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Detection and characterization of rabies virus in Southern Brazil by PCR amplification and sequencing of the nucleoprotein gene

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Summary. Due to the medical and socio-economical importance of both human and animal rabies infection, several studies have suggested the use of molecular techniques such as RT-PCR and DNA sequencing for diagnosis and phylogenetic studies of the rabies virus. Considering the conservancy of the nucleoprotein (N) gene of the virus, we herein describe a RT-PCR assay for rabies diagnosis and characterization. A total of 75 samples obtained from a variety of animal species in the state of Santa Catarina (SC), Southern Brazil, were comparatively studied by fluorescence antibody test (FAT), mouse inoculation test (MIT), cell infection assay and RT-PCR, which revealed itself to be as sensitive as FAT and MIT and less time-consuming than MIT. Direct sequencing of the 5′ end of the N gene allowed the clustering of the SC samples with samples from the vampire bat-related or sylvatic cycle through comparative sequence analysis.

Introduction

Rabies infection is a fatal worldwide encephalopathy causing 35,000–50,000 human deaths, as well as economic losses up to $44 million in livestock in Latin American countries per year [27, 32].

The Brazilian National Health Foundation (Funasa) reported the occurrence of more than 1,300 human cases of rabies infection between 1980 and 2003 [11]. However, it is well established that misdiagnosis of human infection, along with a deficient notification network, may be underestimating the real number of human cases.

Causing almost 30,000 deaths a year, rabies virus infection in Brazilian bovine livestock determines a major economic impact, reaching up to US$ 300,000 only in Santa Catarina State in Southern Brazil [22, 25].

Due to the difficulty of an accurate, specific and sensitive diagnosis of rabies diagnosis, the administration of post exposure treatment (PET) has been used to prevent rabies infection even in people who were not really exposed to the virus. Recent statistics point out that approximately 50% of PET is not associated with animal bites [5], confirming the need for novel, accurate and faster diagnostic tools for rabies infection diagnosis in order to reduce or avoid unnecessary human treatment.

Considered as gold standard methods for rabies diagnosis, the Fluorescence Antibody Test (FAT) [7] and the Mouse Inoculation Test (MIT) [18] are time-consuming and are of variable specificity and sensitivity. Moreover, MIT requires the use of animals for experimentation. In this connection, the use of the RT-PCR for rabies virus detection was proposed [28], which proved to be a suitable technique for rabies diagnosis supported by various researches [6, 14, 17]. Studying 60 samples previously diagnosed by FAT and MIT as positive for rabies virus, Heaton et al. [14] observed a concordance of 93% of the RT-PCR with these classical diagnostic techniques.

Despite the higher sensitivity rates when compared with FAT [14], and the possibility of detecting the presence of the virus in naturally decomposed samples [6], the use of the RT-PCR as a diagnostic tool for rabies infection is not currently endorsed by the World Health Organization [23].

More than a sensitive and specific diagnostic tool, RT-PCR does not require isolation of the virus and allows genetic characterization of the virus, which is of major importance for epidemiological studies of human and/or animal rabies [28]. Moreover, RT-PCR-derived information can identify the virus' origin, even when no bite history is known, and point to the actions to be taken in both control and surveillance phases by health authorities in order to avoid the virus spreading [9].

Since two epidemiological cycles of the rabies virus are concurrent in Brazil (dog-related or domestic, and the vampire bat-related or sylvatic), the use of RT-PCR allows specific detection and characterization of the viruses causing outbreaks, dog- or vampire bat-related viruses, or even new rabies genotypes or variants [15].

Considering the socio-economic importance of the rabies virus infection in humans and animals, the aim of this study was to standardize a RT-PCR assay for rabies virus detection and to comparatively evaluate the technique with FAT/MIT and cell culture isolation. Sequencing of the obtained RT-PCR products allowed a genotype clustering and characterization of the samples as containing the sylvatic (vampire bat-related) rabies virus.

Material and methods

Virus and tissue samples

Brain tissues of Swiss mice experimentally infected with the CVS31.2 strain of the rabies virus, as well as non-infected tissues, were used as control for RNA extraction, cDNA synthesis and RT-PCR assays. The Universidade Federal de Santa Catarina (UFSC) Ethics Committee approved the experiments involving animals (Process 197/2002). Also, tissue samples collected from 75 animals who were suspected of dying from rabies infection were supplied by the Companhia Integrada de Desenvolvimento Agropecuário de Santa Catarina – Cidasc (Table 1). All tissue samples were previously diagnosed by FAT and MIT at Cidasc and then coded prior to RT-PCR assays. Along with CVS31.2, the Pasteur Virus (PV) and Street Alabama Dufferin (SAD) strains of the rabies virus were used as controls in biological assays.

