Sequence analysis of nucleoprotein gene reveals the co-circulation of lineages and sublineages of rabies virus in herbivorous in Rio Grande do Sul state, Brazil

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Received: 17 August 2019 / Accepted: 10 January 2020 / Published online: 17 January 2020
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Abstract

An unprecedented outbreak of rabies occurred in Rio Grande do Sul state (RS) from 2012 onward, resulting in thousands of bovine deaths, important economic losses, and posing risk to human health. This article describes a genetic analysis of 145 rabies viruses (RABV) recovered from herbivorous from RS between 2012 and 2017, based on partial sequence analysis of the nucleoprotein (N) gene. High nucleotide (*nt*) identity (95.5 to 100%) and amino acid (*aa*) similarity (96.7 to 100%) were observed among the analyzed sequences. These sequences displayed a high sequence *nt* identity/*aa* similarity with bovine RABV sequences (96.4–97.9%; 98.1–100%, respectively) and vampire bat RABV sequences (96.3–97.5%; 97.8–99.5%). Phylogenetic analyzes based on the N sequence allowed for the segregation of viruses into two distinct *clusters*. *Cluster 1* comprised RABV sequences covering the whole studied period, whereas *cluster 2* grouped a lower number of viruses from 2013, 2014, 2015, to 2017. In some cases, viruses obtained from the same region within a short period of time grouped to distinct clusters or sub-clusters, indicating the co-circulation of distinct virus lineages in these outbreaks. The segregation into *sub-clusters* was also observed for viral sequences obtained from the same region at different times, indicating the involvement of distinct viruses. In summary, partial sequence analyses revealed a high conservation of N protein and the circulation of two lineages and different sublineages of RABV in the region. In addition, our results confirm the suitability of N gene to study the genetic relationships among RABV isolates.

Keywords *Rhabdoviridae* · Neurological diseases · Cattle · Genetic characterization · Phylogeny

Responsible Editor: Fernando R. Spilki.

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s42770-020-00226-z>) contains supplementary material, which is available to authorized users.

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Introduction

Rabies is a zoonotic disease distributed worldwide, characterized by severe neurological signs of course generally fatal in domestic and wild mammals [1]. The disease is caused by rabies virus (RABV), an enveloped, single-stranded RNA virus of negative polarity, belonging to the genus *Lyssavirus*, family *Rhabdoviridae* [2, 3]. The RABV genome contains genes coding for five proteins: nucleoprotein (N), phosphoprotein (P), matrix (M), glycoprotein (G), and polymerase (L) [3]. The N is the main component of the helicoidal nucleocapsid that encapsidates the genomic RNA and plays a role in the temporal transition between transcription and replication of the viral genome during the replicative cycle [4]. The nucleotide (*nt*) sequence of protein N gene is highly conserved among field RABV isolates and, as such, has been largely used for genetic identification of viral lineages, sublineages, and variants [5–17].

RABV is perpetuated in nature through cycles of infection involving terrestrial carnivores and bats. In South America, the hematophagous bats *Desmodus rotundus* are the main reservoirs and sources of RABV to herbivorous [18]. Cattle and horses are frequent targets for *D. rotundus* feeding and, as such, constitute the main herbivorous species affected by rabies [19]. The control of rabies in endemic areas is based on systematic vaccination of susceptible species and control of bat populations [20, 21].

Historically, rabies has affected bovine herds from several Brazilian regions, leading to significant economic losses to the livestock industry [22]. Official data indicates the occurrence of 37,049 cases of rabies in cattle between 1999 and 2017 in the country. In Rio Grande do Sul state (RS), the southernmost Brazilian state, hundreds of cases have been reported in cattle every year, in addition to countless unreported cases. Official data reported 1990 cases of bovine rabies from 2005 to 2017 [23]. In the last years, in addition to a crescent number of cases, bovine rabies has spread out and extended its geographical range reaching out areas otherwise free of the disease, including neighbor countries as Uruguay and Argentina [24]. The high incidence and the geographical spread of rabies have resulted in drastic economic losses to the cattle industry in the affected regions. In addition, the zoonotic potential of RABV requires that people exposed to affected animals search for medical assistance and, frequently, need to be submitted to post-exposure treatment [25]. Official data indicate that 334,956 anti-rabies medical appointments were performed in RS from 2007 to 2017 [26].

