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ORIGINAL ARTICLE

Trypanosoma cruzi VECTOR INFECTION RATE IS UNDERESTIMATED IN SOME LOCALITIES IN THE STATE OF BAHIA

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ABSTRACT

Bahia was the last Brazilian state declared free of Chagas disease transmission by *Triatoma infestans* in 2006. The program designed to control vector transmission of Chagas is currently active, and all potential triatomines collected by the Bahia State Department of Health officials are most frequently diagnosed as negative for *Trypanosoma cruzi* when analyzed by the conventional parasitological direct method. The aim of the current study was to investigate whether triatomines from Bahia are free of *T. cruzi* infection using a more sensitive diagnostic methodology, namely the kinetoplastid-DNA polymerase chain reaction (kDNA-PCR). With the help of health officials, 51 triatomines were analyzed from peridomicile areas within the central north region of the state of Bahia. The majority (60.8%) were *Triatoma brasiliensis*, 29.4% were *Triatoma pseudomaculata*, and 9.8% were unidentified nymphs. Only one insect tested potentially positive for *T. cruzi* by the conventional parasitological direct method, and 31.4% were positive for *T. cruzi* DNA by kDNA-PCR. Almost half the infected insects (41.9%) were *T. brasiliensis*, a species with high potential for *T. cruzi* transmission. These results demonstrate that the number of infected triatomines with high transmission potential of *T. cruzi* may be greater than expected in four localities in the state of Bahia.

KEY WORDS: Chagas' disease; kDNA-PCR; *Trypanosoma cruzi*; triatomines; Brazil.

RESUMO

A infecção do vetor do *Trypanosoma cruzi* é subestimada em algumas localidades da Bahia

A Bahia foi o último estado brasileiro a ser declarado livre da transmissão da doença de Chagas pelo Triatoma infestans em 2006. O programa designado para controle vetorial da transmissão da doença de Chagas está atualmente ativo, e os potenciais triatomíneos coletados por funcionários do Departamento da Saúde do Estado da Bahia são praticamente todos diagnosticados como negativos para *Trypanosoma cruzi* quando analisados pelo método

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parasitológico convencional direto. O objetivo deste estudo foi investigar se os triatomíneos da Bahia, de fato, não estão infectados por *T. cruzi*, utilizando-se, para isso, uma metodologia de diagnóstico mais sensível, como a reação em cadeia da polimerase do DNA do cinetoplasto (kDNA-PCR). Com a ajuda dos funcionários da área da saúde, foram analisados 51 triatomíneos provenientes de áreas do peridomicílio da região centro-norte do estado da Bahia. Dos insetos analisados, a maioria (60,8%) era *Triatoma brasiliensis*, 29,4% eram *Triatoma pseudomaculata* e 9,8% eram ninfas que não foram identificadas. Apenas um inseto, analisado pelo método parasitológico convencional direto, mostrou-se potencialmente positivo para *T. cruzi* e 31,4% foram positivos para *T. cruzi* pela kDNA-PCR. Quase a metade dos insetos infectados (41,9%) era constituída por *T. brasiliensis*, uma espécie com alto potencial para a transmissão de *T. cruzi*. Estes resultados demonstram que o número de triatomíneos infectados com elevado potencial de transmissão de *T. cruzi* pode ser maior do que o esperado em quatro localidades do estado da Bahia.

DESCRITORES: Doença de Chagas; kDNA-PCR; *Trypanosoma cruzi*; triatomíneos; Brasil.

INTRODUCTION

Chagas disease, caused by *Trypanosoma cruzi*, is reported to affect 7 to 8 million people worldwide, mostly in Latin America, where its prevalence is associated with the distribution of infected triatomine vectors (34). Among the many Triatominae species (Reduviidae, Hemiptera), 25 are found in Bahia, a northeastern Brazilian state. The majority of these species are vectors for *T. cruzi*, including *Triatoma pseudomaculata, T. brasiliensis* and *Panstrongylus megistus*. *Triatoma infestans*, previously the most prevalent species in Bahia and throughout Brazil and considered the main vector for *T. cruzi* transmission (10), is less common now, but may still be found in some areas in Bahia (13, 14). After the national program for control of Chagas disease implemented in 1975-1983, Brazil was declared free of *T. cruzi* transmission by *T. infestans* in 2006 (33). Ten years of insecticide spraying resulted in a 94% reduction in the incidence of Chagas disease in the Southern Cone countries (13, 14). Nevertheless, the disease persists in Brazil, probably because each State has its own epidemiological peculiarities, some of which have not been identified to date (20, 21). The current estimate is that 1.9 million people in Brazil are infected with *T. cruzi* (14). Different species of the vector, including *Triatoma tibiamaculata* and *Panstrongylus geniculatus*, are frequently found to be infected with *T. cruzi* in urban areas, including Bahia capital, Salvador (26).

