Article/Artigo

Genetic characterization of rabies virus isolated from bovines and equines between 2007 and 2008, in the States of São Paulo and Minas Gerais

Caracterização genética de vírus da raiva isolado de bovinos e equinos entre 2007 e 2008, nos Estados de São Paulo e Minas Gerais

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ABSTRACT

Introduction: Rabies is an acute disease of the central nervous system and is responsible for the deaths of thousands of humans, wild animals and livestock, particularly cattle, as well as causing major economic losses. This study describes the genetic characterization of rabies virus variants that circulate in *Desmodus rotundus* populations and are transmitted to herbivores. **Methods:** Fifty rabies virus isolates from bovines and equines in the States of São Paulo and Minas Gerais, Brazil, were genetically characterized and compared with sequences retrieved from GenBank. **Results:** Two clusters (I and II) with mean nucleotide identities of 99.1 and 97.6% were found. The first of these contained nearly all the samples analyzed. Lineages from other Brazilian states grouped in cluster II. **Conclusions**: Analysis of the amino acid sequences of the N proteins revealed the existence of genetic markers that may indicate possible variations between geographic regions, although the biologically active regions are conserved within the species over space and time.

Key-words: Rabies virus. *Desmodus rotundus*. Genetic characterization. Nucleoprotein gene. Cattle.

RESUMO

Introdução: A raiva é uma doença aguda do sistema nervoso central e é responsável por mortes de milhares de humanos, animais silvestres e animais de criação – especialmente bovinos – além de causar elevadas perdas econômicas. Este trabalho descreve a caracterização genética das variantes do vírus da raiva que circulam em populações de *Desmodus rotundus* e são transmitidas aos herbívoros. **Métodos:** Cinquenta isolados de vírus da raiva de bovinos e equinos provenientes dos Estados de São Paulo e Minas Gerais, Brasil, foram caracterizadas geneticamente e comparadas com sequências recuperadas do GenBank. **Resultados:** Dois clusters, I e II, apresentando identidades médias de nucleotídeos de 99,1 e 97,6%, foram obtidos, sendo o primeiro composto de quase a totalidade das amostras analisadas. Linhagens de outros estados do Brasil "clustered" no II. **Conclusões:** A análise das sequências de aminoácidos da proteína N revelou que existem marcadores genéticos que podem determinar uma possível regionalidade embora as regiões biologicamente ativas apresentem-se conservadas dentro das espécies ao longo do tempo e espaço.

Palavras-chaves: Vírus da raiva. *Desmodus rotundus.* Caracterização genética. Gene da nucleoproteína. Bovinos.

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INTRODUCTION

Rabies is an acute disease of the central nervous system that has almost worldwide distribution and can affect all mammals. The rabies virus (RABV) belongs to genotype 1 of the genus *Lyssavirus* in the family *Rhabdoviridae*[1]. Infection by the virus is responsible for the deaths of thousands of humans, wild animals and livestock, particularly bovines, as well as causing major economic losses. In 2007, Brazilian livestock experts calculated that there had been 25,000 cases of bovine rabies in Brazil, when undernotification and clinical diagnoses were taken into account[2]. While traditional viral detection methods can monitor the presence of RABV transmitted to herbivores, only techniques such as the polymerase chain reaction (PCR) and genetic sequencing can determine whether the virus genetic makeup varies with geographic distribution.

In Latin America, practically all cases of rabies in herbivores are transmitted by the hematophagous bat *Desmodus rotundus*. Although the virus can be genetically characterized using samples from hematophagous bats, the rate of positive rabies findings in these animals and in non-hematophagous bats in the State of São Paulo is low (1-2%)[3,4]. Genetic characterization of RABV samples isolated from bovines can provide important information about possible differences in the genetic lineages of the virus circulating in *Desmodus rotundus* populations. These differences are the result of mutations that occur randomly in different geographic regions and over time.

The aim of this study was to genetically characterize the RABV lineages in the States of São Paulo and Minas Gerais in the years 2007 and 2008 that were transmitted by *Desmodus rotundus* and circulating in herbivores. Molecular methods were used to analyze the segment of the genome that encodes the N protein, and the resulting data are expected to be of benefit for studies on the epidemiology and geographic distribution patterns of rabies.