Table 1. Description of the rabies virus samples (code, geographical origin and original host) and results of the Fluorescence Antibody Test (*FAT*), Mouse Inoculation Test (*MIT*) and Reverse-Transcriptase Polymerase Chain Reaction (*RT-PCR*) assay of samples included in this study. *nd* = not determined

Sample code	Original host	Geographical origin	FAT/MIT	RT-PCR	Cell isolation	Sequence
6914	Bovine	Luís Alves	+/+	+	+	+
7051		Vidal Ramos	+/+	+	+	−
7057		Rodeio	+/+	+	+	+
7248		Rio Fortuna	+/+	+	+	+
7454		Ilhota	+/+	+	+	+
7432		Passo de Torres	+/+	+	+	+
7565		Luís Alves	+/+	+	+	+
7574		Imbituba	−/−	+	+	nd
7518		Chapecó	−/−	+	+	nd
7577		Águas Mornas	+/+	+	+	+
7595		Tijucas	+/+	+	+	+
7603		Pedra Grande	+/+	+	+	−
7696		Pedra Grande	+/+	+	+	+
7674		Timbé do Sul	+/+	+	+	+
7669		Timbé do Sul	+/+	+	+	−
7668		Timbé do Sul	+/+	+	+	−
7511		Braço do Norte	−/−	−	nd	nd
7510		Braço do Norte	−/−	−	nd	nd
7570		Indaial	−/−	−	nd	nd
7511		Braço do Norte	−/−	−	nd	nd
7519		São João Batista	−/−	−	nd	nd
7509		Braço do Norte	−/−	−	nd	nd
7503		Tubarão	−/−	−	nd	nd
7676		Xanxerê	−/−	−	nd	nd
7677		Xanxerê	−/−	−	nd	nd
7678		Chapecó	−/−	−	nd	nd
7630		Braço do Norte	−/−	−	nd	nd
7631		Sto. [a]da Imperatriz	−/−	−	nd	nd
7632		Victor Meirelles	−/−	−	nd	nd
7646		Garopaba	−/−	−	nd	nd

(*continued*)

Table 1 (*continued*)

Sample code	Original host	Geographical origin	FAT/MIT	RT-PCR	Cell isolation	Sequence
7651		Tubarão	−/−	−	nd	nd
7652		Pedra Grande	−/−	−	nd	nd
7673		Biguaçu	−/−	−	nd	nd
7609		Itajaí	−/−	−	nd	nd
7601	Dog	Mafra	−/−	−	nd	nd
7670		Schroeder	−/−	−	nd	nd
7663		Gaspar	−/−	−	nd	nd
7654		Palhoça	−/−	−	nd	nd
7662		Chapecó	−/−	−	nd	nd
7672		São José	−/−	−	nd	nd
7622		Campo Belo so Sul	−/−	−	nd	nd
7146		Itajaí	−/−	−	nd	nd
7466		Jaraguá do Sul	−/−	−	nd	nd
7481		Curitibanos	−/−	−	nd	nd
7483		São João do Cedro	−/−	−	nd	nd
7549		Blumenau	−/−	−	nd	nd
7552		Gaspar	−/−	−	nd	nd
7516		Lages	−/−	−	nd	nd
7594		Poço Redondo	−/−	−	nd	nd
7563		São José	−/−	−	nd	nd
7553		Blumenau	−/−	−	nd	nd
7571		Tubarão	−/−	−	nd	nd
7545		Lages	−/−	−	nd	nd
7562		Forquilhinha	−/−	−	nd	nd
7543		Guaramirim	−/−	−	nd	nd
7579		Blumenau	−/−	−	nd	nd
7591		Blumenau	−/−	−	nd	nd
7546	Cat	Araranguá	−/−	−	nd	nd
7606		Ouro	−/−	−	nd	nd
7550		Florianópolis	−/−	−	nd	nd
7502		Canoinhas	−/−	−	nd	nd
7561		Angelina	−/−	−	nd	nd
7619	Unknown	nd	−/−	−	nd	nd
7702		nd	−/−	−	nd	nd
7569		nd	+/+	+	+	+
7484		nd	+/+	−	+	nd
7491		nd	−/−	−	nd	nd
7498		nd	−/−	−	nd	nd
7700		nd	−/−	+	+	nd
7572		nd	+/+	+	+	nd
7485	Vampire Bat	São Joaquim	−/−	+	+	+
7589		Bom Retiro	−/−	−	nd	nd
7584		São Joaquim	−/−	−	nd	nd
7586		São Joaquim	−/−	−	nd	nd
7493		Serro Negro	−/−	−	nd	nd

