

Rabies surveillance in bats in Northwestern State of São Paulo

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ABSTRACT

Introduction: Rabies is an important zoonosis that occurs in mammals, with bats acting as *Lyssavirus* reservoirs in urban, rural and natural areas. Rabies cases in bats have been recorded primarily in urban areas in Northwestern State of São Paulo since 1998. This study investigated the circulation of rabies virus by seeking to identify the virus in the brain in several species of bats in this region and by measuring rabies-virus neutralizing antibody levels in the hematophagous bat *Desmodus rotundus*. **Methods:** From 2008 to 2012, 1,490 bat brain samples were sent to the *Universidade Estadual Paulista* (UNESP) Rabies Laboratory in Araçatuba, and 125 serum samples from vampire bats that were captured in this geographical region were analyzed. **Results:** Rabies virus was detected in the brains of 26 (2%) of 1,314 non-hematophagous bats using the fluorescent antibody test (FAT) and the mouse inoculation test (MIT). None of the 176 hematophagous bat samples were positive for rabies virus when a virus detection test was utilized. Out of 125 vampire bat serum samples, 9 (7%) had levels of rabies virus neutralization antibodies (RVNAs) that were higher than 0.5IU/mL; 65% (81/125) had titers between 0.10IU/mL and 0.5IU/mL; and 28% (35/125) were negative for RVNAs using the simplified fluorescent inhibition microtest (SFIMT) in BHK21 cells. The observed positivity rate (1.7%) was higher than the average positivity rate of 1.3% that was previously found in this region. **Conclusions:** The high percentage of vampire bats with neutralizing antibodies suggests that recent rabies virus exposure has occurred, indicating the necessity of surveillance measures in nearby regions that are at risk to avoid diffusion of the rabies virus and possible rabies occurrences.

Keywords: Rabies virus. Antibodies. Viral detection. *Desmodus rotundus*. Non-hematophagous bats.

INTRODUCTION

Rabies encephalitis is almost always fatal; it is caused by a virus of the *Lyssavirus* genus, and all mammalian species are susceptible^{1,2}. Although it is described as one of the most ancient diseases³, it was only in 1911 that the role of bats as reservoirs and transmitters of rabies in Brazil was first considered⁴. In a study conducted in Brazil in 1931, rabies was diagnosed in hematophagous bats, and the epidemiological importance of these species in disease transmission, especially to herbivores, was recognized⁵.

Bats are the second largest group of mammals, with more than 1,300 species. Among the 178 species that occur in Brazil⁶,

41 have already been found to be infected with the rabies virus (RABV) species of *Lyssavirus*⁷.

Recently, rabies virus infections in bats have become more evident because of the success of measures that have been taken to control rabies in dogs in Brazil. Bats have been responsible for 70% of the human rabies cases over the past decade (2004-2013), followed by dogs, which caused 22% of the human cases^{8,9}.

In Latin America, bats were responsible for 727 known human rabies cases between 1990 and 2013; 243 of these cases occurred in the past ten years (2004-2013). Of these 243 cases, 91.4% were caused by hematophagous bats and 8.6% were caused by either non-hematophagous or non-identified species⁸. The high prevalence of bats as transmitters of rabies to humans is primarily a consequence of outbreak reporting in the States of Pará and Maranhão in Brazil in 2004 and 2005⁸.

From 2001 to July 2013, 1,854 cases of RABV infection in bats were recorded in Brazil. Among these cases, 1,201 (64.8%) were in non-hematophagous species, 327 (17.6%) were in hematophagous species and 326 (17.6%) were in non-identified species^{8,10}. Until 2009, the majority of bat cases were reported

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Received 15 August 2014

Accepted 30 October 2014

in the State of São Paulo⁷, most of them in the Northwest region; among these cases, 50 cases were detected in non-hematophagous species between 1998 and 2007, corresponding to a 1.2% infection positivity rate¹¹.

Bats become infected with RABV and develop rabies primarily through bites resulting from interactions with individuals of the same or different species¹², and rabies infections usually lead to the deaths of infected animals. In hematophagous bats, the disease is identified only when a high percentage of the population is already infected, suggesting that a portion of the animals can be infected without clinical signs¹³ while developing an antibody response¹⁴. Thus, seroprevalence studies can provide valuable information about rabies virus circulation, with the detection of anti-RABV antibodies indicating that seropositive animals were exposed to the virus and that there may have been a recent disease outbreak in the colony^{12,15}.

