




# Rabies Virus Detection in the Common Vampire Bat *Desmodus Rotundus*, Ceará, Brazil

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## Abstract

The common vampire bat, *Desmodus rotundus*, feeds mainly on the blood of mammals and plays a significant role in the life cycle of rabies; this species wreaks havoc on livestock and can affect other domestic animals, including humans. Owing to its role in transmitting rabies, *D. rotundus* has been the focus of studies elucidating the epidemiology of the rabies virus. This study aimed to examine the distribution of the rabies virus and understand its immunological dynamics in *D. rotundus*. One hundred and thirty-four samples were collected from the municipalities of Potiretama, Tauá, and Granja, where cases of rabies in herbivores were confirmed. Only one of the bats diagnosed by Direct Immunofluorescence and Mouse Inoculation tests tested positive by RT-PCR. It seems that *D. rotundus* can limit immune responses, thereby preventing rabies immunopathology.

## Keywords

*Desmodus Rotundus*, Direct Immunofluorescence, Immunological Dynamics, Mouse Inoculation, Rabies

## 1. Introduction

Chiroptera species, a notable order of mammals, possess a remarkable ability to carry pathogens without falling ill for extended periods. Their ability to fly long distances

makes them potential vectors for many diseases. However, little is known about the diseases associated with them, which they succumb to, or reasons for their apparent resistance to numerous pathogens [1]. With over 1400 species, bats comprise the second-largest order (Chiroptera) of mammals, and are the only mammals that execute true self-powered flight [2]. The order Chiroptera includes the vampire bats from the family Phyllostomidae and the subfamily Desmodontinae. These bats consist of three Neotropical species only found in the New World [2]-[5].

The common vampire bat *D. rotundus* is the reservoir for rabies in both humans and livestock [6]. This species has thrived due to environment alteration resulting from increased livestock activity [7]-[9], with implications for rabies transmission [9], which is regarded as one of the key productive activities that have herbivores inevitably exposed to this species and the rabies virus [10]. In 2004 and 2005, vampire bats were the main transmitters of human rabies in Latin America, and Brazil was the country with the highest number of cases—64 humans were infected with the rabies virus by vampire bats [11].

Although not the most represented mammalian order among zoonotic hosts, bats host more zoonotic viruses per species than rodents. Many resulting zoonoses have been high-profile spillover incidents of extreme pathogenicity [12].

Bats, distinct from other mammals, are often recognized for their ability to fly, but are also highly gregarious. This evolution has beneficial consequences for their lifespan and immunological functioning without the manifestation of noticeable disease [13]. Moreover, they act as significant hosts, transmitting approximately 200 different types of viruses, including the rabies virus and potentially harmful bacteria [14] [15]. The role of bats as reservoirs for emerging infectious diseases has increasingly gained recognition [16].

Rabies virus in naturally infected Chiroptera occurs more often in insectivorous bats than in other bat species, including *D. rotundus* [7].

During 2008 and 2009, rabies outbreaks took place in the municipality of Potiretama. This required intervention from the Rabies Surveillance and Control Program (RSCP) of the Health Department of the State of Ceará. Their key objectives were preventing cases of human rabies originating from the wild cycle and monitoring risk factors for the occurrence of rural rabies. As an outcome of their routine work, they discovered only one *D. rotundus* bat roost. They investigated eighteen locations, three of which had deceased animals (cattle, horses, sheep, goats, and lambs) presenting signs of rabies. They captured fifty-two *D. rotundus* bats for rabies diagnosis, with all results returning negative. In 2021 and 2022, rabies outbreaks took place as a result of rural rabies in the municipalities of Tauá and Granja, respectively, requiring interventions from the RSCP, with all rabies diagnosis results returning negative. Rabies outbreaks took place in both municipalities for several years.

