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Major Article

Natural infection by *Trypanosoma cruzi* in triatomines and seropositivity for Chagas disease of dogs in rural areas of Rio Grande do Norte, Brazil

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Abstract

Introduction: Chagas disease is caused by the protozoa *Trypanosoma cruzi*. Its main reservoir is the domestic dog, especially in rural areas with favorable characteristics for vector establishment and proliferation. The aims of this study were to collect data, survey and map the fauna, and identify *T. cruzi* infection in triatomines, as well as to assess the presence of anti-*T. cruzi* antibodies in dogs in rural areas of the municipality of Mossoró, Brazil. **Methods:** An active entomologic research was conducted to identify adult specimens through an external morphology dichotomous key. The analysis of natural infection by *T. cruzi* in the insects was performed by isolation in culture and polymerase chain reaction. The antibody testing for *T. cruzi* in dogs was performed by enzyme-linked immunosorbent assay and indirect immunofluorescence assay. **Results:** A total of 68 triatomines were captured, predominantly the *Triatoma brasiliensis brasiliensis* (Neiva 1911) species. The vector mapping displayed areas with greater risk for parasite transmission. Of the examined triatomines (51 specimens), 41.2% (21/51) were positive on polymerase chain reaction, and all were negative on culture. In the serum testing, 11% (25/218) of dogs were seropositive, but no association was found between the serologic results and the presence and infection by *T. cruzi* in triatomines. **Conclusions:** This study demonstrated the movement of *T. cruzi* in the studied area, by the presence of vectors and naturally infected domestic reservoirs. The mapping of the studied rural area demonstrates the risk of disease transmission.

Keywords: Chagas disease. Vector. Domestic reservoir. Diagnosis. Zoonosis.

INTRODUCTION

Chagas disease (CD) is an anthropozoonosis caused by the protozoan *Trypanosoma cruzi* and is typically transmitted by triatomines, vectors of the Triatominae family (Hemiptera: Reduviidae), known as kissing bugs[1-3].

In Brazil, the triatomine species responsible for CD transmission show wide spatial distribution and invasive potential, by adapting to homes and surrounding areas[4]. Vector presence can also be facilitated by constant alterations to the natural environment, caused by anthropic activities, leading to imbalances in ecosystems and modification of the behavior of insect vectors and wild animals that then enter homes for shelter and food[5,6].

Domestic animals, especially dogs, have an important epidemiological role, because they serve as a link between domestic and wild cycles of *T. cruzi*[7,8]. Furthermore, they are an important disease sentinel, as their infection rates may reveal the potential of disease transmission to humans[9-12].

However, for a better comprehension of the epidemiology of CD, the whole cycle, including vectors, must be assessed. Therefore, identifying the vector species and infection type is crucial to understanding the biology and behavior and to evaluating the risk of disease transmission to humans and domestic animals[4,13]. An important tool used in epidemiological studies of vector-borne diseases is the mapping of the vector's spatial distribution, allowing for the prediction of *T. cruzi* transmissibility between triatomine species from spatial data[14].

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Thus, this study aimed to gather data on animals in a rural area of Northeastern Brazil, as well as to map and investigate natural infection by *T. cruzi* in triatomines. The study also aimed to detect the presence of anti-*T. cruzi* antibodies in dogs in order to evaluate a possible association between infected dogs and the presence of triatomines infected by *T. cruzi.* and to determine environment-related factors.

METHODS

Study area and sample definition

The research was conducted in the rural area of Mossoró, located in the countryside of Rio Grande do Norte, Northeast region of Brazil. According to the State Department of Public Health, this area is considered endemic, because it presents a favorable environment for the vector's survival, due to the proximity of forests and a city dump, as well as an accumulation of debris and organic matter from animal husbandry. A total of 11 rural areas with environmental indicators for the occurrence of triatomines and a history of vector capture between 2008 and 2012, according to the Municipal Health Surveillance Department, were used in this study.

Ethical considerations

All of the 392 residences in these areas were visited between March and July 2014, and informed consent forms were obtained from residents that agreed to participate.

The project was approved by the Ethics Committee on Animal Use of the Federal Rural University of the Semi-Arid (*Universidade Federal Rural do Semi-Árido*) (Ruling 62/2012 - Process 23091.002190/2012-75).

