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Rabies virus distribution in tissues and molecular characterization of strains from naturally infected non-hematophagous bats

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ABSTRACT

Bats are main reservoirs for *Lyssavirus* worldwide, which is an important public health issue because it constitutes one of the big challenges in rabies control. Yet, little is known about how the virus is maintained among bats, and the epidemiological relationships remain poorly understood. The aim of the present study was to investigate the distribution of the rabies virus (RABV) in bat tissues and organs and to genetically characterize virus isolates from naturally infected non-hematophagous bats. The hemi-nested reverse transcriptase polymerase chain reaction (hnRT-PCR) and sequencing using primers to the nucleoprotein coding gene were performed. The results showed a dissemination of the RABV in different tissues and organs, particularly in the salivary glands, tongue, lungs, kidneys, bladder, intestine and feces, suggesting other possible forms of RABV elimination and the possibility of transmission among these animals. The phylogenetic analysis confirmed that different variants of RABV are maintained by non-hematophagous bats in nature and have similar tissue distribution irrespective of bat species and phylogenetic characterization.

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1. Introduction

Rabies is a zoonosis that affects mammals and is endemic throughout the world, with the exception of the Antarctica continent, islands such as Hawaii and New Zealand and some European countries (Fooks et al., 2009). The World Health Organization estimates that 55,000 human deaths occur each year as a result of rabies (WHO, 2007); the majority of cases are reported in Africa and Asia (WHO, 2005). Data obtained from global mortality estimates suggest that every 10 min, one person dies from rabies and approximately 300 others are exposed (Fooks et al., 2009).

RABV genotype 1, the prototype of the genus *Lyssavirus* in the family *Rhabdoviridae*, is the etiological agent of classical rabies that is responsible for the majority of human deaths worldwide (WHO, 2007). Lyssaviruses are enveloped bullet-shaped viruses with a single-stranded and negative-sense RNA genome that encodes five structural proteins: nucleocapsid protein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and the RNA polymerase or large protein (L) (Wunner, 2007).

In Brazil during the last 10 years, human cases of rabies transmitted by dogs have diminished considerably as a result of control measures in domestic animals. However, the number of cases of human rabies acquired by bats has been increasing (Mayen, 2003; Travassos da Rosa et al., 2006; WHO, 1992) and bats have become the main transmitters of human rabies in Brazil (SVS/MS, 2008).

Bats are now the most prominent source of rabies for humans, domestic animals and wild animals in the Americas (Kuzmin and Rupprecht, 2007). Rabies is a complex problem with economic, public health and ecological implications (Cunha et al., 2010). Environmental changes due to urban development may have contributed to the increase in the bat population in urban areas, not only because of the wide variety of shelters but also due to the large food supply and absence of predators (Sodré et al., 2010).

Controlling and understanding bat rabies in natural populations has been a large challenge for researchers worldwide. Given bats' nocturnal habits, ability to fly and variety of species with different habits, studying bat rabies under natural conditions is difficult, and many gaps in the understanding of rabies remain (Klug et al., 2011).

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Colonial bats are gregarious animals. They have close social contact, sometimes fighting or grooming each other, and even mutually feed by regurgitation of ingested blood in the case of vampire bat colonies. These interactions can lead to the transmission of the virus through infected saliva by bites and licking, inhalation of aerosolized saliva, and probably by ingestion of regurgitated blood contaminated with infected saliva (Constantine, 1988; Souza et al., 2009).

As a result of enhanced surveillance, the number of rabid bats detected is increasing each year, especially in non-hematophagous species (Cunha et al., 2010). Bats maintain circulation of specific RABV variants in urban areas, which is a significant public health concern (Rupprecht et al., 1987; Smith et al., 1995).

The transmission and pathobiology of RABV in free-ranging bats is still unclear and remains poorly understood (Carneiro et al., 2010; Steece and Calisher, 1989). Although there is evidence that virus can be transmitted among bats by bite, aerosol, ingestion of virus-infected milk or blood and transplacental infection (Constantine, 1966, 1967a; Constantine et al., 1968; Sims et al., 1963; Souza et al., 2009), the mechanism of virus transmission between bats has not been studied in detail (Kuzmin and Rupprecht, 2007).

The high seroprevalence rates in apparently healthy bats suggest they may be able to control natural infection (Lyles and Rupprecht, 2007). Aguilar-Setien et al. (2005) demonstrated that 3 of 14 vampire bats survived for 2 years after a massive parenteral challenge with RABV and occasionally excreted virus in their saliva. A continuous exposure may explain why the virus is endemic in bats population, a equilibrium between fatalities and exposure to virus (biting, scratches) probably exists and the low virus dose could induce a immune response and the development on virus neutralizing antibodies (VNAs) and clear the virus, while others succumb to disease (Franka et al., 2008, 2009). The apparent low level of virus shedding and the low mortality among experimentally infected bats leave the mechanisms of the transmission cycle in nature a mystery (Vos et al., 2007).

However, it was demonstrated that naturally infected bats do die from rabies (Carneiro et al., 2010; Kuzmin et al., 2008; Freuling et al., 2008). Moreover, a long and variable incubation period is thought to occur in nature, and this may be influencing the maintenance of enzootic rabies in wildlife (Jackson, 2007; Moore and Raymond, 1970).

Considering the great diversity of bats with different feeding habits, their ecology and widespread distribution in the country, improving knowledge about virus circulation among bats will contribute to a better understanding of the bat rabies epidemiology and will provide information to identify the real risk to the human population when in contact with those animals.

Thus, the aim of this study was to analyze the viral distribution by molecular techniques in tissues and organs of naturally infected bats with different habits to look for the presence of virus in organs that may be involved in RABV transmission among bats and possibly to humans and other animals. Additionally, the virus isolates were partially sequenced and subjected to genetic characterization and molecular epidemiological studies.