A study on the isolation of the rabies virus involved six thousand three hundred and eighty-nine bats. Out of those, three hundred and eleven (5.6%) were of the hematophagous species *D. rotundus*, with none of the specimens diagnosed with

the rabies virus in the study area during the specified period. The families with the highest number of instances were insectivorous Vespertilionidae (37/0.57%) and Molossidae (21/0.32%), followed by the Phyllostomidae (18/0.28%) [17].

In their studies [18] [19], it was cited that epidemiological investigations of rabies in wild animals demonstrated how the rabies virus can specifically adapt and transmit to a certain species, becoming less capable of infecting other species. This host-parasite relationship is referred to as the compartmentalization of the rabies virus. Some authors suggest that this compartmentalization exists when the rabies virus is present in a certain bat species and does not exhibit characteristics similar to those of viruses isolated from other bat species.

## 2. Materials and Methods

This quantitative and descriptive study is based on laboratory diagnoses. First, municipalities with occurrences of epizootics caused by *D. rotundus* were chosen, and highlighted roosts held responsible for the occurrence of epizootics were worked on. Specimens of *D. rotundus* were captured and subjected to rabies laboratory diagnosis, and then analysis of the obtained data was performed.

### 2.1. Study Area

The animals were sampled in three municipalities of great epidemiological importance for rabies in the public health and livestock fields in natural roosts in rural areas: Potiretama (5°43'26'' S, 38°09'22'' W), Tauá (6°00'11'' S, 40°17'34'' W), and Granja (3°07'13'' S, 40°49'34'' W). These areas are located to the east, southwest, and northwest of the State of Ceará, respectively. All three municipalities experience a hot semiarid tropical climate with temperature variations between 26°C and 28°C. They also feature similar vegetation types, with open shrubby Caatinga vegetation in Potiretama and Tauá, and Cerrado vegetation in Granja. However, they differ in annual rainfall rates: 790.4, 597.2, and 1039.9 mm, respectively [20].

### 2.2. Ethics Committee

Field procedures commenced only after analysis and authorization from the Ethics Committee for the Use of Animals (ECUA) N° 5495335/2017 and the Biodiversity Authorization and Information System (BAIS) N° 82878/2021.

### 2.3. The Animals

Bats were taxonomically classified visually during captures, and only *D. rotundus* were selected for further analysis. The number of samples from the roosts in the three municipalities was small, and a total of one hundred and thirty-four bats from the hematophagous species *D. rotundus* were captured, adhering to the inclusion criteria for males and females [21]. Reproductive status was determined through visual verification and was divided into categories: scrotal males (young with no visible testicles in the scrotal sac and adult ones with visible testicles in

the scrotal sac) and innate females (young and adult females with normal abdomens and undeveloped breasts). No material was extracted from pregnant females (adult females with a detectable foetus upon abdominal palpation) and lactating females (adult females with fully developed breasts) [22] [23], in order to prevent compromising the species population.

According to [24], colonies of *D. rotundus* are usually small and contain ten to fifty specimens, with a great number of females, which justifies the number of bats captured in the three municipalities. However, groups with one hundred or more bats can occur mainly in regions where control of their populations is not carried out regularly.

#### **2.4. Field Procedures**

Bat captures were conducted specifically for this study in roosts that were worked on during rabies outbreaks near livestock. Capture sessions started minutes before dusk and lasted a few hours until dawn, using mist nets measuring  $7 \times 2.5$  m opened at ground level at the entrances to the roosts for five consecutive nights every six months in each municipality from July 2018 to June 2022. After captures, the bats were housed in metal cages measuring  $40 \times 30 \times 25$  cm, with a maximum of twenty animals per cage until the following morning, when sample collection began. This research did not receive any specific grants from public, commercial, or not-for-profit funding agencies.

