Epidemiol. Infect. (2002), **128**, 503–511. © 2002 Cambridge University Press
DOI: 10.1017/S095026880200688X Printed in the United Kingdom

Genealogical analyses of rabies virus strains from Brazil based on N gene alleles

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(*Accepted 2 January 2002*)

SUMMARY

Thirty rabies virus isolates from cows and vampire bats from different regions of São Paulo State, Southeastern Brazil and three rabies vaccines were studied genetically. The analysis was based on direct sequencing of PCR-amplified products of 600 nucleotides coding for the amino terminus of nucleoprotein gene. The sequences were checked to verify their genealogical and evolutionary relationships and possible implication for health programmes. Statistical data indicated that there were no significant genetic differences between samples isolated from distinct hosts, from different geographical regions and between samples collected in the last two decades. According to the HKA test, the variability observed in the sequences is probably due to genetic drift. Since changes in genetic material may produce modifications in the protein responsible for immunogenicity of virus, which may eventually cause vaccine failure in herds, we suggest that continuous efforts in monitoring genetic diversity in rabies virus field strains, in relation to vaccine strains, must be conducted.

INTRODUCTION

Rabies occurs in two different epidemiological patterns: urban rabies with the domestic dog as the main reservoir and transmitter, and sylvatic or rural rabies, with different wildlife species acting as reservoirs and or transmitters. In South America, the most important reservoir and transmitter of rural rabies is vampire bats, mainly *Desmodus rotundus* [1].

In São Paulo State, southeastern Brazil, the effective control programmes have reduced the number of dog rabies cases and most of the urban areas are free of rabies. However, the number of cases of bovine rabies is increasing along the territory of São Paulo State and the disease remains a serious problem, mainly in northeast and southeast regions of São Paulo State, where at least 837 cases of bovine rabies and 80 cases of vampire bat rabies occurred between 1996 and 1999.

Rabies virus belongs to the genus *Lyssavirus* of the family Rhabdoviridae. Its genome is composed of a single-stranded, negative-sense, non-segmented RNA which encodes for five separate proteins designated as nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and the RNA polymerase (L) [2].

The *Lyssavirus* genus is divided into seven genotypes. Genotype 1 includes the classical rabies virus strains found worldwide. The African rabies-related viruses are classified in three genotypes: type 2, *Lagos* bat virus which is the prototype strain of genotype 2, *Mokola* virus (genotype 3) and *Duvenhage* virus

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https://doi.org/10.1017/S095026880200688X Published online by Cambridge University Press

(genotype 4). The European rabies-related viruses are divided in two genotypes: the European bat lyssaviruses (genotypes 5 and 6). A seventh genotype is Australian bat lyssavirus.

Molecular epidemiology studies based on the RT–PCR (reverse transcriptase–polymerase chain reaction) technique, associated with direct sequencing, is a useful means of classification of animal viruses, including rabies virus, and provides a better understanding of epidemiological relationships [3].

Among the genes of rabies virus, the N gene, in particular, has been successfully employed in molecular epidemiology studies because since its sequences are most conserved [4], and its PCR products are therefore useful for diagnostic purposes. Also, there are many nucleoprotein sequences of all six *Lyssavirus* genotypes available in GenBank [5], which permits comparisons with new sequences and it presents an important role in the host immune response to *Lyssavirus* genotypes [6]. Analysis of the N gene sequences is also very informative in comparisons among related strains [7] and the 400 nucleotides in the 5′ end coding for the N protein can be used to determine the geographical distribution of the major virus lineages and are strongly recommended for phylogenetic analyses [5].

The present work reports the genealogical analyses of different rabies virus lineages using the genealogical methodology [8]. The nucleoprotein N gene sequences of rabies virus from São Paulo State, Brazil, were obtained by the employment of the RT–PCR technique associated with direct sequencing. The analyses were conducted considering three parameters – comparative analyses of rabies viruses isolated from bovine and vampire bats, of rabies virus collected in different localities of the geographical area, of rabies virus samples obtained in different years in the last two decades.

The aim of this work was the genetic characterization of the rabies virus lineages, considering the parameters described above, in order to contribute to health programmes and to elucidate the genealogical history of the lineages.