Molecular and phylogenetic analysis of RABV isolates involved in field outbreaks have become an important tool to determine the origin of the viruses and to understand the temporal and spatial patterns of dissemination/dispersion of colonies of vampire bats [13, 27, 28]. The information gathered from some studies has helped to predict future occurrence of cases and, thus, has allowed for the adoption of prevention and control measures [9, 29]. In general, studies of RABV phylogeny and dispersion have been performed through analysis of the complete G gene, G-L intergenic region, and/or partial or complete analysis of N gene [9, 30]. In this sense, the N gene has been the preferred target for sequence analysis, since it is the most conserved gene among *Lyssaviruses* [31].

Very little information is available on the RABV isolates associated with the recent outbreak reported in RS. The available information is restricted to a few studies analyzing a limited number of viruses, usually originated from restricted areas or outbreaks [29, 32–34]. Thus, the present study aimed at analyzing the N sequences from RABV associated with herbivorous rabies in RS between 2012 and 2017 (77 from this study and 68 obtained from GenBank), trying to identify viral lineages and/or sublineages and to determine possible phylogenetic relationships among the circulating viruses.

Material and methods

This study included brain samples of cattle ($n = 74$), horses ($n = 2$), and sheep ($n = 1$) from 72 farms located in RS. The samples were submitted to the Virology Section of the Federal University of Santa Maria (SV/UFSM), central RS, and to the *Instituto de Pesquisas Desidério Finamor* (IPVDF), Eldorado do Sul, metropolitan RS region, for diagnosis of rabies between 2012 and 2017. All included samples had been tested positive for RABV in a direct fluorescent antibody test (FAT) and by mouse inoculation test. RABV-positive samples were stored at $-20\text{ }^{\circ}\text{C}$ until testing. In addition to the produced sequences, 68 sequences originated from other studies deposited in GenBank (29, 33, 34) were included in the analysis, totalizing 145 sequences from RS.

Fragments of the cortex, hippocampus, thalamus, and medulla of RABV-positive brains (approximately 100 mg) were macerated and submitted to RNA extraction using Trizol® reagent (Thermo Fischer Scientific, Waltham, Massachusetts, USA) according to the manufacturer's instructions. Total RNA was diluted in 40 μL of water DNase, RNase, and pyrogen-free. Total RNA (6 μL) was submitted to in vitro transcription for cDNA synthesis, using the reverse transcriptase enzyme from GoScript™ Reverse Transcriptase (Promega Corporation, Madison, Wisconsin, USA). Following cDNA synthesis, the products were submitted to a PCR for amplification of a segment within the N protein gene coding sequence. The partial N gene (1284 nt) was amplified with primers: forward ATGTAACACCTCTACAATG [35] and reverse TTGACGAAGATCTTGCTCAT [36] according to a reaction described by Carnieli Junior et al. [37]. Each reaction was performed in a volume of 50 μL , with 3 μL of cDNA. Total RNA extracted from RABV negative and positive bovine brains were used as controls.