Active entomologic collection

The entomological collection consisted of a system of notification and collection by the residents of triatomines in their homes. Therefore, this method allowed for the monitoring of infestations by the local community.

Samples of different species of triatomines were presented to the residents to facilitate the recognition and capture of the insects, and they were given a polyethylene bottle containing holes in the lid to store the insects. After capture, the insects were sent to the Oswaldo Cruz Foundation Institute in Rio de Janeiro (FIOCRUZ-RJ), in a specific shipment for biological samples with a deadline of 24 hours, paid for by the institute. Upon receipt, the samples were immediately processed.

Vector identification

Properly packed triatomines were sent to the Laboratory of Leishmaniosis Transmitters of the Forensic Entomology Sector of the FIOCRUZ/RJ and identified by observing their external morphological characters, using the dichotomous key according to Lent & Wygodzinsky[15].

Detection of natural infection by *T. cruzi*

In order to detect *T. cruzi* infection, the intestinal contents of the insects were obtained by abdominal compression and processed for parasite isolation culture and polymerase chain reaction (PCR) techniques in the Laboratory of Immunomodulation and Protozoology of FIOCRUZ/RJ.

For the isolation, the obtained contents were seeded in tubes containing biphasic Novy-MacNeal-Nicolle + Liver Infusion Tryptose culture medium, supplemented with 10% fetal bovine serum and penicillin (10,000U/mL). The tubes were kept in an incubator (FANEM, model 347) at 27°C, and the cultures were examined weekly for four months by light microscopy using an Axioplan 2 (Zeiss®) light microscope to verify positivity.

To detect *T. cruzi* deoxyribonucleic acid (DNA), phenol-chloroform extraction was used to obtain DNA[16], which was then subjected to a PCR with specific primers for *T. cruzi* (5' ASTCGGCTGATCGTTTTCGA 3' and 5' AATTCCTCCAAGCAGCGGATA 3')[17]. The reaction was performed in a Step One Plus™ Real-Time PCR System, using a rapid protocol of 20 seconds at 95°C followed by 40 cycles of 3 seconds at 95°C and 30 seconds at 60°C. Ultrapure water was used as the negative control, and DNA obtained from axenic culture of the *T. cruzi* strain Y was used as the positive control. The amplified products were subjected to agarose gel electrophoresis at 1.5% and stained with GelRed™ (Biotium), using TBE 1x buffer as an electric conductor.

Anti-*T. cruzi* antibody evaluation in domestic dogs

Blood samples from 218 domestic dogs were collected and, after centrifugation, the serum samples were stored in tubes and packed at -20°C until the performance of the enzyme-linked immunosorbent assays (ELISA) and indirect immunofluorescence assays (IIF) at the Laboratory of Immunomodulation and Protozoology of FIOCRUZ/RJ.

For the anti-*T. cruzi* immunoglobulin G (IgG) antibody assay by IIF, immunofluorescent microscope slides with total *T. cruzi* antigen were produced in the Laboratory of Trypanosomatid Biology of FIOCRUZ/RJ. Previously tested positive canine serum and serum of dogs from non-endemic areas were used as positive and negative controls, respectively[18]. An anti-dog IgG (FITC, Sigma™) was used as the conjugate. Serums with titration ≤1:20 were considered positive[19].

A commercial kit produced by Bio-Manguinhos/FIOCRUZ and the anti-dog IgG conjugate (Peroxidase, Sigma™) were used for the ELISA. Samples were considered seropositive when they showed an optical density (OD) greater than the cut line (cut-off) obtained in each reaction.

As recommended by the World Health Organization for the diagnosis of human CD, samples were only considered positive when they reacted in both serological tests.

Data analysis

The data for the statistical analysis were obtained from the identification form of the dogs, the serology results of the dogs' blood samples, and the presence of natural infection by *T. cruzi* in triatomine vectors.

To validate the serological tests, IIF test results were compared with those of a standard test. The analysis of agreement data was based on the sensitivity and specificity, using the statistical program GraphPad Prism version 6.01. The ELISA technique was used as the gold standard[20,21].