2. Materials and methods

2.1. Bats

A total of 26 non-hematophagous bats found by members of the general population or by health agents and sent for definitive rabies diagnosis were used; some of these bats were found dead and other moribund. Bats captured alive were humanely euthanized with ether. The bats were previously identified by the Center of Zoonosis Control, São Paulo-SP as *Artibeus lituratus* (13), *Myotis nigricans* (4), *Eptesicus furinalis* (5), *Eptesicus diminutus* (1), *Lasiurus blossevillii* (1), and *Lasiurus ega* (2). The bats were collected between 2004 and 2009 in 7 cities in the western region of São Paulo state and all were diagnosed as positive for rabies by means of direct immunofluorescence and virus isolation by Dr. Avelino Albas.[2]

2.2. Samples

Fragments of brain tissue, salivary gland, tongue, lung, heart, stomach, liver, spleen, kidney, bladder, intestine, and brown fat were aseptically collected and stored at −80 °C until processing. It was impossible to collect all organs because of the state of conservation of the bat carcasses.

The feces samples were collected from the anus and, when necessary, by opening the final part of the large intestine. Each sample was collected with individual instruments and washed with sterile water 3 times to avoid cross-contamination between tissues from the same animal.

The positive control used for all reactions was the rabies challenge virus strain (CVS), and sterile water (DNAse/RNAse-free) was used as a negative control. Every third sample, one negative control was added.

2.3. RT-PCR

The viral RNA was extracted by the RTP DNA/RNA Virus Mini Kit® (Invitek, Germany) following the manufacturer's instructions. The RT-PCR And hnRT-PCR techniques used was described by Soares et al. (2002); reverse transcription was performed with 7 μl of RNA first denatured for 5 min at 94 °C and chilled on ice and then added to a final volume of 20 μl containing 1 mM of each dNTP (Invitrogen, USA), 20 pmols of primer P510, 1× First Strand Buffer, 1 mM dTT, 200 units M-MLV Reverse Transcriptase (Invitrogen, USA) and 0.01% Nuclease Free Water (IDT, USA). The samples were then incubated for 1 h at 42 °C. Primary amplification were carried out with 5 μl of the reverse transcripted c-DNA template in a final volume of 50 μl, containing 0.2 mM of each dNTP, 25 pmols of sense primer P510 (ATAGAGCAGATTTTCGAGACAGC), 25 pmols of anti sense primer P942 (CCCATATAACATCCAACAAAGTG), 1.5 mM of $MgCl_2$, 1× PCR Buffer, 1.25 units of Platinum Taq DNA Polymerase (Invitrogen, USA) and Nuclease Free Water (IDT, USA). The samples that resulted negative on this primary amplification were submitted to a hnRT-PCR, in order to improve sensitivity. The hnRT-PCR was performed with antisense primer P784 (CCTCAAAGTTCTTGTGGAAGA) and sense primer P510. The cycling conditions for the primary amplification were: initial heating at 94 °C/6 minutes (min), 35 cycles at 94 °C/45 seconds (s), 55 °C/60 s, 72 °C/90 s, followed by a final extending at 72 °C for 10 min. The thermal cycles for the nested assay were the same but with 25 cycles for amplification. Products of PCR were run in 2% agarose gel electrophoresis in standard TBE stained with ethidium bromide 0.5 μg/ml and gels were observed under UV light and photographed. Primer sets P510/P942 and P510/P784 defined 455 and 295 base pairs, respectively.

2.4. Sequencing

The amplicons obtained from rabies virus amplification from brain samples were used for nucleotide sequencing. All isolates were sequenced with a forward (P510) and reverse primer (P942). Twenty isolates had the ideal concentration for sequencing.

The amplified DNA fragment was purified using a commercial kit (Illustra GFXTM PCR DNA and Gel Band Purification Kit® (GE

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Table 1
GenBank accession number/isolates and bats species from which the samples were collected.

Accession number/strain	Bat specie
JF950546/11	*Artibeus lituratus*
JF950539/04	*Artibeus lituratus*
JF950547/12	*Artibeus lituratus*
JF950552/17	*Artibeus lituratus*
JF950554/19	*Artibeus lituratus*
JF950540/05	*Artibeus lituratus*
JF950555/20	*Myotis nigricans*
JF950543/08	*Eptesicus furinalis*
JF950544/09	*Artibeus lituratus*
JF950545/10	*Artibeus lituratus*
JF950541/06	*Lasiurus blossevillii*
JF950536/01	*Lasiurus ega*
JF950548/13	*Eptesicus furinalis*
JF950549/14	*Myotis nigicans*
JF950550/15	*Eptesicus diminutus*
JF950553/18	*Myotis nigricans*
JF950542/07	*Eptesicus furinalis*
JF950537/02	*Eptesicus furinalis*
JF950538/03	*Eptesicus furinalis*
JF950551/16	*Artibeus lituratus*

Healthcare, USA), visually quantified with the Low DNA Mass Ladder® (Invitrogen, USA) in agarosis gel, and finally, sequenced using the BigDyeTM Terminator Kit® (Applied Biosystems, USA) on an automated sequencer (ABI model 377, Applied Biosystems).

2.5. Phylogenetic analysis

First, the raw sequencing data were edited using BIOEDIT v.7.0.5 (Hall, 1999). The complete sequence assemblies were then created with the PHRED/PHRAP (Ewing and Green, 1998) and CAP3 (Huang and Madan, 1999) programs using nucleotide data with quality higher than 20 (http://bioinformatica.ucb.br/electro.html). The derived rabies sequences were aligned using BIOEDIT v.7.0.5 (Hall, 1999).

