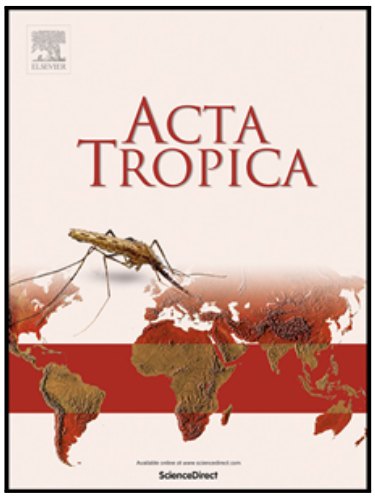

Genotypic Trypanosoma cruzi distribution and parasite load differ ecotypically and according to parasite genotypes in Triatoma brasiliensis from endemic and outbreak areas in Northeastern Brazil

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PII: S0001-706X(21)00233-3
DOI: https://doi.org/10.1016/j.actatropica.2021.106054
Reference: ACTROP 106054

To appear in: *Acta Tropica*

Received date: 17 May 2021
Revised date: 9 July 2021
Accepted date: 10 July 2021

Please cite this article as: Carolina Valença-Barbosa , Paula Finamore-Araujo , Otacilio C. Moreira , Jose Gabriel Vergara-Meza , Marcus Vinicius Niz Alvarez , Juliana R. Nascimento , André Borges-Veloso , Maria Carolina Viana , Maurício Lilioso , Danilo Ciccone Miguel , Fernanda Ramos Gadelha , Marta Maria Geraldes Teixeira , Carlos Eduardo Almeida , Genotypic Trypanosoma cruzi distribution and parasite load differ ecotypically and according to parasite genotypes in Triatoma brasiliensis from endemic and outbreak areas in Northeastern Brazil, *Acta Tropica* (2021), doi: https://doi.org/10.1016/j.actatropica.2021.106054

Running title: *Trypanosoma cruzi* load differs according to parasite genotype

Genotypic *Trypanosoma cruzi* distribution and parasite load differ ecotypically and according to parasite genotypes in *Triatoma brasiliensis* from endemic and outbreak areas in Northeastern Brazil

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Highlights

- In 2016, acute Chagas disease outbreaks were officially registered in four municipalities from Northeastern Brazil.
- *T. cruzi* I was the predominant genotype in *Triatoma brasiliensis* captured in peridomestic ecotopes, whereas TcII was found mainly in sylvatic ecotopes.
- Additional genotypes, such as TcIII, *T. rangeli* A and mixed infections were found in low proportions.
- TcII had more than twice the parasite load of TcI.
- No difference was observed in *T. cruzi* load in investigated areas, both presenting high parasite loads.

Abstract

This study aimed to identify the *Trypanosoma cruzi* genotypes and their relationship with parasitic load in distinct geographic and ecotypic populations of *Triatoma brasiliensis* in two sites, including one where a Chagas disease (ChD) outbreak occurred in Rio Grande do Norte state, Brazil. Triatomine captures were performed in peridomestic and sylvatic ecotopes in two municipalities: Marcelino Vieira – affected by the outbreak; and Currais Novos – where high pressure of peridomestic triatomine infestation after insecticide spraying have been reported. The kDNA-PCR was used to select 124 *T. cruzi* positive triatomine samples, of which 117 were successfully genotyped by fluorescent fragment length barcoding (FFLB). Moreover, the *T. cruzi* load quantification was performed using a multiplex TaqMan qPCR. Our findings showed a clear ecotypic segregation between TcI and TcII harboured by *T. brasiliensis* ($p<0.001$). Although no genotypes were ecotypically exclusive, TcI was predominant in peridomestic ecotopes (86%). In general, *T. brasiliensis* from Rio Grande do Norte had a higher *T. cruzi* load varying from 3.94 to 7.66 x 10^6 *T. cruzi* per insect. Additionally, TcII (median value=299,504 *T. cruzi*/intestine unit equivalents) had more than twice ($p=0.1$) the parasite load of TcI (median value=149,077 *T. cruzi*/intestine unit equivalents), which can be attributed to a more ancient co-evolution with *T. brasiliensis*. The higher prevalence of TcII in the sylvatic *T. brasiliensis* (70%) could be associated with a more diversified source of bloodmeals for wild insect populations. Either TcI or TcII may have been responsible for the ChD outbreak that occurred in the city of Marcelino Vieira. On the other hand, a smaller portion of *T. brasiliensis* was infected by TcIII (3%) in the peridomicile, in addition to *T. rangeli* genotype A (1%), often found in mixed infections. Our results highlight the need of understanding the patterns of *T. cruzi* genotype´s development and circulation in insect vectors and reservoirs as a mode of tracking situations of epidemiologic importance, as the ChD outbreak recently recorded for Northeastern Brazil.