Data were entered into an electronic spreadsheet and transferred to the statistical programs SPSS 21.0 (*Statistical Package for the Social Sciences*) and STATA (Stata Corp., College Station, Estados Unidos) version 13.0, expressed in simple frequency and percentage, as well as prevalence ratio (PR) and confidence interval of 95%, obtained by Poisson regression. A significance level of 5% was considered.

Mapping of triatomine capture areas

The spatial distribution of the triatomines was established from geographic coordinates (latitude and longitude) of the capture sites, using a Global Positioning System (GPS) - Garmin e Trex® in the Universal Transverse Mercator (UTM) coordinate system, using the WGS-84 (or SIRGAS 2000). The free software Quantum GIS 2.8.3 was used to prepare a thematic map of the study area.

RESULTS

The study involved 279 residences, as described in **Table 1**, and insects were captured in 23 of them. A total of 68 triatomines were acquired, involving 54 from inside the residences and 14 from the peridomiciliary areas. In the residences in the study, 135 of them had one or more dogs, and blood samples were obtained from all 218 dogs present.

The captured triatomines corresponded to 25 nymphs of undetermined stage, and 43 adults, identified as 69.7% (30/43) *Triatoma brasiliensis brasiliensis* (Neiva 1911), 23.2% (10/43) *Triatoma pseudomaculata* (Corrêa and Espínola 1964), 4.6% (2/43) *Rhodnius nasutus* (Stal 1895), and 2.3% (1/43) *Panstrongylus lutzi* (Neiva and Pinto 1923). The distribution of these species in the 11 rural localities is shown in **Table 1**. Of the 68 captured specimens, 51 (75%) were under suitable conditions to obtain intestinal contents for *T. cruzi* analysis by isolation culture and PCR, including 24 adult specimens of *T. b. brasilensis*, one of *T. pseudomaculata*, one of *R. nasutus,* and 25 nymphs. Attempts to isolate *T. cruzi* by culture were negative in all triatomines, but 41.1% (21/51) of the samples were positive on PCR.

The results of the serological testing for *T. cruzi* showed that 11% (25/218) of the dogs tested were seropositive for both tests (ELISA and IIF). The sensitivity of the test was 53.2%, and the specificity was 99.4%, with confidence intervals of 38.1 – 67.9 and 96.8 – 99.0, respectively.

There was no association between the serologic results of the dogs and the presence and infection by *T. cruzi* in triatomines. The same was observed for dog-related factors, such as gender, role, and the presence of shelter or other animals (**Table 2**).

Triatomines were present in eight of the eleven rural localities included in this study, as shown in **Figure 1**.

DISCUSSION

The transmission of CD is undoubtedly influenced by socio-cultural, political, economic, environmental, and historical factors[22], because the occurrence of this disease reflects how the population occupies and explores the environment in which it lives[23]. Studies have shown that information about the vectors of CD in endemic areas is lacking, leading to the inefficiency of intervention programs[24]. Control programs of these areas should focus on educational measures adapted to the local context, elucidating the importance of the capture of the vectors and notification of the authorities to control the disease[25].

The species found in this study are also distributed in other states of Brazil[4,26]; however, the Northeast Region of Brazil is considered the epicenter of dispersion of *T. brasiliensis* and *T. pseudomaculata*[27]. Geographical distribution of *R. nasutus* and *P. lutz* is restricted to certain regions of the Northeast region[28], and both species are considered secondary vectors for *T. cruzi* transmission. However, research in Colombia has shown the relevance of these species, demonstrating that 59.4% of the secondary vectors had humans as the main food source[29].

Studies have demonstrated that *T. brasiliensis* is the most important *T. cruzi* vector in Northeastern Brazil, with a high rate of infection[30,31]. This fact is attributed to the adaptability of this species to the hot and dry climate conditions of the Northeast region, where it only rains a few months out of the year[32]. This species of triatomine has high adaptability to a human habitat, corroborating the present study, which noted household and peridomiciliary presence of the same species. *T. brasiliensis* is classified as a domestic species due to the household presence of adults, nymphs, eggs, and molt[32]. This adaptability to the home area can also restrict its access to domestic and synanthropic animals as a food source[33]. Therefore, *T. cruzi* infection in these vectors may be crucial for the establishment of infection in humans and domestic animals.