METHODS

Virus isolates

Twenty-two bovine brains and eight vampire bat (*Desmodus rotundus*) brains from São Paulo State, Brazil, were studied. They were tested for rabies virus by direct immunofluorescent antibody (dIFA) [9]. Table 1 shows the allelic samples of rabies virus analysed according to the hosts (VB for vampire bats and B for bovine), geographic origin, year of isolation and the GenBank access number. Figure 1 presents the geographical distribution of the samples.

RNA extraction and RT–PCR

Total RNA was extracted from infected brain tissue using TRIzol® reagent (GIBCO-BRL, Gaythersburg, MD, USA) as described by the manufacturer. For reverse transcription (RT), 7 µl of extracted RNA was heated at 95 °C for 5 min, and then cooled on ice. RT was carried out in 20 µl of RT mixture containing 4 µl of RT buffer (250 mM Tris–HCl, pH 8·3, 375 mM KCl, 15 mM $MgCl_2$) (Gibco–BRL, USA), 10 mM of dNTPs; 10 pmol of specific nucleoprotein N sense primer (5′-CTACAATGGATGCCGACAAGA-3′, from nucleotide 66 to 86 in relation of PV strain, GenBank X03673), 10 mM of DTT, 200 U of M-MulV RTase reverse transcriptase (Gibco–BRL) and milli-Q distilled water with 0·01% of DEPC as diluent. After incubation at 42 °C for 60 min, 5 µl of cDNA product was added to the total volume of PCR mixture (45 µl) containing 200 mM Tris–HCl, pH 8·0, 500 mM KCl, 50 mM $MgCl_2$, 5 mM dNTPs, 10 pmol of each specific nucleoprotein N primer (sense 5′-CTACAATGGATGCCGACAAGA-3′, and anti-sense 5′-CCTCAAAGTTCTTGTGGAAGA-3′, nucleotides 849–869 in relation of PV strain, GenBank X03673), 1·25 U of *Taq* DNA polymerase (Gibco–BRL) and milli-Q distilled water as diluent.

After an initial heating at 95 °C for 5 min, the amplification reached 35 cycles of 45 sec at 94 °C, 60 sec at 55 °C, 90 sec at 72 °C and followed by a final heating at 72 °C for 10 min. Amplifications were performed in a PTC-200 Peltier Thermal Clycer (MJ Research, Waltham, MA, USA). All PCR products were analysed on 1·2% agarose gels stained by ethidium bromide.

Nucleotide sequencing

Amplified DNA products with expected molecular size (804 bp) were excised from the gel and purified through a commercial kit (GFX™ PCR DNA and Gel Band Purification Kit, Amersham Pharmacia Biotech Inc, Piscataway, NJ, USA). Sequencing reactions were performed by the dideoxynucleotide chain termination method using the BigDye™ TM

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Table 1. *Strains of rabies virus with their respective host species, year and region of isolation*

Strain	Host	Municipality	Year	Region	GenBank
B1	Bovine	Cajatí	1986	Vale do Ribeira	AF357316
B2	Bovine	No information	1986	No information	AF357296
B3	Bovine	Bernardino de Campos	1988	Vale do Ribeira	AF357300
B4	Bovine	Pirajú	1989	Vale do Ribeira	AF357301
B5	Bovine	Pindamonhangaba	1989	Vale do Paraíba	AF357302
B6	Bovine	Pindamonhangaba	1989	Vale do Paraíba	AF357303
B7	Bovine	Pirajú	1989	Vale do Ribeira	AF357304
B8	Bovine	Pindamonhangaba	1989	Vale do Paraíba	AF357305
B9	Bovine	Pindamonhangaba	1989	Vale do Paraíba	AF357306
B10	Bovine	Pindamonhangaba	1990	Vale do Paraíba	AF357286
B11	Bovine	São Joaquim da Barra	1991	No information	AF357287
B12	Bovine	Apiaí	1991	Vale do Ribeira	AF357288
VB1	*Desmodus rotundus*	Taubaté	1992	Vale do Paraíba	AF357309
B13	Bovine	São Roque	1994	Vale do Paraíba	AF357289
VB2	*Desmodus rotundus*	Guaratinguetá	1994	Vale do Paraíba	AF357310
VB3	*Desmodus rotundus*	Guaratinguetá	1994	Vale do Paraíba	AF357311
VB4	*Desmodus rotundus*	Guaratinguetá	1994	Vale do Paraíba	AF357312
VB5	*Desmodus rotundus*	Guaratinguetá	1994	Vale do Paraíba	AF357313
VB6	*Desmodus rotundus*	Taubaté	1994	Vale do Paraíba	AF357314
VB7	*Desmodus rotundus*	Taubaté	1995	Vale do Paraíba	AF357315
B14	Bovine	Taubaté	1998	Vale do Paraíba	AF357290
B15	Bovine	Jacareí	1998	Vale do Paraíba	AF357291
B16	Bovine	Santo Antônio do Pinhal	1998	Vale do Paraíba	AF357292
B17	Bovine	Taubaté	1998	Vale do Paraíba	AF357293
B18	Bovine	Taubaté	1998	Vale do Paraíba	AF357294
VB8	*Desmodus rotundus*	Taubaté	1998	Vale do Paraíba	AF357285
B19	Bovine	Taubaté	1998	Vale do Paraíba	AF357295
B20	Bovine	Jacareí	1998	Vale do Paraíba	AF357297
B21	Bovine	Pariquera-Açu	1999	Vale do Ribeira	AF357298
B22	Bovine	Pariquera-Açu	1999	Vale do Ribeira	AF357299