PCR products were resolved in a 1.5% agarose gel stained by Gel Red® (Biotium, Hayward, CA, USA) and visualized under UV light after electrophoresis (70 V, 25 min). For nt sequencing, PCR products were purified using QIAquick Purification Kit (Qiagen, Hilde, German). Positive samples were sequenced in duplicates in an automatic sequencer ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA), using BigDye reagent. The sequences were analyzed by Staden program [38] to obtain the consensus sequence. The partial N sequence was determined using the complete RABV N protein sequences available in the GenBank database as model. The alignment of the sequences with sequences deposited in GenBank, including nine from Santa Catarina, SC (a neighbor state) [34], 35 from provinces in Argentina (neighbor to RS) [39], and 12 from Uruguay (neighbor to RS) [40], was performed using the program BioEdit Sequence Alignment Editor Software suite, version 7.0.5.3 (<http://www.mbio.ncsu.edu/bioedit/bioedit.html>) [41]. Phylogenetic analyses was performed in the Molecular

Evolutionary Genetics Analysis (MEGA) software X [42], using the method *Maximum Likelihood* with the model of substitution *General Time Reversible* (GTR) [43], and the confiability was evaluated with *bootstrap* of 1.000 repetitions. The sequences were grouped in *clusters* and *sub-clusters* through topology analyses.

The data from the rabies cases from RS (species, origin/location, year, etc.) were obtained from SV/UFSM files, IPVDF files, *Sistema de Defesa Agropecuária* of SEAPDR, and from the literature. The information of the additional sequences was obtained from the literature. The geographic mapping of rabies cases and sequences was performed in Microsoft® Excel (2016). Additional information on the analyzed sequences are available on Online Resource 1.

Results

Sequence analysis of N protein gene coding region

The use of primers forward ATGTAACACCTCTACAATG [35] and reverse TTGACGAAGATCTTGCTCAT [36] allowed for the amplification of part of the N gene (1.284 nt – residues 67 to 1350) of 77 brain specimens. The alignment of sequences produced in the present study ($n = 77$) with sequences deposited in GenBank ($n = 68$) demonstrated the presence of several *nt* changes comparing to the standard RABV CVS-PV (GenBank access number M13215). Some of these changes were aleatory/random, whereas others were frequently identified in each *cluster* or *sub-cluster*. *Nt* changes in 163 sites distributed randomly over the N coding region were present in all examined sequences, distinguishing them from the standard RABV CVS-PV, a RABV originated from carnivorous/dogs. Nonetheless, some *nt* changes were consistently detected according to determined *clusters* and *sub-clusters* and, eventually, determined the segregation of the viruses in the phylogenetic tree (Online Resources 2 and 3). As a whole, most *nt* changes did not result in amino acid (*aa*) substitutions, with exceptions of sequences of *sub-clusters* 1a–1d and 1 h, which presented *aa* changes at residue 50 (Asn → Ser).

The *nt* identity among the sequences varied from 95.5 to 100% and was also high when these sequences were compared with a bovine RABV (96.4 to 97.8%; GU592649) and with a *D. rotundus* RABV (96.3 to 97.5%; GU592648). The

nt identity dropped to 83.2 to 84.4% when comparing these sequences with a domestic carnivore RABV N sequence (KX148217) (Table 1).

The *nt* sequences converted into predicted *aa* resulted in a sequence of 428 residues (residues 23 to 450). The residue position related to RABV virulence in protein N was determined according to Masatani et al. [44, 45] and Shimizu et al. [46] and revealed a high conservation of N sequence among the field RABV viruses, noticeably in residues involved in virulence. The *aa* similarity of the analyzed region varied from 96.7 to 100%. A high similarity was observed between these sequences and a bovine RABV (98.1 to 100%; GU592649) and a *D. rotundus* RABV deposited in GenBank (97.8 to 99.5%; GU592648). A high similarity was also observed with a domestic carnivore RABV sequence (94.6 to 96.4%; KX148217) (Table 1). No *aa* mutation was observed in the residues of N protein putatively involved in RABV virulence: 273 (Phe), 394 (Tyr), and 395 (Phe) [44–46]. Sequences grouped in *sub-clusters* 1a to 1d and 1 h presented a consistent change at residue 50 (Asn → Ser) (Online Resource 4).