Triatoma pseudomaculata is also important for the epidemiological surveillance of CD, since it's the most frequent species after *T. brasiliensis*[27,34]. This species often shelters itself in locations of the residence that receive more sunlight, such as the rooftop[30], and is well suited to the high temperatures observed in the study area[35].

Regarding the testing for the presence of *T. cruzi* in triatomines, the negative results in the culture isolation may be associated with absence of viable parasites in inoculated samples, which is linked to the handling difficulty and death of the insects[36], the contamination of the culture medium inhibiting the growth of the parasite, and the possibility that some strains of *T. cruzi* have a poor growth in culture[37]. However, infection of triatomines was confirmed by the detection of *T. cruzi* DNA by PCR in 41.17% of the samples. Therefore, we do not recommend the use of the isolation technique in culture to investigate the presence of *T. cruzi* in samples from the intestinal tract of insects.

The variation in the rate of infection by *T. cruzi* in this study may be associated with the dominant vector in each region, since each triatomine genera has a preference to certain natural or artificial ecotypes[30,38], which may make them more susceptible to infection. For example, Coutinho et al.[30] observed that there is greater natural infection by *T. cruzi* in triatomines housed in wood piles, because these sites function as burrows of small animals, such as rodents and marsupials, that are wild reservoirs of *T. cruzi*. These animals then have a high capacity to infect the vectors[38], while dogs, cats, and humans show much less infectivity to triatomines[39].

TABLE 1: *Trypanosoma cruzi* evaluation, identification of triatomines, and anti-*T. cruzi* antibody results in dogs from rural localities in the City of Mossoró, Rio Grande do Norte.

Rural locality/ Geographic coordinates	Total number of residences/ Number of infected residences/ Residences with infected triatomines	Triatomine species identified	Total number of dogs/ Number of residences with dogs/ Residences with seropositive dogs
Settlement *Sussuarana* 5º09'43.9''S 37º13'23.8"W	84/6/0	Nymph *T. brasiliensis* *T. pseudomaculata*	38/28/6
Sítio Umari 5°07'52.8''S 37°15'43.8''W	2/2/1	Nymph *T brasiliensis*	7/2/0
Sítio Sombra Grande 5º06'17.5''S 37º10'02.1''W	4/0/0	-	6/3/0
Settlement *Melancias* 5º11'52.6''S 37º14'09.7''W	6/4/1	Nymph *T. brasiliensis* *T. pseudomaculata* *Rhodnius nasutus*	6/4/0
Passagem de Oiticica 5º14'48.5''S 37º21'25.7''W	1/0/0	-	0
Settlement *Laginha* 5º15'14.2''S 37º21'33.7''W	1/0/0	-	3/1/0
Sítio Canto da Farinha 5º22'16.1''S 37º18'39.6''W	1/1/1	Nymph	2/1/0
Settlement *Lorena* 5º17'17.2''S 37º20'03.2''W	28/3/1	*T. brasiliensis*	27/17/1
Sítio Barbadinho 5º22'03.8''S 37º18'32.6''W	3/1/1	Nymph	4/3/0
Settlement *Espinheirinho* 5º26'15.0''S 37º11'58.8''W	43/3/1	Nymph *T. brasiliensis* *Panstrongylyus lutzi*	58/32/6
Settlement *Passagem de Pedra* 5º09'23.9''S 37º17'01.1''W	106/3/0	*T. brasiliensis*	67/44/12
Total	**279/23/6**		**118/135/25**

T.: Triatoma.

The present study utilized serologic tests for canine CD diagnosis because, although the primary infection with *T. cruzi* includes an initial phase with high parasitemia, this phase lasts a short time[21,40], whereas *T. cruzi* antibodies persist at detectable levels for a long time[39,41]. Nevertheless, no serologic test is currently considered the gold standard for CD, so the use of both ELISA and IIF for the diagnosis allows the obtainment of a reliable serology result[42]. The variation in the concordance between serologic tests may occur due to the incidence of reactivity between closely related trypanosomes, such as *Trypanosoma rangeli* and *Leishmania spp*.[12,43].