The aim of this work was to investigate rabies virus circulation in bats in Northwestern State of São Paulo by seeking to identify the virus in the brain in several bat species and by measuring rabies virus neutralization antibodies (RVNAs) in the hematophagous bat *Desmodus rotundus*.

METHODS

Bat samples for serology and virus detection

Sera and brain tissue samples from the common vampire bat, *Desmodus rotundus*, were obtained from January 2009 to July 2012 from shelters in the Araçatuba region of Northwestern São Paulo. The shelters were identified using data from the Center for Hematophagous Bats Control of Penápolis (SP) of the Office of Agriculture Defense [*Escritório de Defesa Agropecuária de Araçatuba* (EDA)].

Ranches with cases of rabies in herbivores in the Cities of Gabriel Monteiro and Guararapes were visited during the study period. Bat colonies previously identified by the Office of Agricultural Defense and farmers with a history of spoliation in their herds were also visited.

All of the activities in this study were officially authorized by the responsible offices (the Ministry of Environment [*Ministério do Meio Ambiente* (MMA)], the Brazilian Institute for the Environment and Renewable Natural Resources [*Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis* (IBAMA)] and the Chico Mendes Institute for the Conservation of Biodiversity [*Instituto Chico Mendes de Conservação da Biodiversidade* (ICMBio)]); protocol numbers 12.751-3/2009 and 27.346-1/2011).

Bats were captured using mist nets of different sizes (7m x 2m, 10m x 2m and 12m x 2m); the nets were placed inside or at the entrances of bat shelters. Dip nets were also used inside the shelters. In colonies with ten or more vampire bats, 20% of the bats were captured; blood was sampled from these bats under anesthesia with ketamine hydrochloride (Ketamina®), after which they were euthanized by cervical dislocation, and their brains were collected. All samples were shipped to the Rabies Laboratory at the *Universidade Estadual Paulista* (UNESP) in Araçatuba for virus detection.

The non-hematophagous bat samples were primarily from urban areas and were sent to the UNESP Rabies Laboratory in Araçatuba by the Zoonosis Control Centers and occasionally sent directly by the population from January 2008 through December 2012. Most of the bats, alive or dead, were found inside or outside houses or on sidewalks, and no information about clinical signs in the bats was available.

Rabies virus detection

Assays to detect the rabies virus were performed in the UNESP Rabies Laboratory in Araçatuba using the techniques recommended by the World Health Organization: the fluorescent antibody test (FAT)¹⁶ and the mouse inoculation test (MIT)¹⁷.

Rabies virus neutralizing antibody research

Blood samples with volumes of at least 500µL were obtained from *Desmodus rotundus* under anesthesia, by intracardiac puncture, and centrifuged. Serum samples were stored at -20°C and inactivated at 56°C for 1h before serial dilution in 96-well plates in minimal essential medium (MEM).

The simplified fluorescent inhibition microtest (SFIMT)¹⁸ was performed in the São Paulo Agency of Agribusiness Technology [*Agência Paulista de Tecnologia dos Agronegócios* (APTA)] Rabies Laboratory in Presidente Prudente. A 200-IU/mL national reference equine serum obtained from the Butantan Institute was used. The results of these tests were expressed in terms of the dilution of a test serum required to achieve a 50% decrease in the number of cells infected with the virus, i.e., the 50% virus neutralization titer.

The resulting titers were then expressed in International Units per mL (IU/mL) by comparing the test results with the results obtained using the reference serum (200IU/mL). The initial dilution of the test samples (1:5) corresponded to a 0.06IU/mL titer, and all samples that resulted in 50% virus neutralization at all dilutions were considered positive¹⁹.

Serum samples were categorized as non-reactive (%), negative (< 0.06IU/mL) and reactive with low (0.06IU/mL to 0.19IU/mL), medium (0.20IU/mL to 0.49IU/mL) and protective (\geq 0.50IU/mL) titers. RABV seroprevalence rate was calculated considering titers \geq 0.50IU/mL to be seroprotective, as previously described^{12,14}.