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA extraction was performed by Trizol® as described by the manufacturer (Invitrogen, Carlsbad) using 100 mg of brain tissue. The reverse transcription reaction was carried out using the M-MLV Reverse Transcriptase (Invitrogen) as proposed by the manufacturer with minor modifications. Instead of the suggested Oligo $dT_{(18)}$ primer, 10 pmol of primers N1 (5′-ATG GAT GCC GAC AAG ATT-3′) and N2 (5′-TTA TGA GTC ACT CGA ATA-3′), directed to the entire coding region of the nucleoprotein (N) gene (genome positions 71–93 and 1423–1406, respectively), as reported by Tordo et al. [31], and 10 μg of total RNA were used.

PCR amplification of the N gene was set up by testing 12 buffers with distinct compositions ($MgCl_2$, KCl, pH) as well as a broad range of annealing temperatures in a Mastercycler Gradient® thermocycler (Eppendorf, Hamburg). A hot start RT-PCR reaction was used as described by Nadin-Davis [24], using approximately 1–2 μg of total cDNA, 2 mM dNTP set (Invitrogen), 20 pmol of each specific primer (N1/N2) and 1U *Taq* DNA polymerase (Cenbiot, Brazil) in a buffer containing 10 mM Tris-HCl pH 9.2, 1.5 mM $MgCl_2$ and 25 mM KCl. Following an initial denaturation step at 94 °C for 5 min, *Taq* DNA polymerase and specific primers were added and the mixture was submitted to 5 cycles of 94 °C/45 s, 55 °C/45 s and 72 °C/90 s, 30 cycles of 94 °C/45 s, 59 °C/45 s and 72 °C/90 s, and a final extension step of 72 °C for 10 min. Furthermore, an RT-PCR reaction with primers N1 and N3 (5′-CTT GTG GTA TGA GAT CTA ATG AC-3′, genome position 636–617) was also standardized using the same process, aiming at the improvement of the reaction's sensitivity [14].

After separation in 1% agarose gel electrophoresis, amplification products were stained with ethidium bromide, observed in a UV transilluminator (Hoefer, San Francisco), and digitally photographed. All pre- and post-amplification procedures were carried out in distinct areas, not sharing reagents and/or equipment in order to avoid contamination.

Sensitivity assays

Brain tissues from Swiss mice experimentally infected with CVS 31.2 strain ($LD_{50} > 10^{-7}$) and with a wild isolated sample (7057) were macerated, submitted to 10-fold dilutions and separately tested by RT-PCR with primers N1/N2 and N1/N3. For comparison, the same material was submitted to virus isolation in neuroblastoma cells [3] and MIT [18], using groups of 5 Swiss mice. All experiments were performed in triplicate.

RT-PCR product sequencing and analysis

After RT-PCR diagnosis of the samples supplied by Cidasc and amplification of the N gene from the CVS31.2, PV and SAD strains as described in 2.2, amplification products of control strains and positive samples were resolved on 1% low melting agarose gel (NuSieve®, Rockland), excised and purified using the QIAquick® Gel extraction Kit (Qiagen, Valencia). The 5′ end of the N gene was directly sequenced in a MegaBACE 1000®. DNA Analysis System using a DYEnamic ET Terminator Kit® (Amershan Biosciences). Sequencing reaction was performed using 100–300 ng of purified RT-PCR products and 3.5 pmol of N2 anti-sense primer with a denaturation step of 25 seconds at 94 °C followed by 30 cycles of 94 °C/20 s, 55 °C/25 s and 60 °C for 1 min. In order to confirm the identity of the amplification products, the multiple sequences obtained for each sample were analyzed by Phred/Phrap/Consed package (www.phrap.org) and the consensus sequences were compared with the GenBank database using the basic Blast tool. The consensus sequences were aligned by ClustalX software, v.1.83 [30], and were analyzed by the bootstrapped neighbor-joining method (1,000 replicates) using the complete deletion and Kimura-2 parameters of the MEGA software package, v.2.01 [19].