#### **2.5. Anesthesia and Euthanasia**

The captured bats were anesthetized and euthanized using a fast-acting inhalation anesthetic, isoflurane 2-chloro-2-(dif)-1,1-trifluoro-ethane at a concentration  $> 1$  MAC (Minimum Alveolar Concentration). This method was suggested for small mammals, according to Resolution N<sup>o</sup> 714, dated June 20, 2022, as proposed by the Federal Council of Veterinary Medicine. The animals were placed in a purpose-chamber, ensuring uniform distribution of the anesthetic. This quick-acting concentration facilitated anesthesia and euthanasia via dose-dependent cardiac and respiratory depression, also leading to increased hypotension, thereby sparing them from any suffering.

#### **2.6. Sample Collection**

The collection of neural material (brain) was performed by aspiration, using 170 mm polypropylene Pasteur pipette with a 3 mm diameter tip, possessing a 3 ml capacity, through the foramen magnum [25]. The head was dissected at the level of the atlanto-occipital joint, and the foramen magnum was then cleared with the help of a small anatomical forceps to remove the atlas vertebra. The pipette's tip was inserted through the foramen magnum to aspirate the brain material. This material was immediately deposited in Eppendorf tubes and refrigerated for later laboratory tests: Direct Immunofluorescence, Mouse Inoculation, and Nucleoprotein and Cytochrome Oxidase Amplification Assay.

## 2.7. Direct Immunofluorescence, Mouse Inoculation and Nucleoprotein and Cytochrome Oxidase Amplification Assay Tests

Direct Immunofluorescence (DIF) and Mouse Inoculation (MI) tests were conducted at the Central Public Health Laboratory of Ceará (LACEN) and the Laboratory of Viral Zoonoses (LVZ), which is part of the Department of Preventive Medicine of the Veterinary College at the University of São Paulo (USP). The aim was to determine whether the captured animals were infected with the rabies virus and whether natural protein antigens were present in their tissues.

When carrying out the DIF test, impressions of central nervous system fragments were placed on glass slides, and the samples were fixed in acetone for at least 30 min at  $-20^{\circ}\text{C}$ . After the fixation and drying processes, the samples were ringed with nail polish on slides that were previously marked with two circles to keep the conjugate in place. These samples were then incubated in a humid chamber for 30 min at  $37^{\circ}\text{C}$ . The slides were rinsed with buffered saline solution (pH between 7.2 and 7.5) and distilled water to prevent the formation of crystals. After additional drying, a drop of immersion oil was instilled for examination [26].

Mice were inoculated with 20% suspensions prepared using one gram of varying central nervous system fragments. This mixture was then macerated, mixed with 4 ml of virus diluents, and followed by centrifugation at 1000 rpm for 15 min, after which the supernatants were removed. The preparations were stored at 2 to  $8^{\circ}\text{C}$  for inoculation into the mice on the same day, primarily via the intracerebral (IC) route. The IC inoculations were administered to mice either 5-day-old (0.01 ml per animal) or 21-day-old mice weighing between 11 and 14 g (0.03 ml per animal). Documentation-wise, identification and reading sheets for the samples were created, with 8 to 10 mice being used per inoculation session. Daily readings continued for 30 days, considering that the samples were collected from hematophagous bats (wild animals). Notes detailing the list of deceased, untreated, and euthanized animals were maintained as well. Animals expiring beyond the fifth day of inoculation underwent the IFD test.

Disposable 1 ml syringes were primarily employed for inoculations, permitting dosages of 0.03 ml, and used needles of 13 mm  $\times$  4.5 mm (length  $\times$  thickness). The animal subjects were euthanized via cervical dislocation once tests were completed, adhering to Normative Resolution N<sup>o</sup> 37 issued on 02/15/2018 by the National Council for Control of Animal Experimentation (CONCEA) to maintain good laboratory practices [26].

The Laboratory of Viral Zoonoses (USP) also carried out molecular diagnosis for rabies virus detection using a Reverse Transcriptase Reaction followed by Polymerase Chain Reaction (RT-PCR) with specific primers for the gene coding the viral nucleoprotein in the single positive result.