Phylogenetic analysis was performed at nucleotide level on the aligned data set, using sequences from the N gene and was carried out by the neighbor-joining algorithm implemented in Mega 5.0 (Tamura et al., 2011). The best evolutive model was determinated by Model test (Posadas and Crandall, 1988) implemented in Mega 5.0. Bootstrap values were calculated on 1000 repeats and the bootstrap value cut off was 65%.

The nucleotide sequence data reported are available in the GenBank databases under the accession numbers listed in Table 1.

2.6. Statistical analyses

The Fisher's Exact Test was used to obtain percentages and to compare the results obtained in the viral distribution among frugivorous and insectivorous bats.

3. Results

The results showed a wide distribution of RABV in the tissues and organs analyzed, with a higher rate of virus positivity in certain samples. All of the bats had detectable virus in the brain and salivary glands (Table 2).

The results of the viral distribution obtained by hnRT-PCR were grouped into frugivorous and insectivorous bats for comparison. According to the Fisher's Exact Test, the difference observed between these two groups was not statistically significant.

The percentages of samples testing positive for virus from frugivorous and insectivorous bats, respectively, were as follows: in tongue, 92% and 85%; in brown fat, 82% and 77%; in lung, 62% and 77%; in heart, 42% and 77%; in stomach, 92% and 64%; in liver, 38% and 67%; spleen, 43% and 27%; in bladder, 73% and 88%; in kidney, 77% and 38%; in intestine, 77% and 38%; in feces, 38% and 42%. All brains and salivary glands tested positive in the primary amplification (Fig. 1).

Table 2
Rabies virus distribution in organs and tissues obtained by hnRT-PCR in naturally infected non-hematophagous bats.

Samples	Positive	Negative
Brain	100% (26/26)	0%
Salivary gland	100% (26/26)	0%
Tongue	88% (23/26)	12% (3/26)
Brown fat	79% (19/24)	21% (5/24)
Lung	69% (18/26)	31% (8/26)
Heart	60% (15/25)	40% (10/25)
Stomach	79% (19/24)	21% (5/24)
Liver	52% (13/25)	48% (12/25)
Spleen	33% (6/18)	67% (12/18)
Bladder	79% (15/19)	21% (4/19)
Kidney	58% (15/26)	42% (11/26)
Intestine	58% (15/26)	42% (11/26)
Feces	40% (10/25)	60% (15/25)

Phylogenetic analysis revealed that RABV isolates were grouped into clusters according to the bat species, and there was evidence of species-specific variants. The sequences were analyzed for the identity of nucleotides and amino acids using BIOEDIT v.7.0.5 (Hall, 1999), which revealed a high similarity among different bat-related RABV from different geographic areas, most of them with 98–99% homology and some with 100% homology.

The sequenced samples formed a phylogenetic tree divided into two major monophyletic lineages: frugivorous (I) and insectivorous (II) samples. Within these larger groups, clusters were formed according to bat species, with group I showing *A. lituratus* clustering together with *Desmodus rotundus* (GenBank: BRDR18, BRDR21, BRDR14) and *Tadarida brasiliensis* (GenBank: IP2136 06–IP185 05/retrieved number). Group II contained subgroups 2 and 3, which were mostly collected from colonial non-migratory bats (*M. nigricans* and *Eptesicus* spp.), and subgroup 4, which contained strains related to migratory and solitary bats (*Lasiurus* spp.) (Fig. 2).

An intra specific transmission has been occurring in different bat colonies. The idea can be confirmed by the sequencing results, virus isolates from the same species have similarities not only in the nucleotides but also in pathogenic characteristics observed by intracerebral and intramuscular experimental inoculation (Wang et al., 2005).

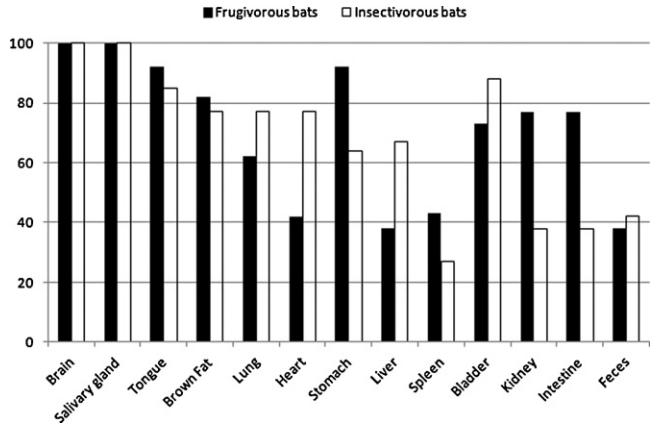

Fig. 1. Comparison of positive results obtained in samples of rabies virus naturally infected frugivorous and insectivorous bats.