Table 2. General characteristics of rabies virus sample sequences retrieved from the GenBank and used as control for comparative sequence analysis in the present study

GenBank accession	Original host	Genotype	Geographical origin	Year of isolation	Reference
AB083793	cat	1	Goiás/Brazil	1999	Ito et al., 2003
AB083795	human	1	Goiás/Brazil	1999	Ito et al., 2003
AB083796	dog	1	Minas Gerais/Brazil	1987	Ito et al., 2003
AB083797	dog	1	São Paulo/Brazil	1989	Ito et al., 2003
AB083804	horse	1	Goiás/Brazil	1998	Ito et al., 2003
AB083807	vampire bat	1	São Paulo/Brazil	1998	Ito et al., 2003
AB083808	sheep	1	São Paulo/Brazil	1992	Ito et al., 2003
AB083811	cattle	1	Tocantins/Brazil	1999	Ito et al., 2003
U22842	bat	2	Nigeria/Africa	1958	Bourhy et al., 1993

After determining the correct reading frame, predicted amino acid sequences were obtained for all sequenced samples by DNAStar software [4] and aligned by ClustalX v.1.83. Sequences of CVS31.2, PV and SAD strains obtained in our laboratory, as well as sequences representing the distinct transmission cycles of rabies virus in Brazil (sylvatic or vampire bat-related and urban or dog-related), were retrieved from the GenBank and included in the comparative sequence analysis (Table 2).

Cell culture assay

Mouse neuroblastoma cells (C-1300; clone A) were obtained from the American Type Culture Collection – ATCC (Rockville) and cultured in Dulbecco's Modified Eagle Medium Nutrient Mixture F-12 (Sigma, St. Louis), supplemented with 2 mM glutamine, 10% (v/v) fetal calf serum (Sigma) and 1× antibiotic/antimycotic solution (Sigma) at 37 °C in a 5% CO_2 atmosphere. Cell infection was performed as described by Bordignon et al. [3] and evaluated by direct fluorescence, after 72 h of incubation, in an Olympus BX40 fluorescence microscope.

Results

Detection of rabies virus by RT-PCR

The herein described RT-PCR protocol revealed amplification products of the expected size (∼1.3 Kb), and no significant differences were observed in the intensity of the amplification products obtained with distinct PCR buffer compositions (1.5 or 3.5 mM $MgCl_2$, 25 or 75 mM KCl and pH from 8.3 to 9.2) among the control strains. PCR annealing temperatures from 55 to 65 °C with increments of 1 °C were tested, and they produced the best results between 55–59 °C (data not shown). The use of a hot start RT-PCR approach clearly reduced the amplification of non-specific products (Fig. 1), especially those observed in control strains.

Except for sample 7484, which had a positive cell culture isolation (data not shown) and showed a false-negative result in RT-PCR, analysis of all other animal samples (Table 1) by this technique revealed products of ∼1.3 Kb (Fig. 2). Four field samples (7574, 7518, 7485 and 7700) which had previous negative FAT and

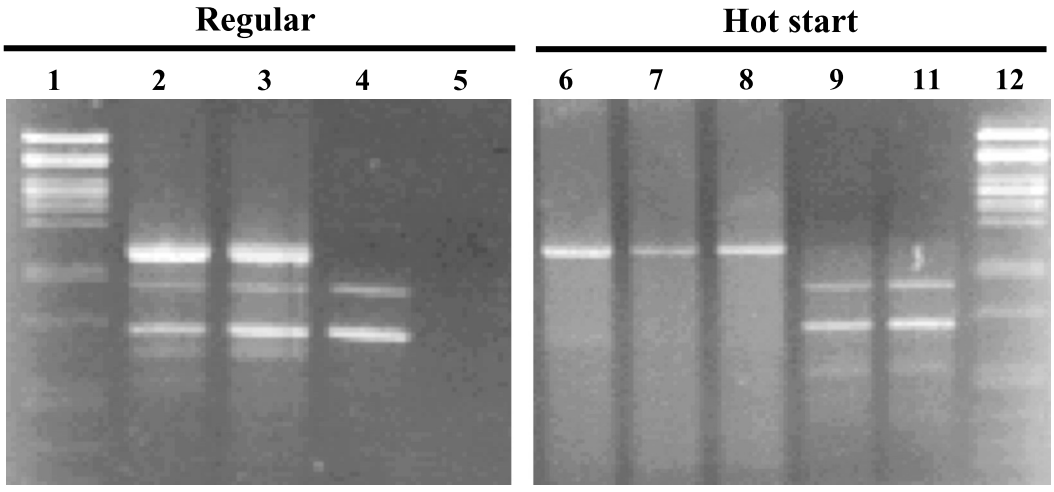

Fig. 1. Comparison of regular and hot start RT-PCR products for detection of the rabies virus nucleoprotein gene in a 1% agarose gel stained with ethidium bromide. *1* and *12* = Molecular weight marker (λ DNA digested with *Pst*I), *2*, *3*, *6*, *7* and *8* = experimentally infected mouse cDNA, *4*, *9* and *11* = non-infected mice cDNA, *5* = negative control (no cDNA added)