Phylogenetic analysis and geographical distribution

The phylogenetic tree built based on N gene demonstrated that all sequences analyzed herein grouped with sequences from herbivorous and vampire bats deposited in GenBank and apart from sequences from carnivores and other wild animals. The herbivorous lineage segregated into two *clusters* (1 and 2), harboring *sub-clusters* (Figs. 1 and 2). This clustering reflected the *nt* changes observed, in which 11 mutations were present in all sequences from cluster 1, and 25 mutations were present in cluster 2 (Online Resources 1 and 2).

Cluster 1 grouped all viruses from 2012 to 2016 and several sequences from 2013, 2014, 2015, to 2017. *Cluster* 2 grouped a lower number of sequences from 2013, 2014, 2015, to 2017, originated from specific RS regions: Porto Vera Cruz and Esperança do Sul (northwestern RS mesoregion), Jaguari and São Pedro do Sul (central-eastern RS mesoregion), and Rolante, Gravataí, Glorinha, Sapiranga, Novo Hamburgo, and Três Cachoeiras (metropolitan mesoregion). These findings indicate that viruses from this cluster may have derived from a common ancestor, different from *cluster* 1 viruses. All sequences from SC and

Table 1 Nucleotide and amino acid identity in the partial sequence of nucleoprotein of RABV recovered from cases of rabies in herbivorous species in Rio Grande do Sul state, Brazil, compared to domestic

carnivore RABV sequence (GenBank access KX148217), bovine RABV sequence (GU592649), *Desmodus rotundus* RABV sequence (GU592648) and among the sequences this study

Identity (%)	Domestic carnivore RABV	Bovine RABV	<i>Desmodus rotundus</i> RABV	Among the sequences
Nucleotide	83.2 to 84.4	96.4 to 97.8	96.3 to 97.5	95.5 to 100
Amino acid	94.6 to 96.4	98.1 to 100	97.8 to 99.5	96.7 to 100

Fig. 1 Phylogenetic tree with Cluster 2 compressed constructed by the Maximum Likelihood method with the General Time Reversible (GTR) substitution model of RABV N gene partial sequences recovered from herbivores in the state of Rio Grande do Sul, Brazil (● RABV from herbivores and vampire bats obtained from GenBank; ■ RABV from carnivores and wild animals obtained from GenBank)

Uruguay and two sequences from Argentina grouped in *cluster 2*. The rest of Argentinean sequences grouped in *cluster 1*. Figure 3 shows the geographical distribution of the 145 analyzed sequences, according to the respective clusters, along with 770 out of 808 cases of rabies (87.5%) diagnosed in herbivorous in RS from 2012 to 2017.

In some cases, viruses from the same county/municipality obtained within a short period of time (0 days to 2 months) grouped to different *clusters* or *sub-clusters* as observed in sequences from Gravataí/2013 (KX090628) and Glorinha/2013 (KX090618), which grouped in *cluster 1*, whereas the others (KX090603, KX090602, KX090604, KX090605, and KX090601) belonged to *cluster 2*. This segregation was also observed in viruses from São Pedro do Sul/2014 in which one sequence (MN103510) grouped to *cluster 1* and the other two (MN103509 and MN103511) with *cluster 2*.

The segregation of viruses originated from the same location in a short period of time into different *sub-clusters* was observed in sequences from Aceguá/2015 (MN103478 in *sub-cluster 1b* and MN103478 in *sub-cluster 1a*), Gravataí/2013 (KX090603 and KX090604 in *sub-cluster 2b* and KX090605 in *sub-cluster 2d*), Hulha Negra/2015 (KX090622 in *sub-cluster 1b* and KX090623 in *sub-cluster 1c*), Pinhal Grande/2012 (KX090636 in *sub-cluster 1b* and the others in *sub-cluster 1f*) and Rio Pardo/2013 (KX090612 in *sub-cluster 1d* and the other in *sub-cluster 1b*). This pattern of segregation suggests that different RABV (from different bat colonies or divergent within the same colony) were probably involved in these cases/outbreaks.