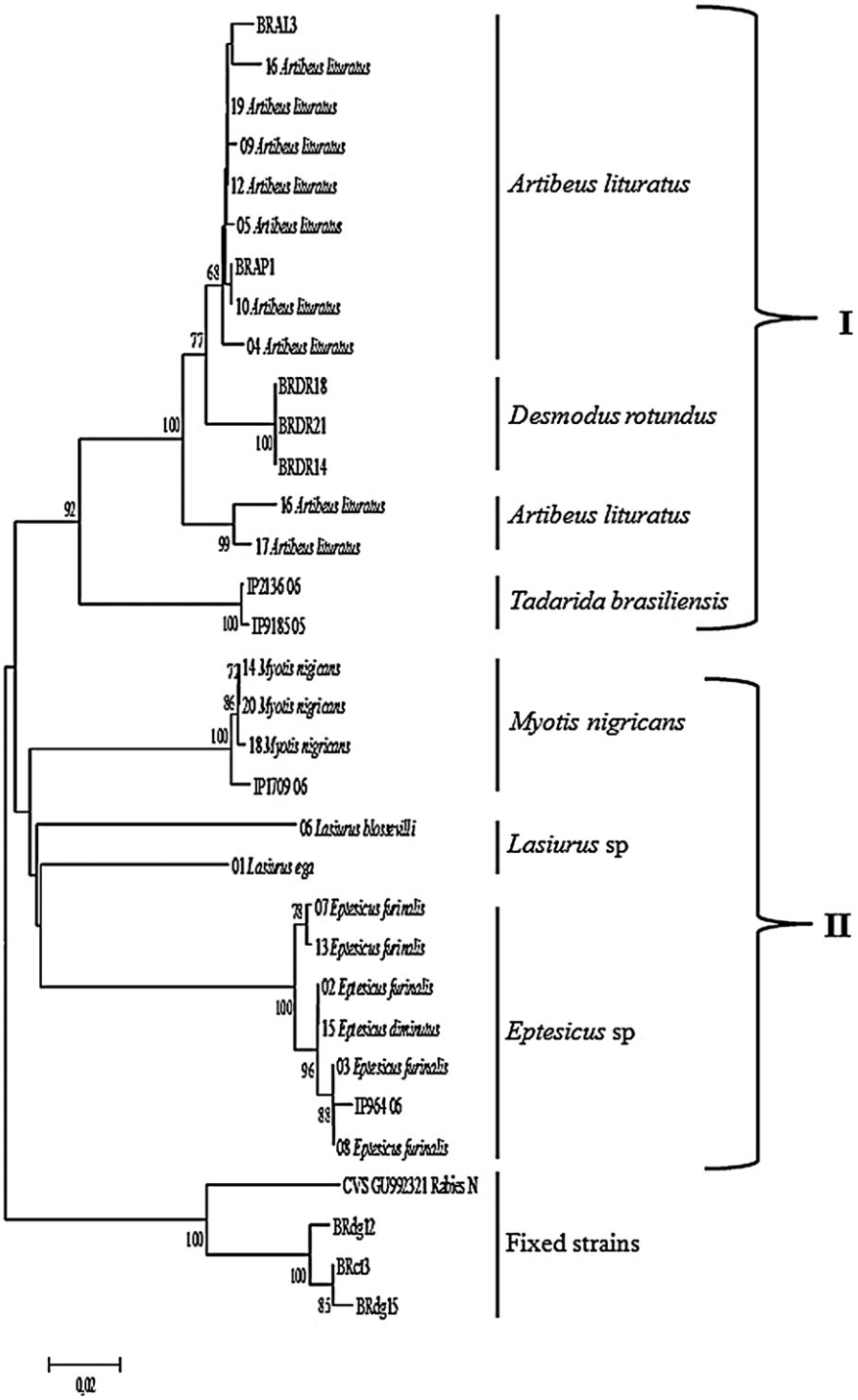

Fig. 2. Phylogenetic tree based on the sequence of 442 bp corresponding to the middle of nucleoprotein gene (N) located between nucleotides 589 and 1031 of the PV virus (GenBank number M13215.1). Phylogenetic analysis was performed using the neighbor-joining method with Tamaru 3 parameter and invariant sites model. The numbers shown at the nodes of the genetic clusters represent the bootstrap values for 1000 replicates, and the RABV fixed strain was used as an outgroup. Phylogenetic tree performed using Maximum Likelihood test yielded similar results to neighbor-joining method (data not shown).

4. Discussion

There is a lack of information involving the virus distribution of classic rabies virus genotype 1, in naturally infected bats. The disease has been investigated on its native host by few researchers. As already suggested, in bats the virus spreads centripetally from the inoculation site to the brain and then centrifugally to other tissues and organs (Freuling et al., 2009). Rabies virus in different organs were detected in our research, in agreement with other studies which indentified the virus in different tissues of infected bats; despite the fact that some samples presented a higher rate of positivity no significant pattern of viral distribution was observed (Franka et al., 2008; Freuling et al., 2009; Johnson et al., 2006).

We could not determine a pattern of viral distribution in the studied bat species, suggesting that is most likely to be related to others factors than with the bat specie or even with the lyssavirus genotype. According to Freuling et al. (2009), the variation on viral distribution in the organs and tissues of the infected bats might

be related to the site of inoculation. This observation could be supported by another study in which *Eptesicus fuscus* were experimentally infected with European bat lyssavirus 1 (EBLV-1), the results revealed that animals who received lower intramuscular (i.m.) virus dose appeared to have a broader organ distribution than bats receiving the higher i.m dose and bats inoculated intracranially (i.c.) (Franka et al., 2008). In addition, the dose and mode of infection among free-ranging bats could lead to an abortive infection and possible be responsible for the naturally occurring antibodies found in bat populations (Davis et al., 2007; Franka et al., 2008).

In our study, RABV was detected in 100% of the salivary glands and in 88% of the tongues analyzed. These data corroborate previous studies that detected high virus positivity rates obtained in the tongues from different bat species (Carneiro et al., 2010; Johnson et al., 2008; Freuling et al., 2009). Although we cannot exclude the possibility of viral contamination through infected saliva, the presence of virus antigen in lingual papilla epithelium associated with the taste buds positivity in salivary gland was characterized by immunohistochemical analysis confirming the presence of rabies virus in these tissues (Freuling et al., 2009).

The release of virus by epithelial cells in the tongue probably occur in infected bats as it was observed with experimentally infected serotine bat (*Eptesicus serotinus*) were the viral antigen could be indentified in lingual epithelium but not be in the secretory epithelial cells of the salivary gland (Freuling et al., 2009). The presence of large amounts of RABV in salivary gland and in tongue regardless of the virus genotype reinforces the knowledge and importance of saliva as a source of RABV elimination in bats irrespective to bat species. As suggested, the transmission through bites is probably the most important mode of infection in free living bats (Franka et al., 2006).