Keywords: Triatomines, Genotyping *Trypanosoma cruzi*, lineages, oral outbreaks, epidemiology

1. Introduction

Chagas disease (ChD) is a neglected tropical illness caused by the protist hemoflagellate *Trypanosoma cruzi* (Kinetoplastida: Trypanosomatidae). Although the incidence has declined over the last decades due to intense programs to eliminate the main domiciliated vector in Brazil, *Triatoma infestans* (Reduviidae: Triatominae), ChD is still considered an important global health problem (WHO, 2015). The infection affects 6 to 7 million people and 25 million are at risk in American countries causing 12,000 deaths per year (WHO, 2020). In Brazil, approximately 1.0 to 2.4% of the population is estimated to be chronically infected, resulting in about 9,000 deaths annually (Dias et al., 2016).

The transmission of *T. cruzi* is highly complex since it is considered an anthropozoonosis involving more than 150 mammal reservoirs and more than 150 triatomine species (Dorn et al., 2018; Oliveira et al., 2018; Lima-Cordón et al., 2019; Nascimento et al., 2019; Alevi et al., 2020; Zhao et al., 2021). Classically, the parasite is transmitted by faeces of infected triatomines, although it may also be transmitted by organ transplantation, blood transfusion, laboratory accidents, congenitally and contaminated food (Rassi et al., 2010). Recently, the latter via has been implicated as the main route of *T. cruzi* transmission, explaining most acute cases of ChD not only in Brazil (Shikanai-Yasuda et al., 1991; Pinto et al., 2008; Roque et al., 2008; Valente et al., 2009; Coura and Viñas, 2010; Souza-Lima et al., 2013; Coura, 2015), but also in Venezuela, Colombia, Bolivia and Ecuador (Santalla-Vargas et al., 2011; Alarcón de Noya et al., 2016; Hernández et al., 2016; Calvopina et al., 2020). In such events, the mortality rate varies from 8 to 35% (Rassi et al., 2010).

Trypanosoma cruzi transmission to humans depends on the local conditions that promote the contact between hosts in sylvatic, peridomestic and domestic cycles. The overlapping of these cycles aggravates the vector control measures, as a result of a complex epidemiological scenario, involving synanthropic mammals in human domestic or peridomestic environments. Such reservoirs may be *T. cruzi*-infected and be sources for triatomine reinfestation of recently insecticide-treated households (Sarquis et al., 2006; Roque et al., 2008; Bezerra et al., 2020, Lilioso et al., 2020). Additionally, the genetic heterogeneity of *T. cruzi* inserts more complexity to understand the transmission cycles of the parasite.

Six different discreet typing units (DTUs; TcI to TcVI) of *T. cruzi*, and the recently proposed Tcbat, have been also described (Zingales et al., 2012, Zingales, 2018). Overall, TcI is widely distributed geographically, occurring from the southern

United States to northern Argentina and Chile and across Brazil (Jansen et al., 2020). It is the main DTU responsible for the ChD in endemic countries located north of the Amazon basin, such as Venezuela, Colombia, Ecuador and Panamá, where TcII have been more sporadically reported (Zingales et al., 2012, Brenière et al., 2016; Anez et al., 2020). The genotype TcII is frequently associated with the domestic transmission cycle in the southern and central regions of South America, strongly associated with ChD in formerly endemic areas from northeastern to southern Brazil (Zingales et al., 2012, 2018). The TcIII genotype has been reported across South America (Argentina, Bolivia, Brazil, Chile, Colombia, Paraguay, Peru and Venezuela), whereas TcIV (the South American lineage) has been mostly associated with sylvatic transmission cycles, being reported in Brazil, Venezuela and Colombia (Anez et al., 2020; Jansen et al., 2020). Finally, TcV and TcVI are generally reported in southern countries of South America (Argentina, Bolivia, Chile, Paraguay) and rarely detected in Brazil, Ecuador and Colombia (Zingales et al., 2012; Brenière et al., 2016; Zingales, 2018). In the Rio Grande do Norte (RN) state, Brazil, TcI, TcII and TcIII have been identified in *T. brasiliensis*, as well as TcI-TcII in humans and TcIII in armadillos (Marcili et al., 2009; Câmara et al., 2010, 2013; Barbosa-Silva et al., 2016; Lima-Oliveira et al., 2020, Honorato et al. 2021). Hence, recognizing the different genotypes of *T. cruzi* and the interaction with their vectors and vertebrates' hosts in each ecotope is crucial for understanding their roles in different transmission cycles.