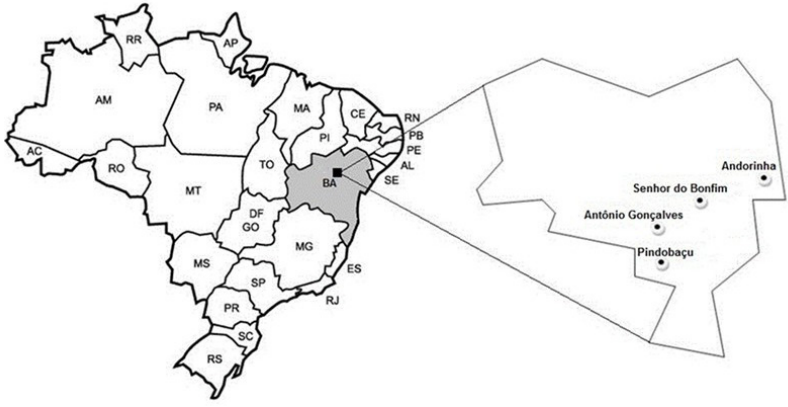
Although Bahia was declared free of *T. cruzi* transmission by *T. infestans* in 2006, the Health Department of Bahia State (HDBS) currently maintains an active epidemiological surveillance program whereby health officials are instructed to conduct triatomine searches in and around houses and to analyze the insect fecal contents by the conventional parasitological direct method (29). In addition, the population is encouraged to collect and submit insects for analyses. The purpose of the study reported here is to work

in cooperation with the HDBS and show that triatomines can be found with *T. cruzi* when analyzed by a technique such as the kDNA-PCR which is more sensitive than the conventional parasitological direct method.

MATERIALS AND METHODS

The four cities included in this study (Andorinha, Senhor do Bonfim, Antônio Gonçalves, and Pindobaçu) are located in the central northern region of the state of Bahia (Figure 1), approximately 400 km from the capital Salvador on the coast of the state. The study area is 400-500 m above sea level, within an area of *Caatinga* biome, with a mild climate (average annual temperature of 23°C) (3). In all four cities, the triatomine capture sites included domestic and peridomestic, natural and artificial environments around underprivileged neighborhoods in the rural area where subsistence agriculture was observed. The homes were made of wood or plaster-covered bricks with tiled roofs. There were often poorly built adobe storage sheds around the houses closed in only by a fence, used to store all sorts of things like animal food and supplies. In the peridomicile there were also hen-houses, pigsties, goat sheds and the animals were often not fenced in, but raised freely. The vegetation was typical of the *Caatinga* biome (semi-arid shrublands), including many of the Cactaceae family and several palm tree species, mainly babaçu (*Orbignya phalerata*). Searches for triatomines were conducted with the help of Bahia health officials, by carefully observing inside the houses (ceiling, walls, cracks in the walls and ceiling, windows, mattresses, and floor) and around the houses (dead tree bark, chicken coops, and any type of construction material). The insects were captured in May of 2012 and analyzed within approximately 10 days at the Federal University of Paraná. Species identification was performed based on external morphology, according to Lent & Wygodzinski (1979).

Figure 1. Map of Brazil indicating in gray the Bahia state, and inset (■)



Among all triatomines captured, the study included only insects for which it was possible to obtain the intestinal contents by abdominal compression, as recommended by the Brazilian Ministry of Health (1981) (29). The conventional parasitological direct method was carried out by examining one drop of the intestinal contents (obtained by abdominal compression) under a light microscope. Total DNA from another 1-3 drops of the intestinal contents was extracted using a commercial kit (Wizard® Genomic, Promega, USA) following the manufacturer's instructions.