METHODS

Samples

To study the nucleoprotein (N) gene in antigenic variant 3 (AgV3) lineages, which are characteristic of *Desmodus rotundus*, a total of 50 central nervous system samples (40 from bovines and 10 from equines, GenBank accession numbers GQ160910 to GQ160959) were sequenced genetically (**Table 1**). These samples had originated from the states of São Paulo (SP) and Minas Gerais (MG) (**Figure 1**) in the years 2007 and 2008 and had been sent to the Pasteur Institute of São Paulo to be analyzed for rabies.

Direct immunofluorescence and mouse inoculation test

The central nervous system samples were diagnosed positive for rabies by inoculation in mice, as described by Koprowski[5], and by the direct immunofluorescence test[6] using fluorescein isothiocyanate-labeled anti-nucleocapsid polyclonal antibodies.

Reverse transcriptase-polymerase chain reaction, DNA sequencing and phylogenetic analysis

RNA was extracted from the 50 samples using TRizol® reagent (Invitrogen), in accordance with the manufacturer's instructions. RT-PCR was carried out using the 21G sense (ATGTAACACCTCTACAATG) and 304 antisense (TTGACGAAGATCTTGCTCAT)[7] primers and the protocol described by Macedo et al[8].

The amplified DNA fragments were purified with GFX PCR DNA and the Gel Band Purification kit (Amersham Biosciences™) and subjected to sequencing reactions using sense and antisense primers with the BigDye Terminator v3.1 cycle sequencing kit (Amersham Biosciences™) in accordance with the manufacturer's instructions. The sequencing was carried out in an Applied Biosystems 3,130 automated DNA sequencer. A 1,320-nucleotide region corresponding to the portion of the nucleoprotein gene located between nucleotides (nt) 30 and 1,350 of the PV strain (GenBank accession number M13215.1) was analyzed. Data from raw sequencing were edited using CHROMAS software (version 2.24 © 1998-2004 Technelysium Pty Ltd), and the final sequences were aligned with sequences present in GenBank (**Table 2**) by the CLUSTAL/W method using BioEdit software[9]. The alignments were used to build neighbor-joining distance-based DNA phylogenetic trees with the Kimura-2 parameter correction model and 1,000 bootstrap repetitions for statistical support using the Mega 2.1 program[10]. The nucleotide and amino acid identities were calculated using BioEdit software.

RESULTS

Direct immunofluorescence and mouse inoculation test

All 50 AgV3 RABV lineages used in this study were positive for FAT and MIT.

Phylogenetic analysis

Phylogenetic analysis was carried out using the sequences corresponding to the N gene (1,320 nt). The lineages segregated into two clusters (I and II), and the first of these was divided into six subclusters (Ia to If) (**Figure 2**). The relationship between the clusters and subclusters and their geographic distribution (areas X1 to X4) can be seen in **Figure 1**.

TABLE 1 - GenBank accession numbers for the sequences of N gene used in this study showing the species from which the AgV3 RABV lineages were isolated, city, state and year in which the sample were obtained.