Positivity of 79% in brown fat was detected in our study, which is in accordance with several reports that detected RABV in the interscapular brown adipose tissue in naturally infected bats (Bell et al., 1962; Villa et al., 1963; Scheffer et al., 2007). In *Myotis* bats, the frequency of isolation of RABV from the brain, brown fat, and salivary glands was 92%, 30% and 17%, respectively. Sulkin et al. (1960) and Sulkin (1962) experimentally infected insectivorous bats (Mexican free-tailed bat and *Myotis lucifugus*) with the objective to understand the mechanism by which these animals serve as reservoirs in nature. The results suggested that the brown fat provides a sit for the storage of RABV during bat hibernation and for virus replication. The virus isolates were quantified by RABV titration in tissue suspensions of the brain, salivary gland and brown fat of mice. These results revealed high virus titers in the interscapular fat, with concentrations similar to those of the brain and salivary glands.

RABV transmission via an airborne route is much less common than by bites but can occur in caves where millions of bats roost (Constantine, 1962; Jackson, 2007). RABV has been observed in the nasal mucosa of naturally infected Mexican free-tailed bats (*Tadarida brasiliensis mexicana*) by virus isolation and fluorescent antibody test, suggesting that the nasal mucosa is a portal of entry and possible portal from which virus is expelled into the air in respiratory mucus particles (Constantine et al., 1972). Another study examined the viral distribution in paralyzed or recently deceased Mexican free-tailed bats collected from caves in Texas. Rabies virus was detected in 130 bats, in 30% which the virus was isolated from lung samples (Constantine, 1967a,b). In this study, from 26 bats studied, the virus was detected in the lungs of 18 (69.2%), suggesting that airborne transmission may occur in natural conditions, especially in caves with many bats and without air circulation, as observed by Constantine (1967b).

In contrast, bats exposed to aerosolized rabies virus under experimental conditions survived and produced VNAs (Davis et al., 2007). Franka et al. (2009) observed that after intranasal (i.n.) inoculation with EBLV-1a all animals survived, none developed VNAs, in contrast to the bats infected parenterally, the authors suggested a possible role for innate immunity in peripheral clearance of rabies virus in bats. The lack of observed morbidity and mortality led the researchers to conclude that an aerosol route of exposure might not play a major role in transmission of rabies among free-living bats.

Although not frequently reported, rabies transmission by an oral route can occur (Jackson, 2007). It has been proved that the ingestion of infected carcasses by carnivores can transmit rabies (Ramsden and Johnson, 1975). It was also demonstrated that the virus remains viable in infected skunk carcasses for 22 days at 10 °C and for 14 days at 24 °C (Schaefer, 1983) and that ingestion of infected tissue may be a mode of transmission to mammalian scavengers (Correa-Giron et al., 1970; Ramsden and Johnson, 1975). Bell and Moore (1971) demonstrated that striped skunks may be fatally infected after eating a single rabid mouse, suggesting that bats could cause rabies infection by an oral route when carnivores feed on them. Assuming that the transmission of rabies can occur by eating infected bat carcasses, we cannot exclude the possibility of rabies transmission to other carnivore species in rural and urban locations, or even to other bats. There are reports that hoary bats (*Lasiurus cinereus*) may prey on Pipistrelles bats (Constantine, 1967a; Bishop, 1947; Orr, 1950). Cannibalism among bats has been observed in captivity and probably occurs in nature more frequently than is reported (Constantine, 1967a).

The high percentage of virus positivity in stomach tissue (79%) could be due to swallowing of the virus, as previously suggested by Johnson et al. (2006). Viral RNA was also detected in bat feces, kidney and bladder. These results suggest that bat urine as well as feces could be a source of infection and a possible mean of rabies transmission, as previously suggested (Constantine et al., 1972; Johnson et al., 2006; Scheffer et al., 2007). However, the epidemiological importance of these excretions must be evaluated more closely in a natural environment in which rabies virus is inactivated by unfavorable ambient conditions.

The low virus positivity rate found in spleen (33%) was similar to other results reported by Carneiro et al. (2010), who suggested that these low levels could be explained by the lack of specific involvement of RABV with the lymphatic system.

It has been demonstrated, through the analysis of partial nucleoprotein gene sequences, that there are different virus variants circulating among bats (Nadin-Davis et al., 2001; Oliveira et al., 2010; Vellasco-Villa et al., 2006). The 400 nucleotides in the amino-terminal coding region of the N gene are recommended for use in phylogenetic analysis because they can determine the geographical distribution of the major virus lineages (Kissi et al., 1995). However, it has been observed that in general, similar conclusions can be made about epidemiological relationships regardless of the specific sequenced region of the target gene (Nadin-Davis, 2007). In our study, the sequenced region corresponded to the middle of the N gene, and phylogenetic analysis revealed that virus variants tended to form groups according to bat species.

Group I, a cluster defined by bootstrap of 91, contained samples from *A. lituratus*, vampire bats and *T. brasiliensis* (numbers retrieved from GenBank) that allowed us to conclude that there is a significant relationship between hematophagous and frugivorous bats. The results obtained here had already been observed by other researchers (Kobayashi et al., 2005, 2007; Shoji et al., 2004), implying that an interaction had occurred between these species; the virus isolated from *A. lituratus* is clearly characterized as vampire bat-related RABV.

There are hypotheses that try to explain the transmission of RABV between hematophagous and frugivorous bats; some researchers have suggested that it, while others posit that some vampire bats may have fed on other species of bats that shared the same roosts, especially during inclement weather, when bats may be confined (Greenhall, 1988). Virus transmission might have

occurred by sharing the same roost, as was observed in Mexico (Forment et al., 1971). Further studies should be carried out to better understand the relationship between these species.