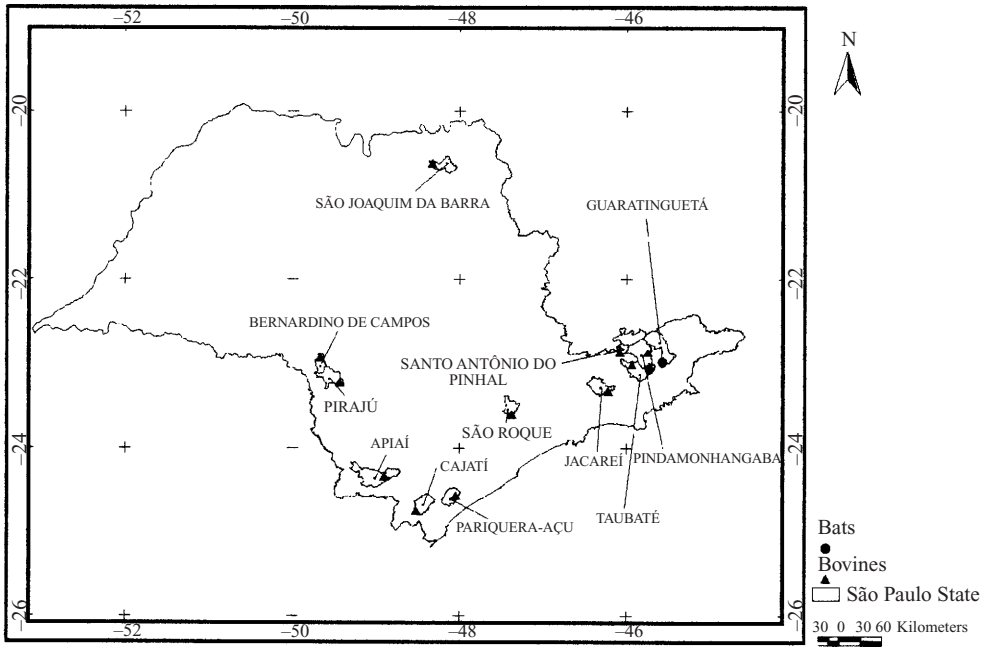

Fig. 1. Localization of the rabies virus isolates from different municipalities from São Paulo State, Southeastern Brazil.

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Table 2. *Nucleotide diversity within the categories analysed*

	No.*	M†	k‡	*V*(*k*)*o*§	*V*(*k*)*e***	*Pi*¶
Bats	7	27	12·857	12·368	31·313	0·02189
Bovines	22	59	12·537	3·169	31·42	0·02129
Year 86/91	12	33	8·439	2·946	14·716	0·01429
Year 92/99	17	49	14·221	5·345	39·675	0·02419
PB	21	52	13·389	3·984	39·508	0·02276
RB	7	25	9·714	7·252	18·488	0·01645

* No., sample size; † *M*, number of mutation; ‡ *k*, average number of nucleotide differences; § *V*(*k*)*o*, observed variance of *k*; ** *V*(*k*)*e*, expected variance of *k*; ¶ *Pi*, nucleotide diversity.

