host, were identified during the period of sampling of this study (from 2012 to 2015). In the state of RS, sublineages 1A and 1B were detected with a predominance (37 out of 59 isolates) of sublineage 1A; cocirculation was suggested by the identification of the sublineages in two municipalities. In the state of SC, circulation of sublineage 1B only was detected.

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Author contributions MES, LLA, JCAR, JCF, SDT and HBCRB conceived and designed the experiments; MESF and ANFG performed practical experiments; MESF, PCJr., JGCK and RNO performed the analysis of sequences; MESF, PMR and HBCRB wrote the manuscript and prepared the figures. All authors reviewed and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This work complies with Protocol No. 01/2015 issued by the Ethics Committee of the Pasteur Institute of São Paulo.

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