Total DNA extracted from *in vitro* cultures of *T. cruzi* (G strain) and *Trypanosoma rangeli* (Choachi strain) reference strains was used as positive control, and ultrapure water, instead of total DNA, was used for the negative controls. The DNA from the *T. cruzi in vitro* culture was extracted using the proteinase K and phenol-chloroform protocol of Sambrook et al. (1989) and *T. rangeli* DNA was extracted using the TELT method described by Medina-Acosta & Cross (1993) with some modifications. Briefly, 1×10^7 cells were centrifuged, washed with 350 µL of TELT buffer (50 mM Tris-HCl, pH 8.0, 62.5 mM EDTA, pH 8.0, 2.5 M LiCl and 4% Triton X-100). The DNA was purified once using phenol/chloroform/isoamyl alcohol (25:24:1, v/v/v), precipitated by adding 100% ethanol (1:2, v/v), washed with 70% ethanol, dried, and suspended in ultrapure water.

The absorbance and quantification of recovered DNA was determined by a spectrophotometer (NanoDrop 2000, Thermo Scientific, USA). The PCR primers selected have been successfully used elsewhere for DNA amplification of *T. cruzi*, Tcru1 (121) (5' -AAA TAA TGT ACG GGK GAG ATG CAT GA-3') and Tcru2 (122) (5' -GGT TCG ATT GGG GTT GGT GTA ATA TA- 3') and *T. rangeli*, TrINT1 (5'CGC CCA TTC GTT TGTCC3') and TrINT2 (5'TCC AGC GCC ATC ACT GAT C3'). With the *T. cruzi* primer set the amplification of a 330 bp fragment from the kinetoplast DNA was expected, whereas a 375 bp fragment from the mini-exon was expected to be amplified when using *T. rangeli* primers (8). Primers specific to *T. rangeli* were used in case an infection by this protozoan was present (allowing for a false positive result by the conventional parasitological direct method). The 25-µL PCR reaction combined 1× reaction buffer (5 mM Tris-HCl [pH 9.0], 5 mM NaCl), 0.2 pmol of each primer, 6 mM $MgCl_2$ for *T. cruzi* and 4 mM $MgCl_2$ for *T. rangeli*, 25 µM of triphosphate deoxyribonucleotides (dNTP), 1 U Taq polymerase (Promega, USA) and approximately 5 ng of the positive control DNA from *T. cruzi* and *T. rangeli*. Total DNA extracted from the intestinal contents of the triatomines was used in serial dilution (ranging from 2 to 100 ng) to test for the presence of inhibitors. The cycling program (Peltier-based thermal cycler; Thermal Cycler PCR-MG96+; Bio-Equip) consisted of an initial denaturation, at 94°C for 2 minutes, followed by 30 cycles of 30 seconds at 94°C, an annealing step of 30 seconds at 54°C for *T. cruzi* and 58°C for *T. rangeli*, and 30 seconds at 72°C. A final extension step was allowed for 7 minutes at 72°C. The PCR

products were visualized by ultraviolet transillumination after 1.8% agarose gel electrophoresis and ethidium bromide staining.

RESULTS

Among all 134 triatomines collected, 51 were analyzed, including five nymphs and 46 adults, all found in the peridomiciliary area, on piled tiles and building debris, tree bark, and tree holes (Table). *T. brasiliensis* was captured mainly within piles of tiles and wood while *T. pseudomaculata* was found in perches and under the bark of dead trees, cashew and "Jurema" (any tree of the genus *Mimosa, Acacia* or *Pithecelobium*) trees, up to 20 m from the domiciles. Palm trees were not searched for triatomines. The majority of the adults (60.8%) were *T. brasiliensis* and 29.4% were *T. pseudomaculata*. The nymphs comprised 9.8% of the analyzed insects and were not identified.

Table. Total number and species of triatomines analyzed from the peridomiciliary areas of four cities in Bahia state and results obtained by the conventional parasitological direct method and kDNA-PCR for detection of *Trypanosoma cruzi*.

City	No. of triatomines	Species	No. of triatomines positive for *T. cruzi*	
			Direct method	kDNA-PCR
Pindobaçu	3	Unidentified	0	0
Senhor do Bonfim	2	Unidentified	0	1
Antônio Gonçalves	15	*T. pseudomaculata*	1	2
Andorinha	31	*T. brasiliensis*	0	13
Total	51		1 (2.0%)	16 (31.4%)

By the conventional parasitological direct method, it was possible to detect a movement suggestive of a flagellate in only one sample, from a *T. pseudomaculata* captured in Antônio Gonçalves. According to the HDBS, when a movement is observed, it is determined to be positive for *T. cruzi*. The identity of this flagellated organism was later confirmed by kDNA-PCR to be *T. cruzi*.