Terminator (Applied Biosystems, CA, USA) and the sequences were determined with an automated sequencer (ABI model 377, Applied Biosystems, CA, USA) according to the manufacturer's instructions. Sequencing was performed in both directions using the cited primers. All sequences were performed at least three times, so as to prevent any distrust about the correct sequences.

Genealogical analysis

The nucleotide sequences of 600 bp in the 5′ end coding for N protein obtained from 30 isolates were aligned by eye using the softwares Clustal X [10], SeqPup v.0.6f [11] and sequence Navigator v.1.01 [12]. The putative amino acid sequence of the PCR products was automatically translated using the programme MacClade v.3.01 [13] for Macintosh.

Statistical data involving nucleotide diversity and sequence variability were obtained using the software DnaSP v.3.51 [14]. Values of the following parameters were estimated: nucleotide diversity (*Pi*), average number of nucleotide substitution *per* site (*Dxy*), number of net substitution *per* site between populations (*Da*) [15], average number of nucleotide differences (*k*) [16, 17]. In the gene flow analyses, the estimators *GammaSt* and *Nm* [18, 19] were employed.

The genealogical analyses were conducted in three major steps. First, the total sample was split into categories according to the hosts (bovines and vampire bats); the total sample was split according to geographic origin (PB for Vale do Paraíba and RB for Vale do Ribeira), except the sample B2 (see Table 1). The total sample was then split according to the isolation date (1986–91 and 1992–9). Since the sample VB8 had shown an unexpected behaviour in the genealogies obtained (see results and discussion), we removed it from the analyses. For the three parameters analysed, the HKA test [20] for silent substitutions was applied in the allelic sample assuming a χ^2 distribution with 1 degree of freedom, with $P = 0{\cdot}05$. After the analyses described above and according to the results obtained, we carried out a similar approach involving two major groups formed by: group (1) CVS, PV, SAD B19 and VB8; group (2) the other sequences.

We employed software PAUP* v.4.0b3a [21] with exhaustive search and equal weighting in the maximum parsimony analyses. The consistency index (CI) [22] was obtained with MacClade v.3.03 [13]. Bootstrap values [23] were obtained using the heuristic search using 500 replicates. Sequences from SAD B19, PV, CVS strains, which are used in vaccines, were obtained from GenBank (M31046, AF357308, AF357308, respectively). The allelic trees were drawn out with the programme TreeView v.1.4 [24].

RESULTS

All samples analysed were rabies virus-positive by dIFA and RT–PCR assays. No gaps were detected in the allelic sequences. The statistical results involving the alleles comparison, both within and between categories, are shown in Tables 2 and 3, respectively. Considering the three categories (hosts, geographical locality and year of isolation), the nucleotide diversity (*Pi*) and the average number of nucleotide differences (*k*) were similar within and between groups. No fixed differences (FD) were found between the compared categories, on the other hand an important number of shared mutations (SM) were observed. Furthermore, the *Pi*, *k*, *Dxy*, *Da*, the high *Nm* values, and the low *GammaSt* results indicated a great similarity between the groups in each category (Tables 2, 3).

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Table 3. *Nucleotide diversity between the categories analysed*

	p1m2	p2m1	FD	SM	*k*(*t*)	*Pi*(*t*)	*Dxy*	*Da*	*Nm*	*GammaSt*
Bovine × Bat	41	9	0	18	12·935	0·02117	0·02156	0·0004	11·12	0·05752
Year 86–91 × Year 92–99	19	35	0	14	13·074	0·02117	0·02179	0·00291	4·53	0·09941
PB × RB	37	10	0	15	12·357	0·02126	0·0206	0·00134	8·22	0·05733

p1m2, comparison between polymorphic sites in group 1 and monomorphic in group 2; p2m1, comparison between polymorphic sites in group 2 and monomorphic in group 1; FD, fixed differences; SM, shared mutation; *k*(*t*), average number of nucleotide differences; *Pi*(*t*), nucleotide diversity; *Dxy*, average number of nucleotide substitution per site; *Da*, number of net substitution per site between populations; *Nm*, Nei's pseudoparameter; *GammaSt*, gamma estimation of gene flow.