The *T. cruzi* parasitemia in the mammalian hosts is one important factor in the parasite infection of the vectors, thus ensuring dispersion and maintenance of parasites in nature (Jansen et al., 2018). Once ingested, *T. cruzi* needs to multiply and colonize the lumen of the vectors' intestine. In this way, the vector species, parasite load and its genotype are important factors to warrant parasite development and the continuity to the transmission cycle. Since microscopic observation/quantification of trypanosomes in the triatomine intestine lacks specificity, sensitivity and accuracy, Moreira et al. (2017) developed a molecular assay based on real-time PCR to quantify *T. cruzi* load in the triatomine gut content. It is a highly sensitive and accurate methodology, raising the possibility of evaluating the triatomine's ability to transfer the parasite during a blood meal. The success of host infection is influenced by multiple variables, such as vectorial abilities, parasite load and genotypes (Verly et al., 2020). Therefore, any information regarding both *T. cruzi* genotypes and parasite load found in triatomine species may

raise important contributions to understanding natural transmission cycles and the risks posed by insect vectors and their *T. cruzi* genotypes the ChD transmission.

In the semiarid Brazilian northeast, there are autochthonous triatomines dispersed throughout the region, mainly *T. brasiliensis* and *T. pseudomaculata*, and small populations of *T. petrocchiae*, *Panstrongylus lutzi,* and *Rhodnius nasutus* (Costa et al., 2003; Lima-Oliveira et al., 2020). Considering the epidemiologic synanthropic behavior, *T. brasiliensis* and *T. pseudomaculata* are the ones adapted to infest artificial ecotopes (Barbosa-Silva et al., 2019). The geographical distribution of the two (peri)domestic species and difficult to control overlaps, playing an important role in the epidemiology of ChD in this region (Alencar, 1987; Diotaiuti, 2007). Also, they are able to recolonize peridomiciles and intradomiciles shortly after insecticide application (Diotaiuti et al., 2000; Abad-Franch et al., 2014; Lima-Oliveira et al., 2020), posing as a challenge for ChD control measures.

Triatoma brasiliensis and *T. pseudomaculata* have been found naturally infected by *T. cruzi* (Costa et al., 2003; Dias, 2007; Barbosa-Silva et al., 2016; Bezerra et al., 2014; Lilioso et al., 2017). However, *T. pseudomaculata* is more associated with birds, which are refractory to *T. cruzi*; consequently, it presents colonies with lower rates of natural *T. cruzi* infection – especially those inhabiting chicken coops (Sarquis et al., 2004). In contrast, *T. brasiliensis* is highly abundant in the studies area, more frequently found infesting domiciles, showing higher rates of natural infection by *T. cruzi.* Therefore, it has been considered the main vector in the semiarid region of Brazil (Costa et al., 2003; Barbosa-Silva et al., 2016; Bezerra et al., 2018, 2020). Dense populations of *T. brasiliensis* in peridomiciles with a high prevalence of natural infection by *T. cruzi* (including insects found inside the houses) in RN state were suggested to be determinants in the ChD outbreaks in RN (Barbosa-Silva et al., 2016, Lilioso et al., 2017; Monsalve-Lara et al., 2021). Thus, due to the wide geographical distribution, high rates of natural *T. cruzi* infection, and the ability to inhabit natural ecotopes and anthropic environments, *T. brasiliensis* has become one of the main priorities of the Ministry of Health aiming to control the main vector in northeastern Brazil (Alencar, 1987; Costa et al., 2003; Sarquis et al., 2010).

In the last decade, most acute ChD cases in Brazil have been associated with food-borne outbreaks. Between 2000 to 2013, the Ministry of Health registered 1,034 cases of acute ChD associated with the ingestion of *T. cruzi* contaminated food, mainly in the Amazon region (Coura, 2015). In northeastern states, one of the poorest and

underdeveloped regions in Brazil, acute ChD outbreaks have been reported since 1991 (Shikanai-Yasuda et al., 1991). The first outbreak recorded by the scientific community occurred in the state of Paraíba, as the result of a meeting at a farm and affected 26 people with one fatal case – probably transmitted through the consumption of contaminated sugarcane juice. The same situation has been reported for an outbreak in Ceará state (Cavalcanti et al., 2009) and Bahia (Bastos et al., 2010). In 2016, an outbreak of acute ChD was officially registered by the Brazilian Health Surveillance System in Marcelino Vieira County, RN state, comprising 18 cases confirmed, including three deaths, probably by ingestion of sugarcane juice contaminated with *T. cruzi*-infected *T. brasiliensis* (Vargas et al., 2018). All acute ChD patients reported they had been ingested sugarcane juice from a farm in Marcelino Vieira municipality. During entomological investigations, *T. brasiliensis* was the most frequent triatomine species (91%) captured in the peridomicile, showing a natural infection rate of 63%-98% nearby the sugar cane mill, where the juice was processed, which suggests that the ingestion of juice infected by *T. cruzi* was the mechanism involved in this outbreak (Vargas et al., 2018, Lilioso et al. 2000. Monsalve-Lara et al. 2021).