5. Conclusion

These results indicate the presence of RABV in several organs of naturally infected non-hematophagous bats, particularly in the salivary glands, lungs, kidneys, bladder, intestine, and feces suggesting other possible forms of rabies virus elimination and the possibility of transmission among these animals. The phylogenetic analysis confirmed that different variants of RABV are maintained by non-hematophagous bats in nature and have similar tissue distribution, irrespective of bat species and phylogenetic characterization. However within the same bat species geographic genotypes exist, confirming a local viral circulation. It was not possible to define a pattern of viral distribution in the analysed samples.

In conclusion, knowing the characteristics of each virus contribute for a better understanding of the relationship between the virus, the animals and the species. The role that the infectious dose, the pathogenicity and the immune innate response plays on rabies infection in wildlife requires more detailed studies.

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References

Aguilar-Setien, A., Loza-Rubio, E., Salas-Rojas, M., Brisseau, N., Cliquet, F., Pastoret, P.P., Rojas-Dotor, S., Tesoro, E., Kretschmer, R., 2005. Salivary excretion of rabies virus by healthy vampire bats. Epidemiol. Infect. 133, 517–522.

Bell, J.F., Moore, G.T., Raymond, G.H., Tibbs, C.E., 1962. Characteristics of rabies in bats in Montana. Am. J. Publ. Health 52, 1293–1301.

Bell, J.F., Moore, G.J., 1971. Susceptibility of carnivore to rabies virus administered orally. Am. J. Epidemiol. 93, 176–182.

Bishop, S.C., 1947. Curious behavior of a hoary bat. J. Mammal. 28, 293–294.

Carneiro, A.J.B., Franke, C.R., Stöcker, A., Dos Santos, F., De Sá, J.E.Ú., Moraes-Silva, E., Alves, J.N.M., Brünink, S., Corman, V.M., Drosten, C., Drexler, J.F., 2010. Rabies virus RNA in naturally infected vampire bats, northeastern Brazil. Emerg. Infect. Dis. 16 (12), 2004–2006.

Constantine, D.G., 1962. Rabies transmission by nonbite route. Public Health Rep. 77, 287–289.

Constantine, D.G., 1966. Transmission experiments with bat rabies isolates. Reaction of certain carnivore, opossum, rodents and bats to rabies virus of red bat origin when exposed by bat or by intra-muscular inoculation. Am. J. Vet. Res. 27, 24–32.

Constantine, D.G., 1967a. Bat rabies in the southwestern United States. Public Health Rep. 81 (10), 867–888.

Constantine, D.G., 1967b. Rabies transmission by air in bat caves. In: Public Health Service Publication 1617. National Communicable Disease Center, Atlanta.

Constantine, D.G., Solomon, G.C., Woodall, D.F., 1968. Transmission experiments with bat rabies isolates: responses of certain carnivores and rodents to rabies virus from four species of bats. Am. J. Vet. Res. 29, 181–190.

Constantine, D.G., Emmons, R.W., Woodie, J.D., 1972. Rabies virus in nasal mucosa of naturally infected bats. Science 175, 1255–1256.

Constantine, D.G., 1988. Transmission of pathogenic microorganisms by vampire bats. Public Health Greenhall. In: Schmidt, A.M.U. (Ed.), The Natural History of Vampire Bats. CRC Press, Florida, pp. 167–189.

Correa-Giron, E.P., Allen, R., Sulkin, S.E., 1970. The infectivity and pathogenesis of rabies-virus administrated orally. Am. J. Epidemiol. 91, 203–215.

Cunha, E.M.S., Nassar, A.F.C., Lara, M.C.C.S.H., Villalobos, E.C.M., Sato, G., Kobayashi, Y., Shoji, Y., Itou, T., Sakai, T., Ito, F.H., 2010. Pathogenicity of different rabies virus isolates and protection test in vaccinated mice. Rev. Inst. Med. Trop. Sao Paulo 52, 231–235.

Davis, A.D., Rudd, R.J., Bowen, R.A., 2007. Effects of aerosolized rabies virus exposure on bats and mice. J. Infect. Dis. 195, 1144–1150.

Ewing, B., Green, P., 1998. Base-calling of automated sequencer traces using phred. II. Error probabilities. Gen. Res. 8, 186–194.

Fooks, A.R., Johnson, N., Rupprecht, C.E., 2009. Rabies. In: Barrett, A.D.T., Stanberry, L.R. (Eds.), Vaccines for Biodefense and Emerging and Neglected Diseases. Academic Press, Italy, pp. 609–630.

Forment, W.L., Schmidt, U., Greenhall, A.M., 1971. Movement and population studies of vampire bat (*Desmodus rotundus*) in Mexico. J. Mammal. 52, 227–228.

Franka, R., Denny, C.G., Kuzmin, I., Velasco-Villa, A., Reeder, S.A., Streicker, D., Orciari, L.A., Wong, A.J., Blanton, J.D., Rupprecht, C.E., 2006. A new philogenetic lineage of Rabies Virus associated with western pipestrelle bats (Pipistrellus hesperus). J. Gen. Virol. 87, 2309–2321.

Franka, R., Wu, X., Jackson, F.R., Velasco-Villa, A., Palmer, D.P., Henderson, H., Hayat, W., Green, D.B., Blanton, J.D., Greenberg, L., Rupprecht, C.E., 2009. Rebies virus pathogeneis in relationship with intervention with inactivated and attenuated rabies virus vaccines. Vaccine 27, 7149–7155.

Franka, R., Johnson, N., Muller, T., Vos, L., Neubert, L., Freuling, C., Rupprecht, C.E., Fooks, A.R., 2008. Susceptibility of north American big brown bats (*Eptesicus fuscus*) to infection with European bat lyssavirus type 1. J. Virol. 89, 1998–2010.