This segregation was also observed for some sequences obtained in different periods in the same location. RABV sequences from Pinhal Grande/2012 grouped predominantly to *sub-cluster 1f*, whereas sequences from 2015, 2016 and 2017 grouped to *sub-cluster 1a*. Similar segregation was observed for viruses from Caçapava do Sul, which were grouped into distinct *sub-clusters* (2015 and 2017 in *sub-cluster 1b* and 2013 in *sub-cluster 1d*). These findings indicate that distinct RABV were involved in the cases/outbreaks occurring in these locations at different times, likely reflecting distinct RABV circulating in different bat colonies or, less likely, resulting from virus evolution.

Discussion

Sequence analysis of part of RABV N allowed for the identification of lineages and sublineages, as well their spatial and temporal circulation in RS and nearby areas between 2012 and

2017. This study included an expressive number of sequences ($n = 145$) originated from all RS mesoregions, differing from previous studies which analyzed a limited number of viruses from localized outbreaks and/or from restricted areas [29, 32–34].

Sequence analysis of RABV N protein has been largely used to classify/group isolates according to their genetic lineage, variant, host species, origin, and/or geographical distribution [9, 10, 14, 17]. Analysis of RABV N protein from Brazil has led to clustering the isolates into two groups, according to the host species: viruses from dogs/carnivores, belonging to the terrestrial epidemiological cycle, and vampire bats, which together with non-hematophagous bats, form the aerial cycle of the rabies in Brazil [9, 10]. The group of viruses associated with vampire bats could be further divided/classified in subgroups according to their geographic origin [9]. Analysis of N sequence also allowed for the identification of a group of RABV associated with non-hematophagous bats from several Brazilian regions [17].

The results presented herein confirmed the high conservation of the N coding sequence among field RABV isolates [35, 47]. Amino acid mutations were randomly observed among the analyzed sequences, yet these changes did not keep an evident pattern nor involved the residues associated with RABV virulence. Residues 273 [Phe], 394 [Tyr], and 395 [Phe] are involved in the evasion of the innate immunity and are important for packaging the viral genome [44–46]. A mutation in residue 50 (Asn → Ser) observed in sequences of *sub-clusters 1a* to *1d* and *1h* and also in sequences recovered from GenBank might lead to structural changes of the N protein and, thereby, may affect the virus biology and pathogenesis [48].

The co-circulation of these RABV lineages and sublineages in RS apparently does not pose risk to the efficacy of FAT-based diagnosis since the antibodies used in such assays are generally polyclonal [49]. These reagents are able to recognize/react with different viral proteins and, as such, seem refractory to minor antigen variations as that observed in N residue 50 (Asn → Ser). On the other hand, FAT assays based on monoclonal antibodies (MAbs) and molecular-based techniques are more prone to failure when used in studies applied to an antigenic/genetic diverse population of viruses [50]. Fortunately, this is not apparently the case.

An interesting finding from the phylogenetic analysis was the segregation of viral sequences from the same location in different sub-clusters according to time. These findings indicated that different RABV lineages were associated with outbreaks occurring at different times in the same locations. A given virus associated with an outbreak in 2012 may have suffered mutations with time, and/or, more likely, a distinct virus may have been introduced in the region by arrival of new bat colonies [20, 27, 29].

In addition to viral divergence observed with time, some viruses recovered from the same area within a short period

Fig. 2 Phylogenetic tree with Cluster 1 compressed constructed by the Maximum Likelihood method with the General Time Reversible (GTR) substitution model of RABV N gene partial sequences recovered from herbivores in the state of Rio Grande do Sul, Brazil (● RABV from herbivores and vampire bats obtained from GenBank; ■ RABV from carnivores and wild animals obtained from GenBank)

(days to months) segregated to distinct *clusters* or *sub-clusters*. These findings indicate a co-circulation of different viral variants in the same region, sharing the same or nearby shelters and/or bat colonies, as described previously in the central mesoregion [29]. On the other hand, the same lineage of RABV may be involved in different outbreaks occurring in the same region in a short period, as described previously in the northeast mesoregion [33].