Table 4. *Nucleotide diversity between the groups analysed*

Groups	p1m2	p2m1	FD	SM	*k*(*t*)	*Pi*(*t*)	*Dxy*	*Da*	*Nm*	*GammaSt*
1 × 2	59	64	52	9	12·935	0·02117	0·01859	0·121	0·43	0·5353

p1m2, comparison between polymorphic sites in group 1 and monomorphic in group 2; p2m1, comparison between polymorphic sites in group 2 and monomorphic in group 1; FD, fixed differences; SM, shared mutation; *k*(*t*), average number of nucleotide differences; *Pi*(*t*), nucleotide diversity; *Dxy*, average number of nucleotide substitution per site; *Da*, number of net substitution per site between populations; *Nm*, Nei's pseudoparameter; *GammaSt*, gamma estimation of gene flow.

Table 5. *Results of HKA test for all categories and groups*

Categories	χ^2*	*P* value†	Significant
Bovine × Vampire Bat	0·005	0·9443	No
Year 86/91 × Year 92/99	0·084	0·7725	No
PB × RB	0·007	0·9347	No
Group 1 × 2	0·269	0·6042	No

* Value of χ^2; † $P = 0{\cdot}05$.

The statistical analyses involving the groups 1 (SAD B19, CVS and PV plus VB8) and 2 (other alleles) are presented in Table 4. Note that this time the number of fixed differences was increased perceptibly if compared to the category analyses. This change was also followed by a decrease of the shared mutations between the groups and of the values of the gene flow estimators. These results indicate that the groups 1 and 2 could be regarded as significantly different groups. The results of the HKA test coroborated the data obtained in the categories and in the groups analyses. According to this test, there is no significant difference between the allelic regions studied (Table 5), thus, the nucleotide variability found could be due to the genetic drift.

In the maximum parsimony analyses of the nucleotide sequences, 20 trees with 362 steps were found. The strict consensus tree was computed from the shortest trees and represents the genealogy involving

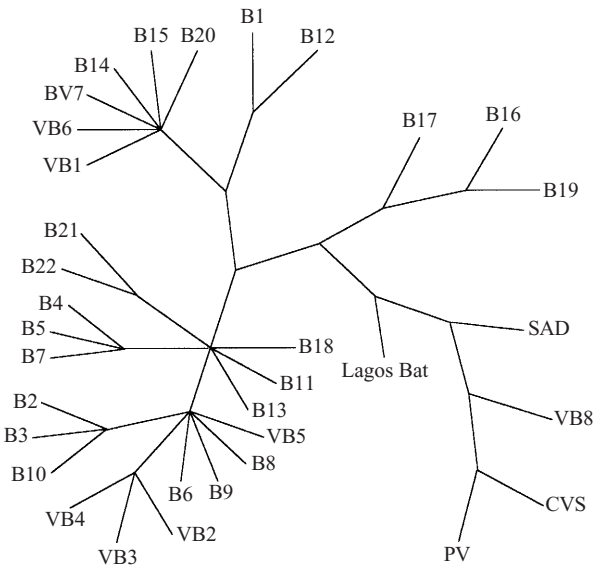

Fig. 2. Maximum-parsimony unrooted tree based on the nucleotide sequence of the nucleoprotein gene of rabies virus. The CI value was equal to 80%.

the N gene alleles. Figure 2 shows the unrooted allelic tree. In the maximum parsimony analyses involving the amino acid sequences, 100 trees with 72 steps were found. After the translation, a conspicuous number of branches collapsed. The strict consensus unrooted amino acid tree is shown in Figure 3.

Bootstrap values of 100% were found in the nodes that separate the strains of rabies virus (genotype 1)

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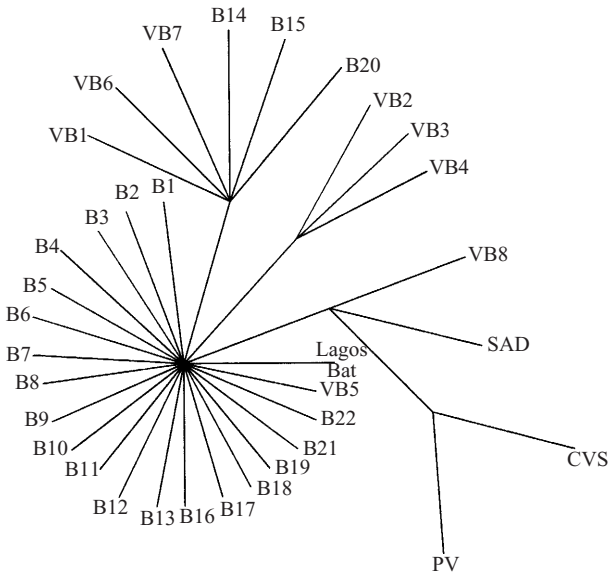

Fig. 3. Maximum-parsimony unrooted tree based on the amino acid sequence of the nucleoprotein gene of rabies virus.

from the external group (genotype 2 – *Lagos* Bat Virus), in the node of the divergence between groups 1 and 2 and in the node that divides the branches of the PV/CVS strains from that of the SAD B19 and VB8 strains.