Nucleic acid was extracted using the QIAquick<sup>TM</sup> Kit (QIAGEN, Valencia, CA, USA), adhering to the manufacturer's instructions. Positive and negative controls were established using rabies virus (RABV) samples derived from mouse brains

and nuclease-free water, respectively [27]. For the synthesis of the complementary cDNA strand, reverse transcriptase was employed, which was then followed by partial amplification of the gene encoding the N7-protein [28]. Three primers were utilized for amplifying the N gene: 21G, 504, and 24-304 [29], plus a pair of primers for amplifying Cytochrome Oxidase 114 (LCO-HCO) [30]. The PCR product was purified using the QIAquick Gel Extraction Kit (QIAGEN, Valencia, CA, USA), and gel-purified bands were acquired with a 1% agarose gel, and the QIAquick® Gel Extraction Kit was used according to the manufacturer's guidance. After purification, the DNA was quantified visually on a 2% agarose gel with a low-mass DNA ladder (Invitrogen-Carlsbad, CA, USA), in compliance with the manufacturer's instruction. After the electrophoresis purification reaction, sequencing was performed to determine the generated sequence. A comparison was then made in PubMed, and it was possible to note the similarity of approximately 100% with the samples commonly found in *D. rotundus*.

## 2.8. Data Analysis

For the data analysis, simple Prevalence (Prev) calculation with 95% Confidence Intervals (CI) and standard deviation (SD) with lower limit (LL) and upper limit (UP) was carried out, determining the proportion of *D. rotundus* specimens with positivity in the diagnosis of rabies.

## 3. Results

Only one male specimen for both Direct Immunofluorescence and Mouse Inoculation tests tested positive by RT-PCR, with a confidence interval between 4.0 and 24.4, meaning that there is 95% confidence in the low and true prevalence of rabies positivity in the studied population within this interval, aligning with the health conditions of male and female specimens analyzed in the two aforementioned reference laboratories (Table 1).

**Table 1.** Simple prevalence with 95% confidence intervals of *Desmodus rotundus* specimens positive for the diagnosis of rabies from the municipalities of Potiretama, Tauá, and Granja, Ceará.

Municipalities	N°	Males						Females					
		N°	+	Prev.	SD	CI		N°	+	Prev.	SD	CI	
						UL	LL					UL	LL
Potiretama	85	25	0	0	0	0	0	60	0	0	0	0	0
Granja	30	7	1	14,28	5,2	24,4	4,0	23	0	0	0	0	0
Tauá	19	2	0	0	0	0	0	17	0	0	0	0	0

## 4. Discussion

Leukocyte profile studies in Neotropical bats like *D. rotundus* state that environmental conditions can shape the host's immune defense, in addition to being important in understanding which wild populations may be more susceptible or re-

sistant to pathogens, significantly influencing epidemiology in preventing disease risk with anthropogenic disturbances and climate; in this study [6], both municipalities experienced the same climate and temperature variations and also feature similar vegetation types with low circulation of rabies virus, with few or no specimens positive for rabies. Epidemiologically, the results of this study are worrying, as bats have low or no circulation of viruses in colonies; they cause epizootics with high risks of transmitting the disease to humans, needing to reveal some particularities regarding the immunology of the species.

The diversity of viruses associated with bats has led many studies to focus on understanding how these animals can carry numerous viruses that are potentially pathogenic in other species without becoming ill [31] [32]. It is hypothesized that the ability to fly could be the key to explaining these animals' resistance to viruses and other pathogens [31].

During flight, metabolism increases, consequently raising the levels of free oxygen radicals. This, in turn, generates more molecules that damage DNA. To prevent unwanted inflammatory responses to damaged DNA, bats have evolved mechanisms to suppress inflammation [31].

The observation of the low prevalence of the rabies virus in the species *D. rotundus* has been consistent over the years. Studies have discovered a relatively low ratio of rabies virus isolation in *D. rotundus* bats. Indeed, merely 11 (2.21%) out of 496. The specimens studied were from areas where rabies was present in the State of São Paulo [33].