The identification of two *clusters* and their *sub-clusters* of the vampire bat RABV lineage demonstrates their co-circulation in RS in the examined period. These lineages may have been originated from distinct bat colonies or, alternatively, may have been maintained concomitantly along time in the same bat colonies [29]. These findings reinforce that the development of efficient strategies for the control of bat colonies depends upon the understanding of the dynamics of virus transmission within and between colonies [51]. The efforts for virus elimination or mitigation should be, therefore, coordinated spatially aiming at defining the dynamics of virus transmission within enzootic regions [52].

The present study included sequences obtained from 48 counties, covering all RS mesoregions and comprising a diversity of biomes, including remains of the Atlantic forest and

Pampa biomes. This sampling covered a significant area containing many of the approximately 2600 bat shelters cataloged by the RS Official Veterinary Service and subjected to periodic control of bat populations [54, 55].

Co-circulation of two distinct RABV lineages in RS has already been reported by Fernandes [34] and Cargnelutti et al. [32] testing a smaller number of samples. Our data support and extend these findings indicating that these lineages are likely established in the RS ecosystem. Most of the sequences analyzed in the present study grouped in *cluster 1*, which presented a wide geographic distribution (Fig. 3) and were genetically related to viruses from nearby states (SC) and countries/provinces (Uruguay and Argentina). On the other hand, viruses belonging to *cluster 2* showed a more restrict geographic pattern but were related to sequences from SC and from a neighbor Uruguayan province.

Sequence analysis of RABV has already been used for mapping out the geographic dispersion of isolates [9, 27, 30, 53]. Unfortunately, the data from the present study did not allow for the identification of particular dissemination patterns for each lineage/sublineage. Analysis of a larger number of samples recovered from a more comprehensive area during a longer period may be required to achieve such an intent.

Historically, herbivorous rabies related to vampire bats has been endemic in several RS regions, noticeably those presenting environmental conditions to favorably shelter bat populations. Southern municipalities/counties such as Aceguá, Bagé, and Caçapava do Sul were historically free of rabies in spite of the presence of vampire bat populations. From 2011 onward,

Fig. 3 Cases/outbreaks of herbivorous rabies in the state of Rio Grande do Sul, Brazil. Farms that had viruses grouped in cluster 1 in the phylogenetic tree are highlighted in green, and farms that had viruses in

cluster 2 are in red. Farms where the rabies diagnosis was performed by the immunofluorescence test, biological test, or RT-PCR from 2012 to 2017, but whose N sequences were not obtained are highlighted in yellow

the occurrence of herbivorous rabies moved southward, reaching out these regions, close to the Uruguayan border (Fig. 3) [56]. Natural or manmade ecological changes occurring in the last years may have contributed for this dispersion/dissemination, probably by affecting the behavior and dispersion dynamics of bat colonies [20].

In summary, partial sequence analysis of the N gene from RABV recovered from RS (2012–2017) demonstrated a high conservation of this gene/protein, indicated the co-circulation of two lineages and sublineages, and reinforced the suitability of N sequence to study the genetic relationships among RABV isolates.

Acknowledgments We thank the *Secretaria da Agricultura, Pecuária e Desenvolvimento Rural do Rio Grande do Sul (SEAPDR/RS)* for the leaving period conceded to GLA and for *Instituto de Pesquisas Veterinárias Desidério Finamor (IPVDF)* for providing some bovine brains and information concerning the viral sequences and to Nilton Rossato and Wilson Hoffmeister, coordinators of the Herbivore Rabies Control Program in Rio Grande do Sul, for their support and encouragement for this study.

Funding information EFF and RW are recipients of CNPq fellowships (Conselho Nacional de Desenvolvimento Científico e Tecnológico). This study was founded in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Finance Code 001 and by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (47337/2014–9).

Compliance with ethical standards

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

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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