DISCUSSION

Economic losses in Brazil related to rabies in herbivores are around US$15000000·00/year, and almost 40 thousand bovines are lost each year [25, 26]. The uncontrolled increase in the *Desmodus rotundus* population, the main species involved in rabies transmission to bovines, due to the migration of the population and to the increase in the number of natural and artificial shelters, has contributed to the increasing number of outbreaks in the state of São Paulo, since the beginning of the 1980s [26].

The allele sequences of the nucleoprotein N gene obtained from rabies virus strains revealed the presence of specific sites that may offer information for the assessment of genetic correlation between the strains, enabling the reconstruction of their genealogy.

The unrooted strict consensus nucleotide tree (Fig. 2) presented the consistency index (CI) equal to 80%. Since the CI evaluates the degree of homoplasy of a tree, the present results indicate that the data are well-adequated to the proposed tree topology. The polytomies in the strict consensus tree could be evidence of a high evolutionary conservation of the encoding genes analysed. Alternatively, it could be indicating simultaneous dispersion centres and/or events.

The topology of the tree obtained based on the protein sequences (Fig. 3) shows a large amount of collapsed branches, probably as a result of the occurrence of synonymous mutations in the gene sequences. Since non-synonymous mutations may alter structural protein structure and function in a deleterious way, in the present case, the great number of polytomies in the amino acid tree was expected.

In the maximum parsimony unrooted tree based on the amino acid sequence (Fig. 3) the group made up of strains CVS, PV, SAD B19 and VB8 (group 1) could be separated from the rest of the strains (group 2). In relation to group 2, although we may separate the strains into three subgroups, no segregation was observed in relation to geographic region, time or species. No difference between the strains circulating in the categories of bovines and bats was expected. The bat is, in the state of São Paulo, the main agent in the transmission of rabies to bovines, which are located at the end of rabies transmission chains in rural areas [1, 27].

Therefore, it is expected that the spectrum of strains that infect bats may be related to the one that infects bovines. This supposition is reinforced by data obtained in the intercategory analyses, which produced *GammaSt* (0·0572) and *Nm* (11·2) (Table 3) estimated values that indicate that the virus strains should be considered as a single evolutionary unit.

The value of the estimator *Dxy*, which informs the average number of nucleotide substitutions per site between the categories, was equal to 2·15%. This is an expected value for categories that behave as a panmictic unit [28]. *Dxy* values estimate the probability of allele identity by lineage, that is, the result (Table 3) is the probability that the alleles of each category originated by direct lineage within the same category, and not due to 'migration'. This fact leads to the conclusion that a single evolutionary unit is being analysed. The absence of fixed differences between these two categories associated with the low value of the number of net nucleotide substitutions *per* site (Table 3) reinforce the evidence that the strains from these groups form a single evolutionary group.

Results obtained in this trial, in relation to the circulation of the strains in the hosts, are similar to those obtained by Mattos et al. [29], who observed that 12 strains isolated from bovines in Venezuela were closely related (98% homology) to isolates from

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vampire bats in Latin America. Tordo et al. [30], working with Brazilian strains, distinguished two groups, one comprising wild rabies (bovines and bats) and the other, dog strains (urban rabies). These authors concluded that there are two distinct variants, one for the wild cycle and the other for the urban cycle.

Favoretto et al. [31], who also studied Brazilian strains using monoclonal antibodies determined that 100% of the strains isolated from bovines belong to the *Desmodus rotundus* variant, what is in agreement with the results of the present study.

No differences were observed between the different strains isolated in the different time periods (1986–91 and 1992–9 (Table 3)). The gene flow estimator *Nm* equal to 4·53 indicates that there are no significant differences between the two categories. The results of the present trial corroborate those reported by several authors [5, 7, 32–34], who, in general, did not observe differences along the time between the distinct isolates of rabies virus.