Therefore, considering the recent ChD outbreak in the Northeast of Brazil and the potential vector involved (*T. brasiliensis*), we selected *T. cruzi*-infected *T. brasiliensis* to analyze the distribution of *T. cruzi* genotypes and the parasitic load in two municipalities of RN. The first (Marcelino Vieira) one was the main affected municipality by the outbreak ChD and the second (Currais Novos) was chosen because it exhibits high rates of triatomine infestation after insecticide spraying (Lilioso et al., 2017). We used a multi-dimensional approach (qualitative, quantitative and spatially dynamic) aimed to detect the parasitic load and *T. cruzi* genotypes in *T. brasiliensis* in geographic and ecotypic scales. Additionally, we assessed *T. cruzi* DTUs and parasitic load in insects captured in the two studied areas aiming for a better understanding of the factors that could be involved in outbreaks of ChD in Northeastern Brazil.

2. Materials and Methods

2.1. Study area and field-collected triatomines

The survey was conducted in a rural area in the semiarid zone of the State of RN located in northeastern Brazil. This state is divided into 167 counties distributed in four mesoregions: East, Agreste, West and Central. The climate is hot and dry, with temperatures ~23–33°C (absolute range, 16–38°C) and rainfall <850 mm/year with

periodic severe droughts. The predominant biome is the Caatinga – where this study was conducted. It is characterized by a mosaic of xerophytic, deciduous, semi-arid thorn scrubs, and forest, in addition to uncovered soil in a considerable portion of its extension (Leal et al., 2003).

Fieldworks were conducted in Currais Novos (CN; -06^{o} 15'39,6", -36^{o} 30'54") and Marcelino Vieira (MV; -06^{o} 17'42", -38^{o} 09' 57") municipalities in the dry season. The distance between both municipalities is ~180 km. We were assisted by technicians hired by municipal health departments and followed their guidelines for entomological captures. We divided the sampled insects into two groups: the ones found in artificial (man-made) and the ones caught in natural ecotopes (rocky outcrops). Accordingly, artificial ecotopes were represented by dwelling's unities (DUs), which comprise the domestic and peridomestic environment. For each DU, distinct peridomestic ecotopes were searched for triatomines, such as chicken coops, goat and pig corral and piles of timber and bricks. Natural ecotopes were represented by the primary *T. brasiliensis*' ecotope – so-called the sylvatic ecotope, which are represented by rocky outcrops commonly located more than 200m apart to human domiciles (Lent and Wygodzinsky, 1979), cacti (Valença-Barbosa et al., 2014a), bird nests and palms. In both types of ecotopes, manual searches were adopted using tweezers and flashlights. Captures were performed by exhaustion: all bugs seen were caught with the aid of tweezers/gloves by three researchers and one technician for 4 hours. Triatomines were taxonomically identified based on morphology, following Jurberg et al. (2014) and Dale et al. (2018). Insect populations were defined as a group of insects collected in the same ecotope and at the same elapsed four hours.

2.2. Assumptions to select the sampling

We discarded the starved insects. We also privileged nymphs in the last stages (N4-N5; 80% of the total selected). Therefore, immature insects comprised 96% of the total sampling. The criterium to select non-starving insects was based on the visualization of the blood meal (a black mass with more than 20–25 mg) during dissection. This strategy aimed to decrease bias regarding some variables, as the post-feeding period and evolutionary stage of insects.

2.3. Molecular assays

2.3.1. DNA isolation

All collected insects were stored individually in an Eppendorf tube at −20 °C in ethanol absolute until processed. The gut of each insect was macerated in liquid nitrogen by using a sterile crusher. DNA was isolated with DNeasy Qiagen® kit, according to the manufacturer's protocol. DNA integrity was assessed by electrophoresis on 0.8% agarose gels and quantification in a NanoDrop® 1.000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA).