Ethical considerations

All sampling and laboratory procedures were performed in accordance with the ethical principles of the Brazilian Committee on Animal Experimentation [*Colégio Brasileiro de Experimentação Animal* (COBEA)], (protocol number 00858-2012).

RESULTS

A total of 1,490 bat samples were analyzed. Of these, 1,314 (88.2%) were from non-hematophagous species and 176 (11.8%) were from *Desmodus rotundus*, which is the principal hematophagous species found in Northwestern São Paulo. All vampire bat brains were negative for the rabies virus, according to the FAT and MIT results. Among the samples from

non-hematophagous species, 26 (2%) brains were positive, the majority of which were from 2009 and 2010 (nine cases per year in both of these years; **Table 1**). Overall, 1.7% of all the samples analyzed were positive for the rabies virus.

Insectivorous bats from the Molossidae (38.5%) and Vespertilionidae (50%) families represented 88.5% (23/26) of the total positive cases among non-hematophagous bats. Among the rabies virus-positive bats, 43.5% (10/23) were of the genus *Molossus*, with a predominance of *Molossus rufus*; 39.1% (9/23) were of the genus *Eptesicus*, with a predominance of *Eptesicus furinalis*, and 17.4% (4/23) were of the species *Myotis nigricans*. Only 11.5% (3/26) of the RABV-positive bats were of the frugivorous species *Artibeus lituratus* (Phyllostomidae; **Table 2**).

Evidence of rabies virus infection was found in bats from six cities in the Northwestern region (**Figure 1**). The most cases were recorded in Penápolis (12), followed by Araçatuba (5), Guararapes (4), Birigui (3), Pereira Barreto (1) and Coroados (1). In Guararapes, rabies was found in bats (September and October 2009) and in one head of cattle (April/2009), whereas in Gabriel Monteiro, rabies was only found in two heads of cattle (May and June/2011).

Serum samples from 125 (71%) of the 176 hematophagous bats captured in the region were submitted to simplified

fluorescent inhibition microtest (SFIMT). From this total, 86 were from a roost in a tree hollow in Araçatuba, 33 were from a tree in Gabriel Monteiro, four were from a tunnel on Marechal Rondon Road in Rubiácea, and two were from an abandoned shed on a farm in Guararapes.

According to the criteria defined above, 72% (90/125) of the serum samples were positive, and of these, 39% (35/90) were from males and 61% (55/90) were from females. Low and medium titers (0.10IU/mL to 0.50IU/mL) were observed in 90% (81/90) of the bats, and titers ≥ 0.50 IU/mL were observed in 10% (9/90), which translates to a seroprevalence of 7.2% (9/125; **Figure 2**). Only three samples with titers ≥ 0.50 IU/mL were from bats captured in shelters close to properties with confirmed bovine rabies cases; these bats were captured in Guararapes (1), Rubiácea (1) and Gabriel Monteiro (1). The six remaining samples were from animals captured in a tree in Araçatuba, a city without any recorded bovine rabies cases (**Figure 1**).

DISCUSSION

In Northwestern São Paulo, clinical rabies infections or evidence of RABV infection in bats was identified and reported only in non-hematophagous species, which represented

TABLE 1 - Detection of rabies virus in bats in Northwestern State of São Paulo, Brazil, from 2008 to 2012.

Year	Feeding habits						Positivity (% of total)
	non-hematophagous			hematophagous (<i>Desmodus rotundus</i>)			
	positive	negative	total	positive	negative	total	
2008	1	237	238	0	0	0	0.4
2009	9	363	372	0	16	16	2.3
2010	9	349	358	0	37	37	2.3
2011	1	113	114	0	71	71	0.5
2012	6	226	232	0	52	52	2.1
Total	26	1,288	1,314	0	176	176	1.7

TABLE 2 - Distribution of rabies virus-positive bats according to species and feeding habits in Northwestern State of São Paulo, Brazil, from 2008 to 2012.