GenBank number	Species	Mucicipality	State	Year
GQ160910	cattle	Andradas	MG	2007
GQ160911	cattle	Andradas	MG	2007
GQ160912	cattle	Itapeva	SP	2007
GQ160913	cattle	Itirapuã	SP	2007
GQ160914	equine	Mococa	SP	2007
GQ160915	cattle	Patrocinio Paulista	SP	2007
GQ160916	cattle	Pedra Bela	SP	2007
GQ160917	cattle	Pocos de Caldas	MG	2007
GQ160918	cattle	Pocos de Caldas	MG	2007
GQ160919	cattle	Socorro	SP	2007
GQ160920	cattle	Andradas	MG	2008
GQ160921	cattle	Belo Horizonte	MG	2008
GQ160922	equine	Braganca Paulista	SP	2008
GQ160923	cattle	Caconde	SP	2008
GQ160924	equine	Caconde	SP	2008
GQ160925	cattle	Divinolândia	SP	2008
GQ160926	cattle	Espirito Santo do Pinhal	SP	2008
GQ160927	cattle	Extrema	MG	2008
GQ160928	cattle	Extrema	MG	2008
GQ160929	cattle	Extrema	MG	2008
GQ160930	equine	Extrema	MG	2008
GQ160931	equine	Franca	SP	2008
GQ160932	equine	Franca	SP	2008
GQ160933	cattle	Iacri	SP	2008
GQ160934	cattle	Iacri	SP	2008
GQ160935	equine	Joanopólis	SP	2008
GQ160936	cattle	Lindoia	SP	2008
GQ160937	cattle	Lindoia	SP	2008
GQ160938	cattle	Marilia	SP	2008
GQ160939	equine	Pedra Bela	SP	2008
GQ160940	cattle	Pedregulho	SP	2008
GQ160941	cattle	Piracaia	SP	2008
GQ160942	cattle	Piracaia	SP	2008
GQ160943	cattle	Salesopolis	SP	2008
GQ160944	cattle	São João da Boa Vista	SP	2008
GQ160945	cattle	São João da Boa Vista	SP	2008
GQ160946	cattle	São Sebastião da Grama	SP	2008
GQ160947	cattle	São Sebastião da Grama	SP	2008
GQ160948	cattle	São Sebastião da Grama	SP	2008
GQ160949	equine	São Sebastião da Grama	SP	2008
GQ160950	cattle	São Sebastião da Grama	SP	2008
GQ160951	cattle	São Sebastião da Grama	SP	2008
GQ160952	cattle	Serra Negra	SP	2008
GQ160953	cattle	Serra Negra	SP	2008
GQ160954	cattle	Socorro	SP	2008
GQ160955	cattle	Socorro	SP	2008
GQ160956	cattle	Três Corações	MG	2008
GQ160957	equine	Vargem	SP	2008
GQ160958	cattle	Vargem	SP	2008
GQ160959	cattle	Vargem	SP	2008