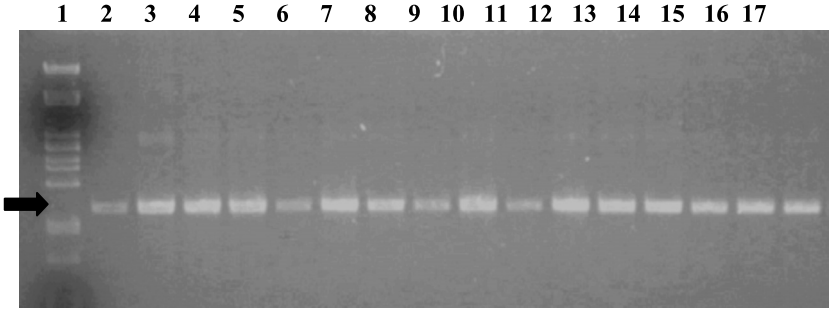

Fig. 2. One percent agarose gel stained with ethidium bromide showing the purified 1.353 bp RT-PCR products (arrow) obtained from positive field samples with primers N1/N2 (Table 1). *1* = Molecular weight marker (λ DNA digested with *Pst*I), *2–4* = strains CVS31.2, PV and SAD, *5–17* = samples 7696, 7577, 7674, 7595, 7485, 7565, 7574, 7569, 6914, 7454, 7057, 7248 and 7432

MIT results turned out to be positive in both cell culture assays and in the RT-PCR assay in two independent analyses. Based on these results, RT-PCR revealed 98% of specificity and 95% of sensitivity when compared with FAT and MIT for rabies virus detection. Analysis of discordant samples by RT-PCR using primers N1/N3 did not alter the results obtained with primers N1/N2 (data not shown).

Sensitivity assays

Using RNA extracted from each of the 10-fold dilutions of Swiss mouse brain experimentally infected with CVS31.2 strain, MIT was the most sensitive test

under our experimental conditions, detecting the presence of the rabies virus in dilutions of up to 10^{-7} in comparison with RT-PCR and cell infection assays which were able to detect the presence of the virus in dilutions of up to $10^{-5}/10^{-6}$ and 10^{-5}, respectively. Considering the field sample (7057), MIT showed a sensitivity of 10^{-5} in contrast with 10^{-3} obtained with RT-PCR and isolation in cell culture. RT-PCR assays carried out with the same samples employing N1–N3 primers increased the sensitivity of the assay to 10^{-7} for CVS31.2 strain and to 10^{-5} for sample 7057, the same levels observed for MIT.