Bats are well equipped to control viral infections through mechanisms that limit inflammation and, consequently, the incidental damage that these responses might cause in their bodies [31] [34]. Not only have mutations been observed, but also suppression of expression and low activity of molecules involved in inflammatory responses in bats. This allows their immune system to manage viruses without triggering an overblown inflammatory response, which could result in tissue damage and deteriorating health conditions. This may be a critical mechanism that explains the longevity and status of bats as virus reservoirs [2] [31] [34].

The mechanisms of inflammatory limitations in bats are primarily related to viral pattern recognition receptors and the initiation of signaling events. These result in the production of cytokines involved in viral evasion of the host's interferon (IFN) response [31] [35] [36].

Endosomal Toll-like Receptors (TLRs) 3, 7, 8 and 9 have, for the most part, evolved under similar functional constraints to those in other mammals. Among these, *D. rotundus* exhibits classical genetic characteristics. TLRs 3, 7, and 8 recognize viral RNA, while TLR 9 recognizes viral, bacterial, and protozoan DNA [37].

MicroRNA clusters that evolve rapidly seem to target genes involved in aging, virus-host interactions in bats, dampening inflammatory responses. This process limits both immunopathology and possibly energy expenditure. These genes include those that are active in antiviral immunity, the DNA damage response, apoptosis and autophagy. One example of this is found in the black flying fox

*Pteropus alecto*, which serves as a natural reservoir for the human pathogens *Hendra virus* and *Australian Bat Lyssavirus* [37].

The recent discovery of viral endogenous elements in animal genomes suggests that the immune system may be able to accept pathogens as intrinsic parts of its organism. However, this pathogen tolerance in bats is not universal, as severe morbidity and mortality in bats can result from infection by certain viral, bacterial, and fungal pathogens. In the majority of these cases, it is the host's immunopathological response rather than the pathogen itself that is primarily responsible for mortality. A possible major exception to this is rabies-related mortality, where the virus can cause direct pathology in the central nervous system while completely evading immune detection [12] [32].

In addition to the molecular patterns leveraged by the host to identify viral infections, there is evidence suggesting that bats have evolved adaptive intracellular mitochondria to alleviate the oxidative stress accumulated during metabolically intensive activities such as flying. Current research emphasizes the increasing recognition of mitochondria's crucial role in cellular signaling and defense. It is proposed that bats could control pathogenesis in microbe-invaded cells via autophagy and apoptosis processes, which initially evolved to manage metabolic stress, thereby evading immunopathological consequences. However, these control mechanisms are restricted to intracellular pathways, rendering bats susceptible to the immunopathological ramifications of attempted extracellular infections [12].

## 5. Conclusion

Although significant circulation of rabies virus has not been observed among *D. rotundus* in the municipalities that have experienced rabies epizootics with animal deaths due to spoliation by vampire bats, and a low number of specimens testing positive for rabies leads us to believe that bats have beneficial consequences for their lifespan and immunological functioning without the manifestation of noticeable disease and that they may involve mechanisms to suppress inflammation in a rabies virus infection, according to reports in the scientific literature; therefore, this research may serve as an important tool to understand the maintenance and circulation of rabies virus within the *D. rotundus* species.

## Authors' Contributions

Francisco Bergson Pinheiro Moura: Conceptualization, Methodology, Data curation, Writing—original draft preparation.

Maria Fátima da Silva Teixeira and Bruno Marques Teixeira: Visualization, Supervision, Validation, Reviewing, and Editing.

Meylling Mayara Linhares Magalhães, Paulo Eduardo Brandão, Felipe Rodrigues Jorges, Washington Carlos Agostinho, and Sueli Akemi Taniwaki Miyagi: Performance of the laboratory diagnosis. Maria Mariza de Lima e Silva, Antonio Robério Soares Vieira, José Cleonardo da Costa Filho: Performance of field pro-

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## Conflicting of Interests

The authors declare no conflicts of interest.

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