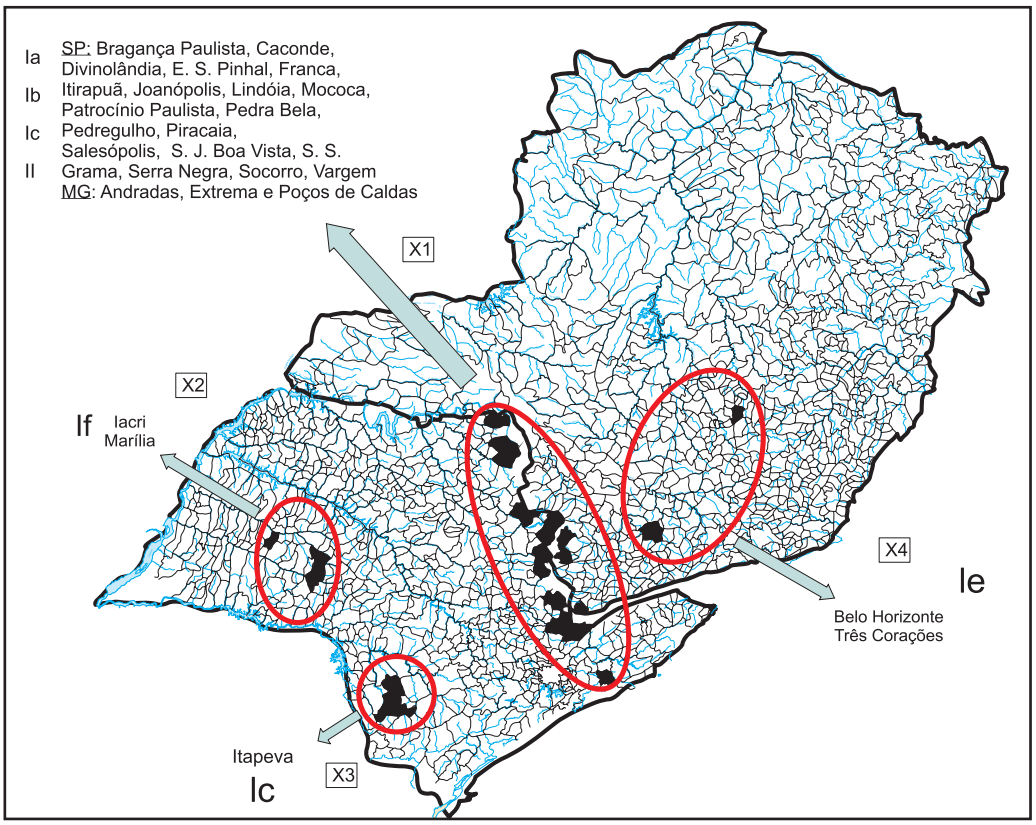

FIGURA 1 - Map of the States of São Paulo and Minas Gerais. The cities from which the 50 rabies virus lineages originated are shown grouped into four separate areas - X1, X2, X3 and X4 -identified by circles. Ia to If: subclusters in cluster I, II: cluster II.

TABLE 2 - GenBank accession numbers for the reference sequences of N gene used in phylogenetic analysis in this study showing the species from which the AgV3 RABV lineages were isolated, municipality, state and year in which the sample were obtained, and bibliographic references.

GenBank number	Species	Municipality	State	Year	References
AB083803	cattle	Morrinhos	GO	1999	Kobayashi et al[8]
AB083805	cattle	São Roque	SP	1994	Kobayashi et al[8]
AB083811	cattle	Colinas	TO	1999	Kobayashi et al[8]
AB083813	cattle	Alto Taquari	MT	1999	Kobayashi et al[8]
AB083817	cattle	Piraju	SP	1989	Kobayashi et al[8]
AB083818	cattle	Corumbaia	GO	1999	Kobayashi et al[8]
AB201802	*Artibeus lituratus*	Dracena	SP	2002	Kobayashi et al[8]
AB201804	*D. rotundus*	Lindóia	SP	2000	Kobayashi et al[6]
AB297634	*D. rotundus*	Itaperuna	RJ	1997	Kobayashi et al[6]
AB297636	*D. rotundus*	Guarulhos	SP	2000	Kobayashi et al[6]
FJ649082	cattle	Extrema	MG	1999	Carnieli et al[1]
FJ649085	cattle	Joanópolis	SP	1999	Carnieli et al[1]
FJ649087	cattle	Piracaia	SP	1999	Carnieli et al[1]
FJ649092	cattle	Piracaia	SP	1999	Carnieli et al[1]
FJ649098	cattle	Joanópolis	SP	1999	Carnieli et al[1]
FJ649103	cattle	Socorro	SP	1999	Carnieli et al[1]
FJ649115	cattle	Caconde	SP	2000	Carnieli et al[1]
FJ649138	cattle	Socorro	SP	2000	Carnieli et al[1]
FJ649142	cattle	Lindóia	SP	2000	Carnieli et al[1]
FJ649148	cattle	Pedra Bela	SP	2000	Carnieli et al[1]
FJ649154	cattle	Bragança Paulista	SP	2000	Carnieli et al[1]
FJ649159	cattle	Caconde	SP	2001	Carnieli et al[1]
FJ649170	cattle	Espírito Santo do Pinhal	SP	2001	Carnieli et al[1]
FJ649172	cattle	Mococa	SP	2001	Carnieli et al[1]
FJ649183	cattle	Vargem	SP	2000	Carnieli et al[1]

Cluster I, which was made up of sequences from samples that originated in SP and MG and a single sample from Goiânia, State of Goiás, had 99.1% mean nucleotide identity. Subclusters Ia to If had mean identities of more than 99%, and the identities between them ranged from 99 to 99.7%. Cluster II consists of sequences from the States of Goiás (GO), Mato Grosso (MT), Tocantins (TO) and Rio de Janeiro (RJ), as well as some sequences from São Paulo (SP). The mean identity within this cluster was 97.7%, and the mean identity between clusters I and II was 96.8%.

The changes in the nucleotides resulted in few changes in the amino acid (aa) sequences analyzed. Comparison between the predicted sequences of amino acids in the N protein in this study and the putative aa alignments revealed a region that characterized clusters I and II.

The aa identified at the position corresponding to position 50 of the complete coding of the N gene was histidine (H), and it was therefore this amino acid that characterized the sequence in this cluster, with the exception of subcluster If, in which this position was occupied by asparagine (N). Asparagine (N) was also identified in cluster II at the same position in two sequences from SP (municipalities of São Roque and Mococa) and in the sequences from GO and RJ. In all the other sequences from SP, as well as those from MT and TO, the amino acid serine (S) was identified at the same position. The amino acids cited above thus represent the genetic markers for clusters I and II (**Figure 3**).