Freuling, C., Grossmann, E., Conraths, F.J., Schameitat, A., Kliemt, J., Auer, E., Greiser-Wilke, I., Muller, T., 2008. First isolation of EBLV-2 in Germany. Vet. Microbiol. 131, 26–34.

Freuling, C., Vos, A., Johnson, I., Denzinger, A., Neubert, E., Mansfield, K., Hicks, D., Nuñez, A., Tordo, N., Rupprecht, C.E., Fooks, A.R., Muller, T., 2009. Experimental infection of serotine bats (*Eptesicus serotinu*) with European bat lyssavirus type 1a. J. Virol. 90, 2493–2502, doi:10.1099/vir.O.O11510-0.

Greenhall, A.M., 1988. Feeding behavior. In: Greenhall, A.M., Schmidt, U. (Eds.), Natural History of Vampire Bats. CRC Press, Boston, pp. 111–131.

Hall, T.A., 1999. Bioedit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/nt. Nucl. Acids Symp. Ser. 41, 95–98.

Huang, X., Madan, A., 1999. CAP3: a DNA sequence assembly program. Genome Res. 9, 868–877.

Jackson, A., 2007. Pathogenesis. In: Jackson, A.C., Wunner, W.H. (Eds.), Rabies. Academic Press, San Diego, pp. 341–381.

Johnson, N., Wakeley, P.R., Brookes, S.M., Fooks, A.R., 2006. European bat lyssavirus type 2 RNA in *Myotis daubentonii*. Emerg. Infect. Dis. 12, 1142–1144.

Johnson, N., Vos, A., Neubert, L., Freuling, C., Mansfield, K.L., Kaipf, I., Denzinger, A., Hicks, D., Nuñez, A., Franka, R., Rupprecht, C.E., Muller, T., Fooks, A.R., 2008. Experimental study of European bat lyssavirus type-2 infection in Daubentonĭs bats (Myotis daubentonii). J. Gen. Virol. 89, 2662–2672.

Kissi, B., Tordo, N., Boourhy, H., 1995. Genetic polymorphism in the rabies virus nucleoprotein. Virology 209, 526–537.

Klug, B.J., Turmelle, A.S., Ellison, J.A., Baerwald, E.F., Barclay, R.M.R., 2011. Rabies prevalence in migratory tree-bats in Alberta and the influence of roosting ecology and sampling method on reported prevalence of rabies in bats. J. Wildl. Dis. 47 (1), 64–77.

Kobayashi, Y., Sato, G., Shoji, Y., Sato, T., Itou, T., Cunha, E.M.S., Samara, S.I., Carvalho, A.A.B., Nociti, D.P., Ito, F.H., Sekai, T., 2005. Molecular epidemiological analysis of bat rabies viruses in Brazil. J. Vet. Med. Sci. 67, 647–652.

Kobayashi, Y., Sato, G., Kato, M., Itou, T., Cunha, E.M., Silva, M.V., Mota, C.S., Ito, F.H., Sakai, T., 2007. Genetic diversity of bat rabies viruses in Brazil. Arch. Virol. 152 (11), 1995–2004.

Kuzmin, I.V., Rupprecht, C., 2007. Bat rabies. In: Jackson, A.C., Wunner, W.H. (Eds.), Rabies. Academic Press, San Diego, pp. 259–307.

Kuzmin, I.V., Niezgoda, M., Franka, R., Agwanda, B., Markotter, W., Beagley, J.C., Urazova, O.Y., Breiman, R.F., Rupprecht, C.E., 2008. Lagos bat virus in Kenya. J. Clin. Microbiol. 46, 1451–1461.

Lyles, D.S., Rupprecht, C.E., 2007. Rhabdoviridae. In: Knipe, D.M., Griffin, D.E., Lamb, R.A., Strauss, S.E., et al. (Eds.), Fields Virology. Lipincott, Philadelphia, pp. 1363–1408.

Mayen, F., 2003. Haematophagous bats in Brazil, their role in rabies transmission, impact on public health, livestock and alternatives to an indiscriminate reduction of population. J. Vet. B: Infect. Dis. Vet. Pub. Health 50, 469–472.

Moore, G.J., Raymond, G.H., 1970. Prolonged incubation period of rabies in naturally infected insectivorous bat, *Eptesicus fuscus* (Beauvois). J. Wildl. Dis. 6, 167–168.

Nadin-Davis, S.A., Huang, W., Armstrong, J., Casey, G.A., Bahloul, C., Tordo, N., Wandeler, A.I., 2001. Antigenic and genetic divergence of rabies viruses from bat species indigenous to Canada. Virus Res. 74, 139–156.

Nadin-Davis, S.A., 2007. Molecular epidemiology. In: Jackson, A.C., Wunner, W.H. (Eds.), Rabies. Academic Press, San Diego, pp. 69–122.

Oliveira, R.N., De Souza, S.P., Lobo, R.S.V., Castilho, J.G., Macedo, C.I., Carnieli Jr., P., Fahl, W.O., Achkar, S.M., Scheffer, K.C., Kotait, I., Carrieri, M.L., Brandão, P.E., 2010. Rabies virus in insectivorous bats: implications of the diversity of the nucleoprotein and glycoprotein genes for molecular epidemiology. Virology 405, 352–360.

Orr, R.T., 1950. Unusual behavior and occurrence of a hoary bat. J. Mammal. 31, 456–457.

Posadas, J., Crandall, K.A., 1988. Model test: testing the model of DNA substitution. Bioinformatics 14, 817–818.

Ramsden, R.O., Johnson, D.H., 1975. Studies on the oral infectivity of rabies virus in carnivore. J. Wildl. Dis. 11, 318–324.