A much higher number of positive results for *T. cruzi* was obtained through kDNA-PCR, including 16 (31.4%) of the 51 samples. An expected product of 330 bp was detected, (in triplicates for the same DNA sample) and

confirmed the presence of *T. cruzi* DNA (Figure 2). All samples were also tested for the presence of *T. rangeli* DNA and were all negative (for example, sample A1, Figure 2). Some samples, including the positive control for *T. cruzi*, amplified an additional band of approximately 600 bp. According to Desquesnes & Dávila (2002) some primer sets allow for the presence of several bands due to the amplification of more than one tandem repeat; nonetheless, it is sufficient to say that the sample is positive when at least one expected product is observed.

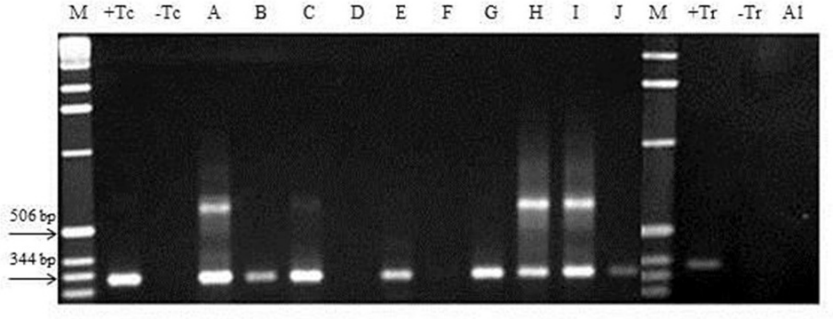


Figure 2. *Trypanosoma cruzi* DNA amplification by PCR from the intestinal contents of triatomines. Samples A through J were tested for *T. cruzi*; A, B, C, E, G, H, I, and J are positive; samples D, and F are negative. Sample A1, tested for *T. rangeli*, was negative. (M) Molecular weight marker 1 Kb (Invitrogen®). (+Tc) *T. cruzi* positive control, 330 bp product. (+Tr) *T. rangeli* positive control, 375 bp product. (-Tc and -Tr) *T. cruzi* and *T. rangeli* negative controls. Photograph of a 1.8% agarose gel stained with ethidium bromide and visualized under ultraviolet transillumination.

DISCUSSION

In the 1970s and 1980s, Bahia state struggled with a high prevalence of Chagas disease and the presence of the main triatomine vectors throughout the State. At the time, it was estimated that 4.2% of the Brazilian population was infected with the parasite, and approximately 100,000 new cases were reported each year (14). At some point, during those decades, the National Program for Chagas Disease Control was implemented in the country and efficiently reduced the number of vector-transmitted cases as the program invested in eliminating *T. infestans* (14). Despite all the hard work and measures that are still currently active in Bahia, there are areas with constant high mortality rates due to Chagas Disease and at least two outbreaks have been reported possibly caused by the accidental ingestion of sugar cane juice contaminated with the excrement of infected triatomines (1, 11, 21). In an epidemiological investigation, *Triatoma sordida* was found infected with *T. cruzi* at the site of both outbreaks (1, 11). Therefore, all epidemiological data collected in

Bahia is of great relevance for the State health officials to be able to monitor the situation and map the prevalence over time to constantly improve control measures.

Partially due to its favorable biome of open areas within the *Cerrado* and *Caatinga*, Bahia is the Brazilian state with the largest number of triatomine species (14). Among the 25 species, some were detected infected in different towns, including urban areas like the capital Salvador (9, 11, 14). *T. sordida, T. pseudomaculata, T. tibiamaculata, T. melanocephala, Triatoma lenti*, and *T. brasiliensis* are the species found infected with *T. cruzi* in Bahia, in the wild, rural, or urban centers, in the domicile or peridomicile areas (4, 9, 11, 14, 24, 26, 30).