Wunner[11] provided descriptions of biologically active areas of the N protein, and these were analyzed in the present study. For this purpose, we used the vaccine strain PV (GenBank accession number M13215) as a reference. A mutation was found in antigenic site III between amino acids 313 and 337. In the lineages studied here, a threonine residue was found at position 332, while in the PV lineage, alanine (nonpolar) was found at this position.

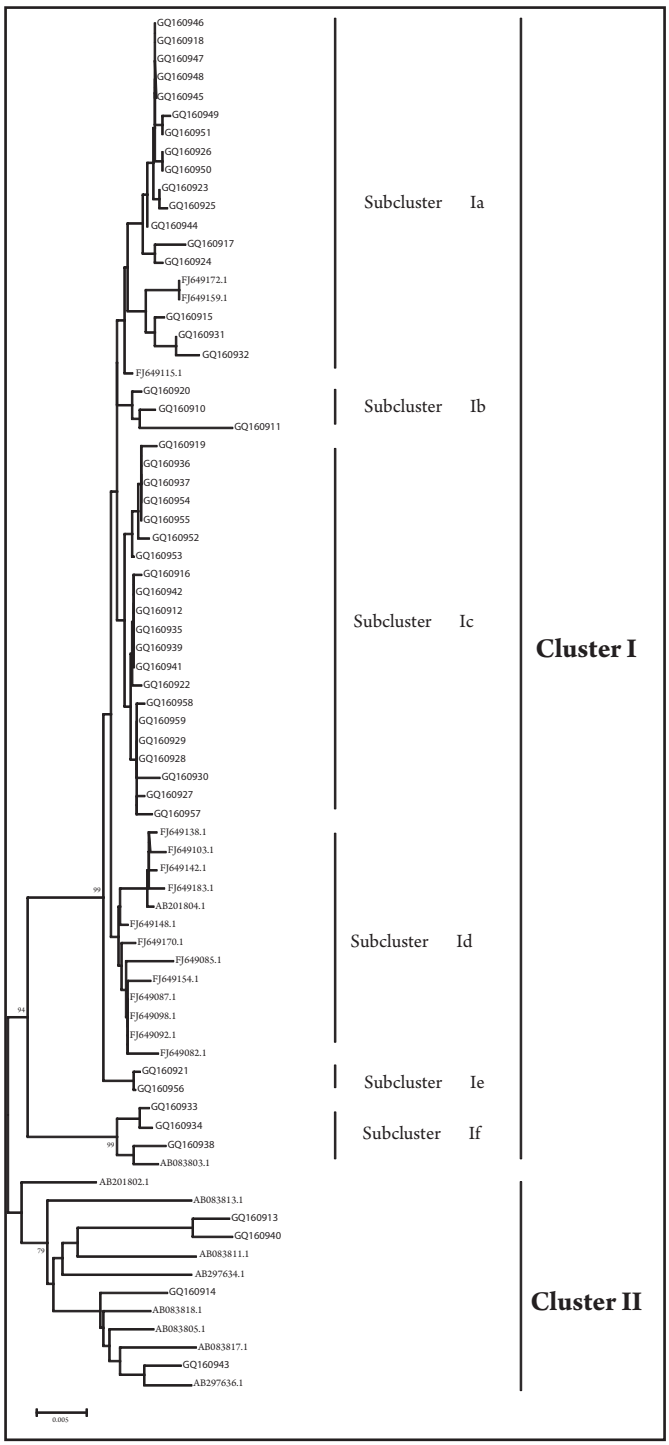

FIGURE 2 - Neighbor-joining tree constructed with sequences from the N gene of AgV3 RABV lineages isolated from cattle and equines in Brazil.

The regions from aa 358 to 367 in antigenic site I and from aa 359 to 366 in antigenic site IV were conserved, while the region from aa 375 to 383, also in antigenic site IV, contained three mutations: alanine, glutamic acid and threonine at positions 377, 378 and 379, respectively, instead of threonine, aspartic acid and valine, which are found at these positions in the PV.