The seropositivity of dogs in this study (11.5%) was lower than that found in areas with the presence of triatomine vectors known to be infected, such Colombia, where 71.6% of dogs were seropositive through ELISA and IIF techniques, and the State of Ceará, Brazil, where Bezerra et al. found 38% seropositivity using the same tests[44]. However, similar results to this study were observed in Peru, where the seropositivity in dogs was 12.3% by ELISA and TESA-blot techniques, and the main vector for the parasite identified in the region was *Triatoma infestans*[45]. In the State of Piauí, Brazil, the seropositivity obtained by ELISA and IIF was 7.7%, with the species *T. brasiliensis* reported as

TABLE 2: Prevalence of the variables function, gender, presence of shelter, presence of other animals, and presence and infection by *T. cruzi* in triatomines with seropositive dogs for Chagas disease in rural areas.

Variable	Total number of dogs	Prevalence of seropositive dogs (%)	PR	95%CI
Vector presence				
yes	18	1 (5.6)	0.27	0.04 – 1.86
no	116	24 (20.7)	1	
Vector infected by *T. cruzi*				
yes	03	0 (0.0)	-	-
no	131	25 (19.1)	1	
Male dogs				
yes	104	18 (17.3)	0.74	0.34 – 1.61
no	30	07 (23.3)	1	
Dog function: companion				
yes	50	13 (26.0)	1.82	0.90 – 3.67
no	84	12 (14.3)	1	
Dogs function: guard				
yes	80	13 (16.3)	0.73	0.36 – 1.47
no	54	12 (22.2)	1	
Dogs function: hunting				
yes	21	4 (19.0)	1.02	0.39 – 2.68
no	113	21 (18.6)	1	
Own shelter				
yes	33	8 (24.2)	1.44	0.68 – 3.03
no	101	17 (16.8)	1	
Number of dogs in residence				
one	79	12 (15.2)	0.64	0.32 – 1.30
more than one	55	13 (23.6)	1	
Presence of production animals				
yes	69	13 (18.8)	1.02	0.50 – 2.07
no	65	12 (18.5)	1	
Presence of domestic animals				
yes	100	22 (22.0)	2.49	0.80 – 7.81
no	34	3 (8.8)	1	
Presence of wild animals				
yes	15	3 (20.0)	1.08	0.37 – 3.19
no	119	22 (18.5)	1	

PR: prevalence ratio; **95%CI**: 95% confidence interval.