In relation to the categories based on geographical distribution, it was not possible to demonstrate differences between the strains from Vale do Paraíba and Vale do Ribeira. As presented in Figure 3, several strains segregate together, regardless of their origin. This observation is reinforced by data from population analyses between the two categories, in which the *Nm* was 8·2 (Table 3). This value indicates that these two categories also form a single evolutionary unity.

Although data obtained here do not indicate variability related to the different geographical locations, there are reports which demonstrate that this may not be the general rule. Nadin-Davis et al. [35, 36], did not find variations between lineages from different hosts, but demonstrated that there was a very clear and consistent difference between rabies viruses from different geographical regions in Ontario province, Canada. Kissi et al. [5] and Smith et al. [33] also differentiated rabies virus strains according to their geographical distribution throughout the world.

Souza et al. [37] studied the serological and biological aspects of the strains isolated from *Desmodus rotundus* captured in Vale do Paraíba (SP). Two strains of their study were also analysed in the present trial (VB2 and VB3). Those authors reported that the strains, when serologically compared with fixed strains of rabies, presented differences in reactivity, that is, antisera from strains PV and CVS could only neutralise strains VB2 and VB3 in low dilutions. In spite of the present focus on nucleoprotein N and the neutralization had being attributed mainly to antibodies directed against glycoprotein G, our results are in agreement with those obtained by Souza et al. [37]. As shown in the amino acid tree, the strains VB2 and VB3 segregate separately (Fig. 3).

Group 1 (PV, CVS, SAD B19, VB8) is significantly separated from the other strains and presents a monophyletic profile. This is very intriguing, since strain VB8 is an isolate from a vampire bat. Nucleotide variability data from groups 1 and 2 (Table 4) indicate the occurrence of 52 fixed mutations, that shows a large absolute difference between the strains from both groups. An *Nm* value of 0·43 and a *GammaSt* value of 0·53 show that the groups 1 and 2 could not be considered as a single evolutionary unity.

Reasonable hypotheses to explain the position of strain VB8 in the proposed genealogy could be either that the VB8 strain is a new variant, and its strain is originated from a recent common ancestry of the strains analysed, or VB8 strain is a variant from a non-vampire bat. The hypothesis is reinforced by Tordo et al. [38], who identified strains isolated from a non-vampire bat (*Tadarida brasiliensis*) in Mexico, which were very close to the canine variants. Favoretto et al. [31], typing rabies virus strains isolated from non-vampire bats using monoclonal antibodies observed cross-reactivity with strains isolated from *Desmodus rotundus* (variant 3), *Tadarida brasiliensis* (variant 4) and *Lasiurus cinereus* (variant 6). They also demonstrated the occurrence of four antigenic profiles not compatible with the existing ones. These data confirms the great diversity of the variants found in non-vampire bats, and therefore the VB8 strain may originate from an insectivorous bat.

Alternatively, the VB8 isolate may be a dog variant that has transmitted to a bat. For this, an intimate interaction between a wild or domestic dog and a vampire bat would be needed. However, this hypothesis lacks credibility because it relies on the complicated process involving the survival of a vampire bat after a dog bite followed by the elimination of rabies virus and then the continuation of virus transmission to other vampire bats.

Results of the HKA test (Table 5), performed with all artificial groups, including groups 1 and 2, show that there is no significant difference between the groups in relation to nucleotide substitution. These results indicate that the differential selective pressure hypothesis in the different regions of the sequences

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studied (5′ end coding for N protein) should be rejected, and that variability observed is probably due to genetic drift. Similar results were obtained by Kissi et al. [5], who suggested that there is no evidence of cumulative effects for the non-synonymous mutations and eventual changes in the epitopes, certainly due to the intense conservation in protein N evolution [39].

Final analyses of data presented here indicates the need for continuous efforts in monitoring gene diversity in field strains, in relation to vaccine strains, since changes in genetic material may produce modifications in the proteins responsible for immunogenicity of virus strains, which may eventually cause vaccine failures in herds.

ACKNOWLEDGEMENTS

We wish to thank Dr Arthur Gruber and Dr Fernando Ferreira for technical assistance. This work was supported by FAPESP (grant no. 96/07723-0).

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