In addition to the antigenic sites analyzed, the N protein has immunodominant helper T-cell epitopes between aa 404 and 418. In the lineages analyzed here, a mutation (a methionine residue instead of the isoleucine residue present in the PV strain) was found in this region at position 410. The serine residue at position 389, which is phosphorylated after binding with the viral RNA, was conserved in the lineages analyzed.

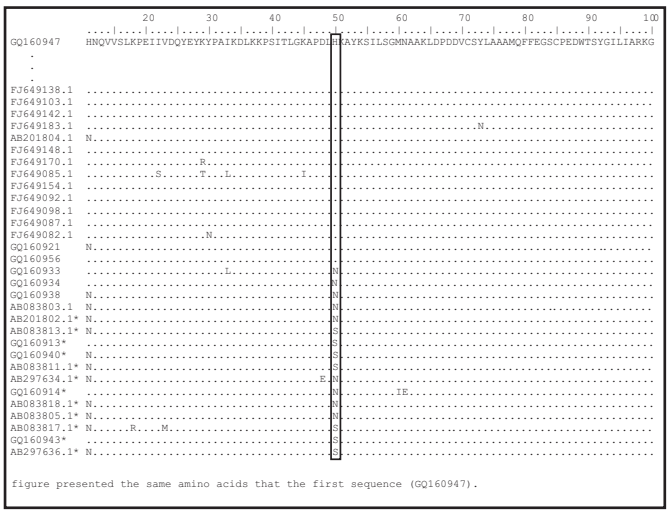

FIGURE 3 - Comparison of the predicted sequences of amino acids in the N protein with the putative amino acid alignments revealed a region corresponding to position 50 that characterized clusters I (Lineages GQ160947 to AB083803.1) and cluster II (Lineages AB201802.1 to AB297636.1 - identified by *). Sequences not showed in

DISCUSSION

In this study, we analyzed AgV3 RABV lineages from SP and MG collected in 2007 and 2008. These lineages were compared with lineages from SP and MG collected between 1999 and 2001[12] and from SP, RJ, MT, GO and TO collected between 1989 and 2006[13,14].

Two clusters were identified in the study (I and II). Cluster I was made up of AgV3 RABV lineages from SP and MG collected between 1999 and 2001 and between 2007 and 2008 and a single lineage from GO collected in 1999. Cluster II consisted of lineages from SP, RJ, MT, GO and TO collected between 1989 and 2000 and four lineages from SP collected between 2007 and 2008.

A high degree of identity between lineages was observed for cluster I (greater than 99%), and cluster II was found to have 97% identity. The mean identity between clusters I and II was 96.8%. These results are in agreement with data obtained in Latin America by other authors[12,14-16], who rarely found more than 2-3% divergence between AgV3 RABV lineages.

We also found that, although the distributions of some clusters overlapped, variations between lineages from different geographic regions could be observed, as was the case with subclusters Ie and If. In addition, the lineages used for comparison, which were mostly from other states, segregated into a different cluster from the majority of the lineages from SP and MG. This variation with geographic region has also been observed by other authors[12,15,17,18].

The analysis on the amino acid sequences of the N protein of the AgV3 RABV lineages studied here revealed that this site provided information that helped differentiate between the lineages. While the amino acid residue at position 50 in cluster I in almost all lineages was histidine, the amino acids at the same position in the lineages in cluster II were serine or asparagine. Carnieli et al[12] found differences in the same site, thus characterizing lineages from distinct areas.

Comparing the biologically active areas of the N protein, we found that the amino acids at these positions were conserved in the AgV3 lineages, as can also be seen from studies by Carnieli et al[12] and

Oliveira[19], and to a certain extent from a study by Velasco-Villa et al[16], who only analyzed the C-terminal region of the N gene.

Epidemiological surveillance using molecular methods such as that described here is very important in planning actions to control rabies. In this study, we analyzed RABV lineages isolated from economically important herbivores. Despite exhibiting genetic markers that could indicate variation with geographic region, these lineages, which were typical of those isolated from the hematophagous bat *Desmodus rotundus*, had biologically active regions that were conserved within the species over space and time. These findings may be of benefit when planning actions aimed at regional control of rabies in bovines.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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