In this study, searches for triatomines were conducted in the domiciles and peridomiciles of rural areas in four localities from the central north of Bahia state. *T. brasiliensis* and *T. pseudomaculata* were captured from peridomiciliary areas; 13.3% of the *T. pseudomaculata* and 41.9% of the *T. brasiliensis* were infected with *T. cruzi*, by kDNA-PCR. *T. pseudomaculata* is of medium importance as a *T. cruzi* vector but is the second most frequently captured species in Bahia while *T. brasiliensis* is the third most captured species but it is of high importance in addition to being the main *T. cruzi* vector in northeastern Brazil (5, 15). Both species are sympatric and have been found in northern Bahia and in the states of Piauí, Ceará, Rio Grande do Norte, Paraíba, and Pernambuco (7, 28, 32). When using the conventional parasitological direct method in prevalence studies, in Pernambuco, 9.8% of the triatomines were found infected with *T. cruzi*, including *T. brasiliensis, T. pseudomaculata, P. lutzi, P. megistus*, and *T. melanocephala* (32). In Ceará, 12.4% to 25% of the triatomines were found infected in the wild, peridomicile, and intradomicile of rural areas, including *T. brasiliensis, T. pseudomaculata*, and *Rhodnius nasutus* (27). In another entomological study conducted in rural areas of Ceará, about 2% of the *T. brasiliensis* and 11% of the *R. nasutus* were found infected in peridomiciles; interestingly, *T. pseudomaculata* was captured but none were infected (28). In contrast to the previous study, *T. pseudomaculata* was the most prevalent species captured from suburban areas in Ceará, 69% of them were infected; while *T. brasiliensis* was basically absent (18). Among 11 triatomine species captured in the state of Piauí, *T. brasiliensis* and *T. pseudomaculata* were the most common while *P. megistus*, *Panstrongylus lutzi, Rhodnius pictipes* and *Rhodnius neglectus* were the most infected, in a 0.8% general prevalence rate (13). From the above entomological studies it is clear how the prevalence rates vary and are still relatively high, considering they were obtained through the conventional parasitological direct method. In other parts of the country, the rates are somehow lower, and, as expected, with distinct triatomine species (19, 23, 24).

Despite being broadly distributed in the *Caatinga* biome *T. brasiliensis* and *T. pseudomaculata* exploit distinct ecotopes and microhabitats (14). While

T. brasiliensis prefers rock outcrops in association with small mammals, *T. pseudomaculata* prefers to colonize the bark of trees near bird nests, and both can be found frequently in peridomiciles (5, 14). This study corroborates Gurgel-Goncalves et al. (2012a) and Sarquis et al. (2012), where *T. pseudomaculata* was found in its natural habitat, under the bark of dried trees and *T. brasiliensis* was found in piles of tiles and wood, in peridomiciles. The devastation of their natural habitats in the wild, the use of wood in domiciles and to build sheds and fences favors their presence in the peridomicile area (15). The sole presence of adults in the peridomiciles presents a high colonization potential (12) especially considering that the peridomicile provides an appropriate environment with domestic animals serving as food sources (6, 18).

In the present study, the prevalence of infected triatomines detected by PCR (31.4%) was much higher than what would have been detected by health officials using the conventional parasitological direct method (2.0%). A prevalence of 31.4% is also much higher than other prevalences detected in northeastern Brazilian states, as shown above, and this is due to the superior sensitivity of the PCR technique over microscopy (2, 31). This discrepancy is not only due to the fact that the conventional parasitological direct method is less sensitive and specific, but it could also be due to the time span between capture and analyses, failures during the analyses, likely connected to the poor analytical conditions known to exist in northeastern Brazil (10). Because molecular techniques, such as PCR, are not currently used in Brazil due to the high costs involved, it is important to revise the direct methodology and invest in professional training and infrastructure to guarantee more efficient diagnosis.

In Brazil, the triatomine natural infection percentages were substantially reduced from 1983 to 1993/1999 (4), especially when observing *T. brasiliensis*; however its vast geographic distribution, high incidence in some areas, and high natural infection rates, as observed in this study, suggest that the number of *T. cruzi* infected triatomines is rising. Part of this, in northeastern Brazil, can be explained by the fact that this region is socially underprivileged, presenting high poor housing rates that favor triatomine colonization; it is also a geographical area that maintains two hard-to-control species, *T. brasiliensis* and *T. pseudomaculata*; and the control measures have decreased in contrast to when they started (9). The results here reported indicate that the four areas surveyed maintain natural and artificial characteristics that favor the incidence of triatomines and the risk of *T. cruzi* transmission to the local population. It is recommended that the community clear peridomicile areas, especially regarding piles of wood and tiles, and dead or dried trees that offer favorable conditions for triatomine colonization. On the other hand, these control measures will be difficult to implement as the storage of local wood is a common habit (12). In addition to the artificial microhabitats existent in the studied area, the domestic animals (chickens, pigs, goats) that move freely in

the peridomicile also favor triatomine colonization and could be maintaining the peridomestic cycle of *T. cruzi* (18, 28). The studied localities require more investigation to determine what animals are infected, the triatomine blood food sources, and the *T. cruzi* lineages circulating in the triatomines, to evaluate the distribution and dynamics of the parasite. It is evident that the area needs sustained entomological surveillance and stronger control measures.

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