Rupprecht, C.E., Glickman, L.T., Spencer, P.A., Wiktor, T.J., 1987. Epidemiology of rabies virus variants. Differentiation using monoclonal antibodies and discriminant analysis. Am. J. Epidemiol. 126, 298–309.

Schaefer, J.M., 1983. The Viability of Rabies in Carrion., http://digitalcommons.unl.edu/cgi/viewcontent.cgi?article=1287&context=gpwdcwp.

Scheffer, K.C., Carrieri, M.L., Albas, A., Santos, H.C.P., Kotait, I., Ito, F.H., 2007. Rabies virus in naturally infected bats in the state of São Paulo, Southeastern Brazil. Rev. Saúde. Publ. 41, 389–395.

Sims, R.A., Allen, R., Sulkin, S.E., 1963. Studies on the pathogenesis of rabies in insectivorous bats. III. Influence of the gravid state. J. Infect. Dis. 112 (1), 17–27.

Shoji, Y., Kobayashi, Y., Sato, G., Itou, T., Miura, Y., Mikami, T., Cunha, E.M., Sâmara, S.I., Carvalho, A.A., Nocitti, D.P., Ito, F.H., Kurane, I., Sakai, T., 2004. Genetic characterization of rabies viruses isolated from frugivorous bat (*Artibeus* spp.) in Brazil. J. Vet. Med. Sci. 66 (10), 1271–1273.

Smith, J.S., Orciari, L.A., Yager, P.A., 1995. Molecular epidemiology of rabies in the United States. Semin. Virol. 6, 387–400.
Soares, R.M., Bernardi, F., Sakamoto, S.M., Heinemann, M.B., Cortez, A., Alves, L.M., Meyer, A.D., Ito, F.H., Richtzenhain, L.J., 2002. A heminested polymerase chain reaction for the detection of Brazilian isolates from vampires bats and herbivores. Mem. Inst. Oswaldo Cruz 97 (1), 109–111.
Sodré, M.M., Gama, A.R., Almeida, M.F., 2010. Updated list of bat species positive for rabies in Brazil. Rev. Inst. Med. Trop. Sao Paulo 52, 71–81.
Steece, R.S., Calisher, C.H., 1989. Evidence for prenatal transfer of rabies virus in the Mexican free-tailed bat (*Tadarida brasiliensis mexicana*). J. Wildl. Dis. 25 (3), 329–334.
Souza, M.C., Nassar, A.F., Cortez, A., Sakai, T., Itou, T., Cunha, E.M., Richtzenhain, L.J., Ito, F.H., 2009. Experimental infection of vampire bats *Desmodus rotundus* (E. Geoffroy) maintained in captivity by feeding defibrinated blood added with rabies virus. Braz. J. Vet. Res. Anim. Sci. 46, 92–100.
Sulkin, S.E., Krutzsch, P.H., Allen, R., Wallis, C., 1960. Studies on the pathogenesis of rabies in insectivorous bats. I. Role of brown adipose tissue. J. Exp. Med. 112, 595–617.
Sulkin, S.E., 1962. Bat rabies: experimental demonstration of the 'reservoiring mechanism'. Am. J. Public Health 52, 489–498.
SVS/MS – Secretaria da Vigilância Sanitária do Ministério da Saúde, 2008. http://portal.saude.gov.be/portal/saude.
Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S., 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol., doi:10.1093/molbev/msr121.
Travassos da Rosa, E.S., Kotait, I., Barbosa, T.F.S., Carrieri, M.L., Brandão, P.E., Pinheiro, A.S., Bergot, A.L., Wada, M.Y., Oliveira, R.C., Grisard, E.C., Ferreira, M., da Silva Lima, R.J., Montebello, L., Medeiros, D.B.A., Souza, R.C.M., Bensabath, G., Carmo, E.H., Vasconcelos, P.F.C., 2006. Bat transmitted human rabies outbreaks, Brazilian Amazon. Emerg. Infect. Dis. 12, 1197–1202.
Vellasco-Villa, A., Orciari, L.A., Juarez-Islas, V., Gómez-Sierra, M., Padilla-Medina, I., Flisser, A., Souza, V., Castillo, A., Franka, R., Escalante-Mañe, M., Sauri-Gonzáles, I., Rupprecht, C.E., 2006. Molecular diversity of rabies virus associated with bats in Mexico and other countries of the Americas. J. Clin. Microbiol. 44, 1697–1710.
Villa, B.R., Alvarez, B.A., Dominguez, C.C., 1963. Presencia y persistencia del virus de la rabia en la glandula inter-escapular de algunos murciélagos mexicanos. Ciência 22 (5), 137–140.
Vos, A., Kaipf, I., Dezinger, A., Fooks, A.R., Johnson, N., Muller, T., 2007. European bat lyssavirus – an ecological enigma. Acta Chiropt. 9, 283–296.
Wang, Z.W., Sarmento, L., Wang, Y.X.-q., Dhingra, V., Tseggai, T., Jiang, B., Fu, Z.F., 2005. Attenuated rabies virus activates, while pathogenic rabies virus evades, the host innate immune response in the central nervous system. Virology 79, 12554–21256.
World Health Organization, 1992. Expert Committee on Rabies. Eight Report. WHO Technical Report Series No. 824. World Health Organization, Geneva.
World Health Organization, 2005. Expert Consultation on Rabies. First Report WHO Technical Report Series No. 931. World Health Organization, Geneva.
World Health Organization, 2007. Rabies vaccines, WHO position paper. Wkly. Epidemiol. Rec. 82, 425–435.
Wunner, W.H., 2007. Rabies virus. In: Jackson, A.C., Wunner, W.H. (Eds.), Rabies. Academic Press, San Diego, pp. 23–68.