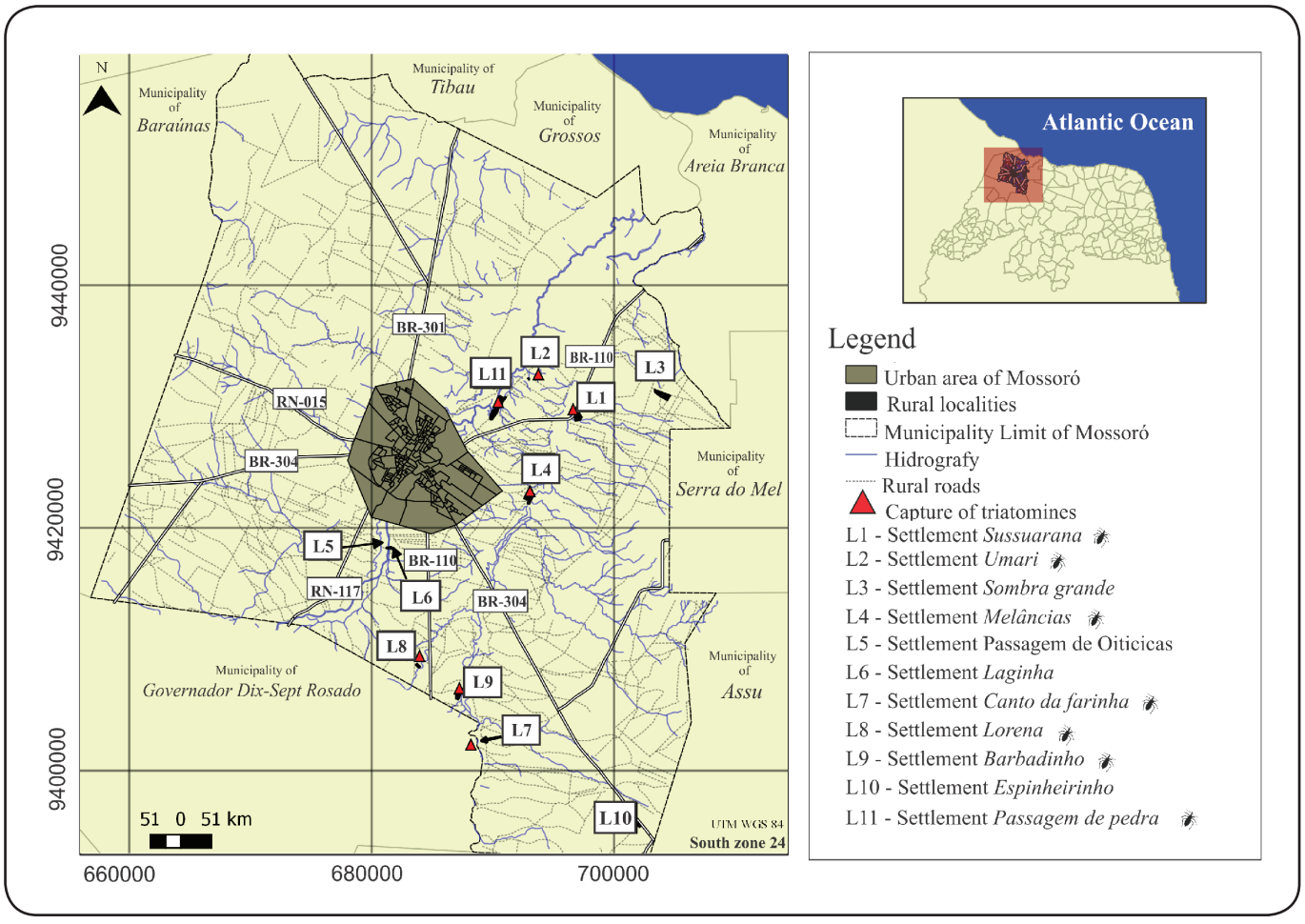

FIGURE 1: Mapping of rural localities of Mossoró, Rio Grande do Norte with triatomine capture history between 2008 and 2012, highlighting localities with active entomologic capture of triatomines and infection by *Trypanosoma cruzi*.

the main vector[46], as in the present study. The difference in seropositivity between the studies may be associated with the abundance of certain vector species in each region, based on ideal climatic, environmental, and geographic conditions for the survival and reproduction of each species[47]. Thus, the triatomine species, the *T. cruzi* prevalence, and the behavior of the dogs are factors that may influence the risk of infection in dogs[48].

In regards to seropositivity in dogs, the health status of the dogs, due to poor nutrition and immunosuppression, may be directly reflected in the antibody levels[39,40]. Therefore, the differences between regions need to be considered when evaluating the transmission cycle of *T. cruzi* in home areas, since socioeconomic indexes and infection risks are directly related[49].

No relation between the presence of seropositive dogs and that of infected triatomine vectors was observed in this study (**Table 2**). These factors cannot be discarded, however, since the domiciliation of triatomines and the circulation of *T. cruzi* between humans and domestic and wild animals are crucial to the establishment of infection. The availability of domestic hosts in households may directly affect the vector's choice of food source, reducing human-vector contact rates and transmission of the parasite[50].

The mapping of vectors (**Figure 1**) allows the spatial observation of areas with *T. cruzi* infection and helps the local health services in the control and surveillance of these areas[14]. Geoprocessing techniques have been widely used to understand epidemiological aspects of CD vectors, including characterization of the areas where the disease is present[51-53] and the relation between its distribution and reservoirs[29,34]. This reinforces the importance of the data observed in this study.

The spatial arrangement of triatomines reaffirms the presence of CD vectors in the environment in some rural areas and confirms the presence of natural infection by *T. cruzi* in these insects, along with diagnosing seropositive dogs. Although no association between seropositive dogs and the presence and infection of triatomine vectors was found, a domestic cycle of the disease is still possible. These results emphasize the importance of epidemiologic monitoring and surveillance of CD, since the health of domestic animals, especially dogs, and their close relationship with humans reflect the health and the level of exposure of local residents to the vector.

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Conflict of interest

The authors declare that there is no conflict of interest.

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