Family/species	Feeding habits	Positive
Phyllostomidae	<i>Artibeus lituratus</i>	3
Molossidae	<i>Molossus molossus</i>	3
	<i>Molossus rufus</i>	7
Vespertilionidae	<i>Eptesicus diminutus</i>	2
	<i>Eptesicus furinalis</i>	7
	<i>Myotis nigricans</i>	4
Total		26

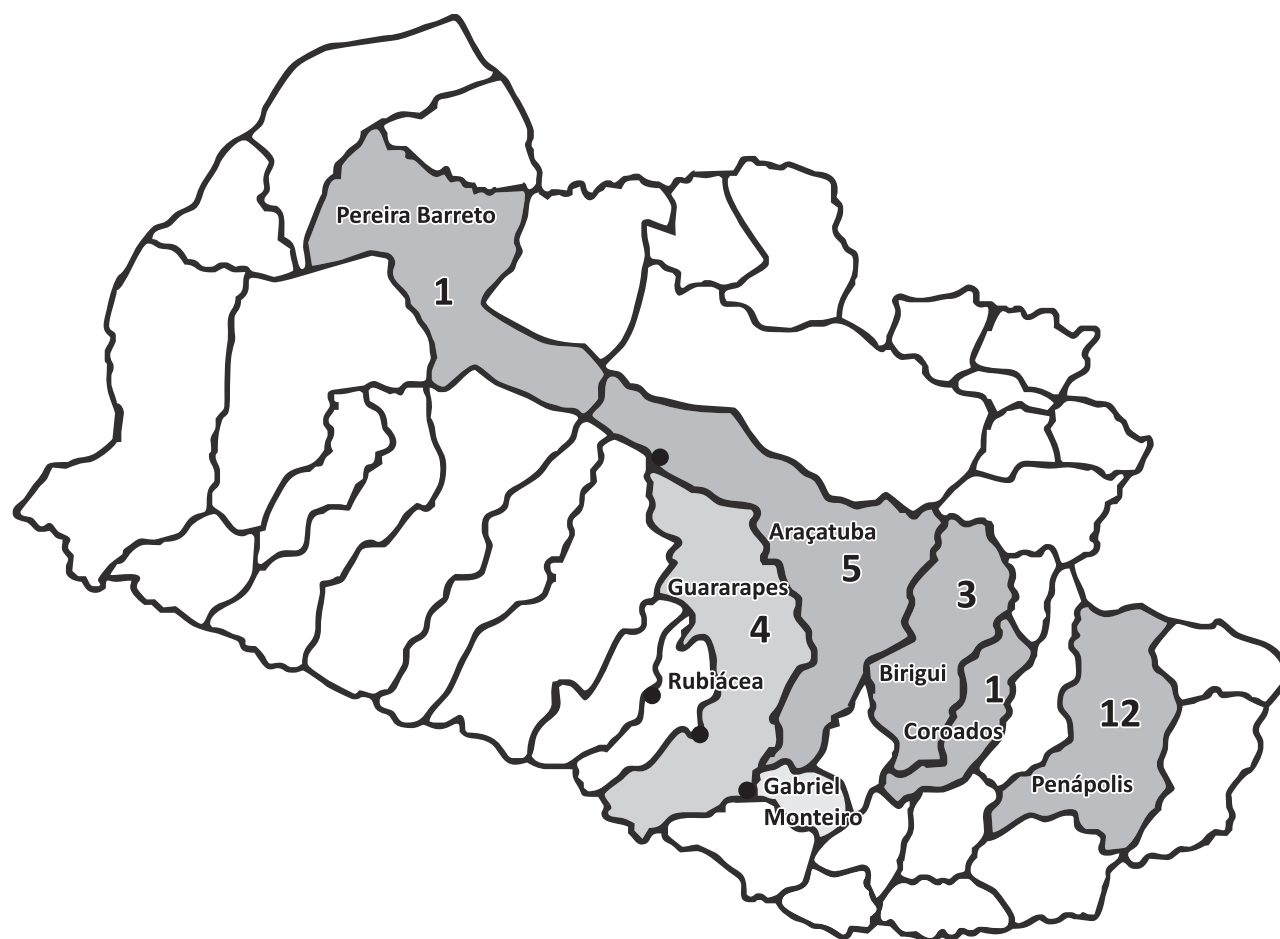


FIGURE 1 - A map of the Araçatuba region showing the counties in which only bat cases of rabies were diagnosed (dark grey), both bat and bovine cases (medium grey) and only bovine cases (light grey). The numbers indicate the numbers of bat cases, and the dots indicate the locations of shelters in which bats with rabies virus neutralizing antibodies were found.