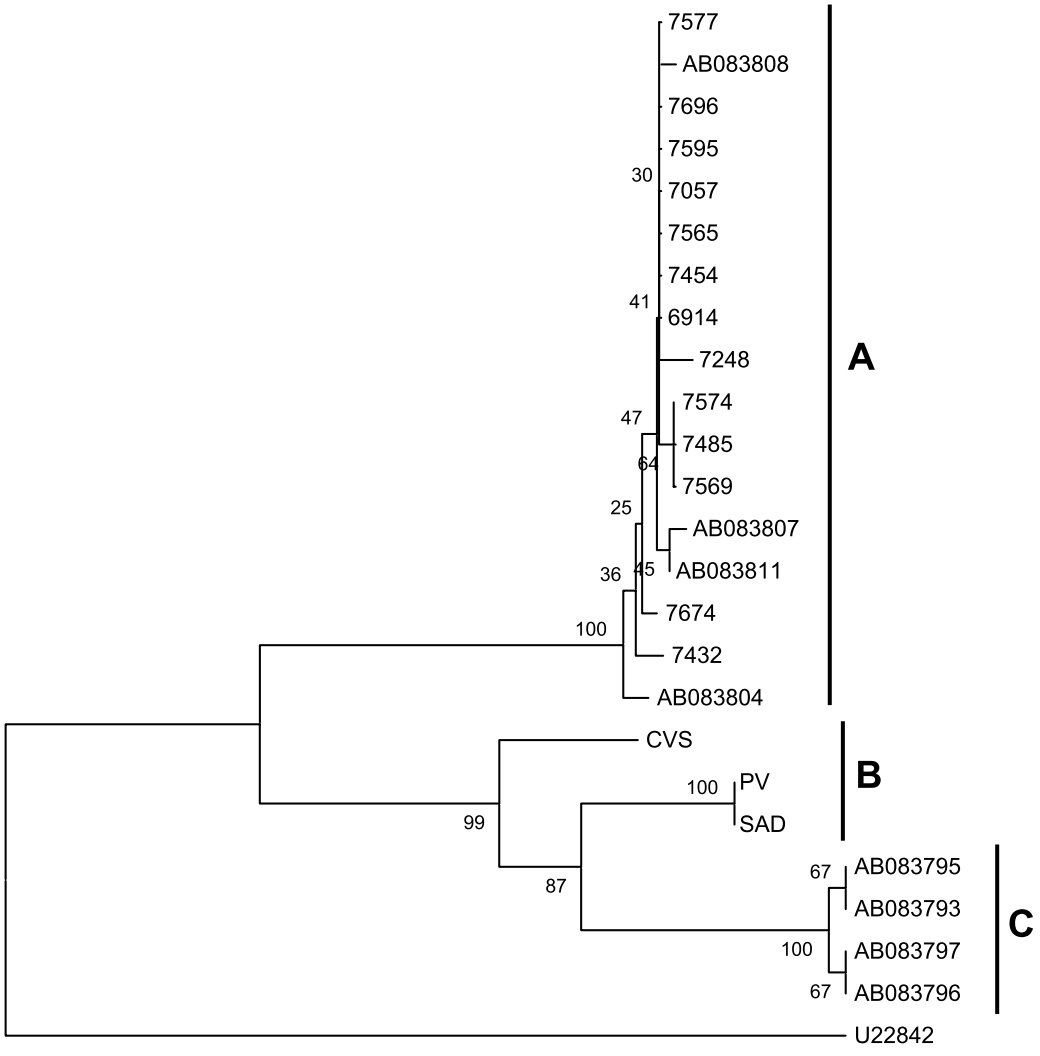

Fig. 3. A neighbor-joining tree resulting from the sequence analysis of the studied samples and strains by MEGA v. 2.01 using the genotype 2 Lagos Bat virus sequence as outgroup (GenBank Accession U22842). A 95% bootstrap cut-off was used to determine the consensus tree. Groups A, B and C were formed by vampire bat-related (sylvatic cycle), control strains and dog-related (domestic cycle) samples, respectively. Strains and sample codes are shown in Tables 1 and 2

Sequence analysis

From a total of 75 samples, we directly sequenced the 5′ end of the N gene from 13 purified RT-PCR products. The direct sequencing strategy proved to be effective, resulting in consensus sequences of 333 bp with Phred quality >20.

Alignment of rabies sequences retrieved from the GenBank database (Table 2) with the herein studied sequences by MEGA v.2.01 confirmed that all 13 studied samples from Santa Catarina State were closely related to strains from the sylvatic cycle of the virus (data not shown). The identity of the samples was also confirmed by comparative sequence analysis of the Santa Catarina State samples with control strains (CVS31.2, PV and SAD) and isolates representing the distinct epidemiological cycles of rabies in Brazil [16] (Fig. 3). Our results clearly demonstrate that all the Santa Catarina samples were clustered, with high bootstrap values, along with samples from the vampire-bat (sylvatic) – related cycle (Fig. 3). The standard strains CVS31.2, PV and SAD formed a distinct and intermediate group with high bootstrap values, not clustering with strains from urban (dog-related) or sylvatic (vampire bat-related) cycles (Fig. 3).

Comparing the similarity of the amino acid sequence between dog-related viruses and control strains (CVS31.2, PV and SAD) the variation was from 98.2% to 97.3%. These values decreased to 93.7% when samples from Santa Catarina State were compared with the same control strains.

Interestingly, comparative sequence analysis carried out with the 5′ end of samples from Santa Catarina State and other vampire bat-related samples of Brazil showed a complete match at the amino acid level (Fig. 4). However, the same analysis revealed clear differences in both NIII (genome position 313–337) and NI (genome position 374–383) antigenic sites of the N gene described on the