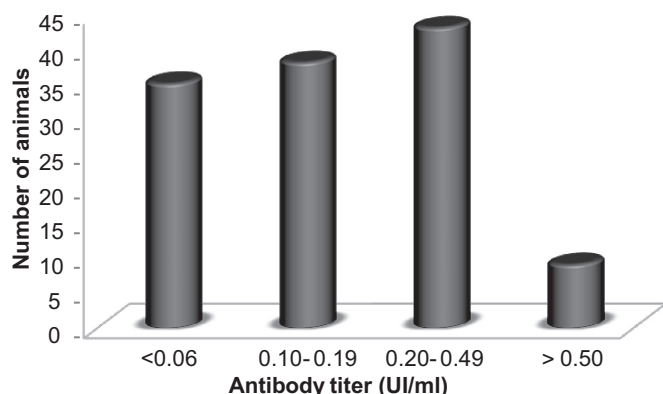


FIGURE 2 - The distribution of rabies virus neutralizing antibodies titers in *Desmodus rotundus*. The numbers of bats with negative (≤ 0.06 IU/mL), low (0.10 to 0.19 IU/mL), medium (0.20 to 0.49 IU/mL) and protective titers (≥ 0.50 IU/mL) are shown.

88% of the bats sent for diagnosis from 2008 to 2012. However, bovine cases were also recorded in the region. This pattern of RABV detection has been observed since 1998 in the Northwestern region^{11,20,21}, where rabies in bats, cattle, dogs and cats was diagnosed in relation to vampire bat variants. However, in these previous studies, the virus was not isolated from the brains of this bat species.

The difficulty of finding hematophagous bats with clinical signs of rabies and/or RABV brain infections has been reported in other studies, even in regions considered epidemic for rabies in cattle. Sugay and Nilsson²² investigated the prevalence of rabies virus in the Vale do Paraíba region, SP, and reported that only 3.8% of bats were positive for RABV in an epidemic region; this prevalence was considered to be low, given the number of bovine cases in the area. Souza et al.¹⁹ observed a similarly low prevalence of RABV in the Vale do Paraíba region; only 5.1% (7/138) of samples from hematophagous bats captured in caves, all from the same capture site, were positive for RABV.

Rabies virus circulation or the risk of rabies occurrence in vampire bats is usually monitored by active surveillance, i.e., by capturing bats in shelters. The prevalence rate in those cases is usually lower than 1%, considering that apparently healthy

bats are monitored²³. Information about the confirmed *Desmodus rotundus* rabies cases describes the bats feeding during the day or being found dead in places with cattle or in caves with a large concentration of individuals, in areas with high numbers of bovine cases^{19,22}. Only one case of a *Desmodus rotundus* bat positive for rabies in an urban area has been described in the literature; this bat was found in Ubatuba, State of São Paulo²⁴.

The circulation of rabies virus in non-hematophagous bats was investigated in the present study by passive surveillance in urban areas, and 1.7% (26/1,490) of the samples that were collected were found to be RABV-positive. This detection rate was greater than the 1.3% (98/7393) reported by Cunha et al.²⁰ from 1997 to 2002 for Northwestern State of São Paulo. Considering only the Araçatuba region, the positivity rate found in the present study exceeded the 1.2% (50/4035) found for samples collected from 1993 to 2007¹¹ and the 1.1% found in Campo Grande, State of Mato Grosso do Sul from February to December 2001²⁵. In the present study, the highest positivity rate, 2.3% (9/388) in 2009, did not reach the maximum positivity rate of 3.3% (10/304) recorded in this geographical region, in 2001¹¹.