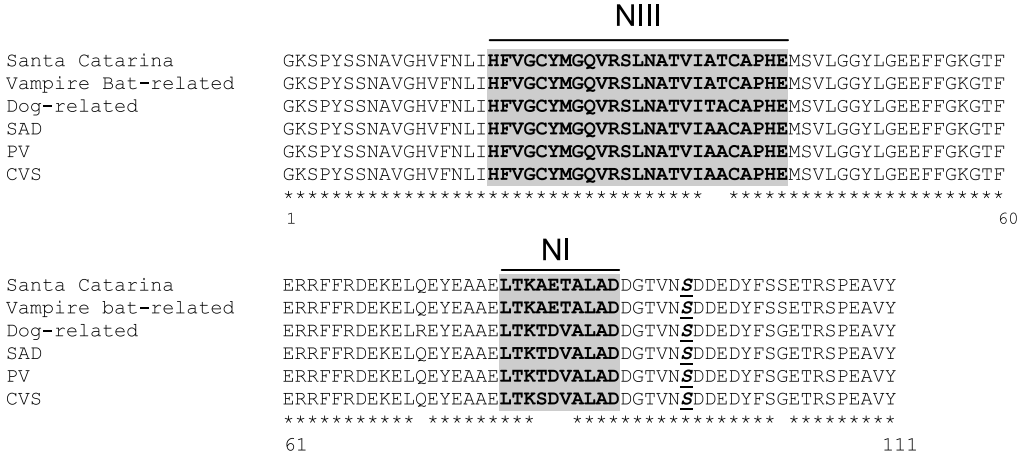

Fig. 4. Amino acid alignment of the consensus sequences of Santa Catarina State samples, dog-related and vampire bat-related viruses and vaccinal strains (CVS31.2, PV and SAD) obtained by DNAStar program. The antigenic sites NIII and NI are in bold/shaded, and the putative phosphorylation site mapping to serine (genome position 389) is marked in bold/underlined. Asterisks underneath the sequences indicate full sequence consensus

basis of the ERA strain sequence [8, 20] between the Brazilian strains, the control strains (CVS31.2, PV and SAD), and the dog-related sample sequences (Fig. 4). Our results revealed a total of 7 amino acid differences between dog- and vampire bat-related viruses among the 111 amino acids analyzed, 5 of which were within the NIII and NI antigenic sites (Fig. 4). A full length sequencing of the N gene of both Brazilian and control strains is underway and will be soon available at the GenBank.

Discussion

Specific and accurate rabies virus detection is essential for both human and animal infection as well as for the epidemiological studies in a specific region or country. These aspects are of major importance to vaccine development and may have a crucial impact on vaccine efficacy, on the treatment of patients involved in accidents with animals suspected of carrying rabies and on the costs of post-exposure treatment [29]. Thus, the development and use of novel diagnostic approaches is relevant for disease control and prevention.

In the present work, we have comparatively tested classical methods of rabies diagnosis (FAT and MIT) with RT-PCR, having used this molecular approach for the characterization of samples collected from animals in the state of Santa Catarina. Our results have confirmed the circulation of the rabies virus of the vampire bat-related cycle in this state in southern Brazil.

Comparative sensitivity assays showed that the herein described RT-PCR protocol was less sensitive than MIT, but that it was as sensitive as the FAT and cell isolation methods for rabies virus detection. RT-PCR was able to detect four false-negative samples in MIT and FAT, which was confirmed by positive isolation in neuroblastoma (N2A) cells. These results reveal the advantage and the sensitivity of the RT-PCR method to detect the virus' presence in decomposed samples, which is in accordance with former reports [6] showing that RT-PCR was able to detect the rabies virus in brain tissues of naturally-infected animals (foxes, cows and dogs) incubated under laboratory conditions at 37 °C for 36 days.

Using the same approach, [14] observed that FAT was able to detect the virus only up to 72 h after incubation at 37 °C in comparison with the RT-PCR which was able to detect the virus in experimentally decomposed brain tissue up to 360 h after the animal's death.

Nevertheless, the failure of the RT-PCR in detecting the virus in sample 7484 reinforces the need to design more specific primers based on sequences from Brazilian isolates and further tests of sensitivity and specificity.

Several strategies to enhance the sensitivity and/or specificity of diagnostic PCR have been used. In this work, the use of a hot start in RT-PCR reaction (Fig. 1) proved to be efficient and it certainly improved the specificity of the reaction as formerly reported [24].

Another method is the so-called 'hemi' or 'semi-nested' PCR, which was used to comparatively study the RT-PCR with FAT/MIT, obtaining an increment of the agreement between the methods from 93% to 100% [14]. Having tested two

distinct primer sets (N1/N2 and N1/N3) for direct rabies virus diagnosis in animal tissues, the use of an N1/N3 primer set increased the sensitivity of the reaction by approximately 100 times when compared to the N1/N2 primers, but did not alter the number of false-negative results obtained with primers N1/N2 (data not shown). Thus, our group is currently working on a semi-nested PCR with primers N1/N3 using the RT-PCR products generated by primers N1/N2 as a template.