Variations between years are observed when these percentages are compared with those observed for other regions in State of São Paulo. The positivity rate increased in the metropolitan São Paulo region from 0.7% (1988-1992)²⁶ to 0.8% (1993-2003)¹¹, reaching 2.2% and 1.4% in 2002 and 2003, respectively. However, in other studies, the rabies virus was not detected in bats from urban areas, including Japurá, Paraná²⁷ and municipalities in Roraima²⁸. Positivity rates ranging from 0 to 0.9% in the same geographical area have also been observed, for example in the Botucatu region, SP^{14,29,30}.

In the present study, 11.5% of rabies virus cases detected during the study period (2008-2012) were in the frugivorous species *Artibeus lituratus*. This percentage represented a decrease from 30% of rabies virus cases being detected in these bats in the period from 1998 to 2007¹¹. The positivity rate in insectivorous bats from the Vespertilionidae family remained unchanged at 50% (13/26). However, *E. furinalis* was the predominant species affected in this family, rather than *M. nigricans*, as was observed from 1998 to 2007. The positivity rate for bats of the Molossidae family increased from 20% (1998 to 2007) to 38.5% (2008 to 2012), whereas *M. rufus* was the predominant RABV-positive species in both periods. Five of the seven positive *E. furinalis* cases came from the same shelter in Penapolis, with three- to four-month intervals between cases.

All bats with RVNA titers $\geq 0.10\text{IU/mL}$ were considered to have reactive titers (the lowest titer for which fluorescence inhibition was observed), in contrast with titers $\geq 0.50\text{IU/mL}$, which are required for a sample to be considered seropositive. This criterion for seropositivity of a titer $\geq 0.50\text{IU/mL}$ was also adopted in a serological surveillance of terrestrial wild mammals³¹. The positivity rate in our study was 72%; seropositivity indicates a previous contact with RABV in the colony or a recent disease outbreak^{15,31}, even though no ill animals were observed. Similar situations have been described in the literature by Souza et al.¹⁹ for Vale do Ribeira, which had a 6.7% positivity rate, and by Langoni et al.¹⁴ in Botucatu, in which 65.2% of all bats examined had reactive antibody titers.

RABV seroprevalence was 7.2% in this study, if only those bats with titers $\geq 0.50\text{IU/mL}$ were considered to be seropositive, as has been reported by some authors. This result is similar to the 7.4% positivity rate found among hematophagous bats¹⁴ in Botucatu, State of São Paulo, and higher than the 5.9% seroprevalence rate observed in the metropolitan São Paulo region for bats of a variety of species, including hematophagous bats¹².

Serological studies have been conducted in bats and terrestrial wild species in Brazil and other countries as a means of determining the risk of rabies occurrence and the need for RABV surveillance^{14,31-35}. All of these studies detected the presence of RVNAs; although the OIE recommends the standardization of serological techniques for the detection of RVNAs³⁶, seroprevalence studies conducted using a variety of methods are still important tools for investigating viral activity levels in wild populations.

No virus was detected in the *Desmodus rotundus* samples, and the rabies risk in herbivores in Northwestern São Paulo varied from medium to negligible, according to the municipality³⁷. However, the high prevalence of RVNA-reactive bats (72%) and the substantial seroprevalence (7.2%) of RVNAs in vampire bats from shelters close to sites with bovine rabies cases indicate that rabies virus infections occur in *Desmodus rotundus* in the Araçatuba region of Northwestern State of São Paulo.

The detection of RABV in non-hematophagous bats in urban areas demonstrates the roles and importance of these species as transmitters of RABV. The presence of vampire bats with RVNAs indicates recent exposure to RABV antigens or to the virus itself, confirming that even in the absence of virus detection, seroprevalence studies can provide important epidemiological data on rabies infections in bats and can generate valuable RABV surveillance data for the affected species.

ACKNOWLEDGMENTS

To Dr. Vladimir de Souza Nogueira Filho, Coordinator of the State Program of herbivores rabies control (EDA Bauru - CDA-SAA / SP) and vampire bat control teams of CDA for providing the data on shelters of bats registered in Northwestern São Paulo.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

FINANCIAL SUPPORT

National Council for Scientific and Technological Development [Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq)] - Proc. 578281/2008-2; São Paulo Research Foundation [Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP)] - Proc 2008 / 00976-0 and Coordination of Improvement of Higher Education Personnel [Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES)] for master scholarship to Daiene KACasagrande.

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