Considering the sensitivity and specificity values and the ability of the RT-PCR approach to specifically detect the presence of the rabies virus, even in decomposed samples, we must conclude that the use of FAT and cell-culture virus isolation, along with the RT-PCR in routine diagnosis will be of outstanding importance, providing means for further sample characterization.

Provision of suitable material for the molecular typing of samples is probably the major advantage of RT-PCR detection of the rabies virus [14]. Analysis of RT-PCR products can be performed by a variety of techniques such as restriction fragment length polymorphism – RFLP [16] and sequencing of the amplification products [15, 28]. These analyses can provide important data about the genetic variability of the virus, which is of utmost relevance in vaccine development and efficacy. As an example, Gould et al. [12] and Arai et al. [1], based on molecular genotyping, have suggested the existence of two new genotypes of rabies virus.

Comparison of 333 bp of the 5′ end of the N gene sequence (genome position on PV strain, 885–1218 bp) of Santa Catarina samples (Table 1) with sequences that were representative of other rabies virus cycles (Table 2) confirmed the occurrence of classical genotype 1 rabies in the state of Santa Catarina, clustering the samples with others from the sylvatic or vampire bat-related cycle (Fig. 3). Furthermore, amino acid sequence comparison carried out using consensus sequences of the same samples demonstrated that the vampire bat-related viruses of Santa Catarina are closely related to the Brazilian sample sequences, but that they are quite divergent from the laboratory strains and dog-related sample sequences in both NIII and NI antigenic sites (Fig. 4).

Distinct protection levels against rabies virus are observed, depending on the vaccine preparation method and/or the origin of the challenging strain [33]. Former reports indicate that experimentally immunized animals challenged with samples isolated from the vampire bat-related (sylvatic) cycle show reduced protection levels when compared with animals challenged with vaccinal or laboratory maintained strains [13, 33].

The herein described changes to the antigenic sites of the N gene (Fig. 4) could play an important role in the above-mentioned vaccine efficacy, since it is well established that the N gene is relevant in eliciting the cellular immunity response against rabies infection [8]. Lodmell et al. [21] demonstrated that anti-nucleoprotein antibodies were able to inhibit the rabies virus replication *in vitro* and that they protected against the virus challenge *in vivo*, confirming the relevance of the N gene product in rabies immunity. It should be emphasized, however, that those data were obtained *in vitro* and in experimental animals that usually are not naturally infected by rabies virus. Studies in naturally susceptible animals,

including humans, should be performed to confirm the real status of N protein in protection afforded by vaccination. In any case, the reported variability of the N gene could be an explanation, at the genomic level, for the observation of Hayashi et al. [13] and Zanetti et al. [33], corroborating that virus diversity studies are of fundamental importance for vaccine design.

Since the occurrence of new variants or genotypes has been reported in Brazil [2, 10, 14, 26], the use of the above-described protocol for specific detection and characterization of these samples is of major importance for the comprehension of rabies virus epidemiology.

As an example, the transmission of a rabies virus from a simian species (*Callitrix jacchus*) to humans [2, 10] and the presence of the rabies virus genotype 5 in a naturally infected bat [14] have been reported in Brazil. However, no sequence or molecular information about these samples is available for further characterization assays or comparative analysis.

Non-vampire bat species (insectivorous and frugivorous) are also important reservoirs of the rabies virus, being responsible for human infection in Brazil as well as for human deaths throughout Latin America [9]. Considering the wide geographical area of rabies virus circulation in Brazil and the already-reported transmission of the virus among non-human primates, insectivorous or frugivorous bats and wild animal species, the existence and circulation of new rabies genotypes or variants may be expected.

Therefore, we strongly believe that the use of the RT-PCR methods, along with the FAT and the cell isolation method, is as efficient as the use of FAT and MIT in diagnosis for both human and animal rabies, with the advantage of i) avoiding the use of animals for experimentation, ii) allowing the identification and characterization of both known and unknown rabies virus variants, iii) permitting the detection of the virus even in decomposed tissue samples, and iv) reducing the time of confirmatory diagnosis and PET.

Our results also indicate that RT-PCR diagnosis of rabies virus infection through detection and sequencing of the N gene is a useful tool for molecular epidemiology, providing relevant data and information for health authorities in rabies control and surveillance as well as for vaccine development.

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