2.3.2. T. cruzi diagnosis via PCR

DNA samples (N = 152) from the intestinal contents of *T. brasiliensis* were submitted to PCRs for *T. cruzi* diagnosis. The set of primers used in the conventional PCR assays were: forward 121 (5' AAATAATGTACGGG(T/G)GAGATGCATGA 3') and reverse 122 (5' GGTTCGATTGGGGTTGGTGAATATA 3') designed by Sturm et al. (1989) and Wincker et al. (1994). The PCR reaction was performed in a 20 µl volume with the final mix containing: 2 µl 10× Taq DNA polymerase buffer, 0.2 mM dNTPs, 3.5 mM $MgCl_2$, 10 pmoles of each primer, 0.04% of bovine serum albumin (BSA), 1U of Taq polymerase and 3 µL of DNA template. PCR cycles consisted of an initial cycle at 95ºC for 5 min, 35 cycles including denaturing at 95ºC for 45 s, annealing at 65ºC for 45 s, extending at 72ºC for 45 s, with a final extension of 10 min at 72°C. PCR products were electrophoresed in 2% (w/v) agarose gel in Tris-borate ethylenediamine tetraacetic acid (EDTA) buffer, stained with Gelred (Biotium Inc., Hayward, CA, USA) and visualized under UV transillumination. The presence of a PCR amplicon of approximately 330 bp indicates positivity for trypanosomes. All PCR reactions were run with two positive controls for *T. cruzi* (Dm28 and Y strains) and *T. rangeli* (Macias strain) and a reagent negative control (without DNA).

2.3.3. Fluorescent fragment length barcoding (FFLB)

We performed the fluorescent fragment length barcoding (FFLB) method for genotyping of trypanosomatids. The technique was first developed for the barcoding of African trypanosomiasis and later proposed for American trypanosomiasis (Hamilton et al., 2011), which had been better standardized in our laboratory (ICB-USP). This procedure allows discriminating trypanosomatid species based on length polymorphism in specific regions of the 18S and 28S ribosomal RNA genes. This method has proved to be accurate and able to detect the TcI, TcIII, TcIV, TcV and Tcbat, whereas TcII and

TcVI are not distinguishable). Additionally, it enables to distinguish *T. rangeli* (Tr) lineages. Moreover, FFLB is considered a quick, highly sensitive technique, without the need to perform *in vitro* cultivation – which may lead to *T. cruzi* lineages selection (Lima et al., 1995). Another advantage of FFLB is the ability to detect mixed infections, as demonstrated by the identification of more than five different species/genotypes in tsetse flies (Garcia et al., 2018).

2.3.4. Absolute quantification by real-time PCR assays (qPCR)

The qPCR was performed according to a methodology previously proposed by Moreira et al. (Moreira et al., 2017), using the multiplex TaqMan system targeting *T. cruzi* nuclear satellite DNA (SAT-DNA) (Piron et al., 2007) and the 12S ribosomal subunit gene of triatomines (Moreira et al., 2017). Both targets were used to estimate the normalized parasite load, according to the DNA amount recovered from field-collected triatomine samples. As conducted for FFLB, the technique was applied to insects considered positive for *T. cruzi* (N = 124). Reactions were carried out in a final volume of 20 μL, containing 2 μL DNA, 2×FastStart Universal Probe Master Mix (Roche Diagnostics GmbHCorp, Mannheim, Germany), 600 nM Cruzi1/Cruzi2 primers and 250 nM Cruzi3 probe (FAM/NFQ-MGB) and 300 nM P2B primer, 500 nM P6R primer and 150 nM Triat Probe (VIC/NFQ-MGB) (Applied Biosystems). The cycling conditions were as follows: 50 °C for 2 min, 95 °C for 10 min, followed by 45 cycles at 95 °C for 15 s and 58 °C for 1 min. Amplifications were performed in the Viia 7 Real-Time PCR system (Applied Biosystems). Standard calibration curves were constructed by serially diluting total DNA obtained from non-infected triatomine intestine samples (fifth instar nymphs of *R. prolixus* from insectary) spiked with 10^6 *T. cruzi* epimastigotes (Dm28c clone, TcI). The resulting DNA was serially diluted to a range of 10^6 to 10^{-1} *T. cruzi* equivalents and 2 to 0.002 triatomine intestine unit equivalents, also enabling the evaluation of the technical sensitivity. Each 96-well reaction plate contained the standard curve and two negative controls. Negative controls consisted of a reaction with *T. cruzi*-specific primers without DNA or DNA extracted from non-infected triatomines. Regarding qPCR assays, all experiments were performed in an experimental duplicate. Results were expressed as the mean of *T. cruzi*/intestine unit equivalents, a quantitative measure to normalize the parasite load. Intestinal content was weight before DNA isolation. All DNA samples were extracted from the same amount of intestinal material.

2.4. Statistical analysis

Statistical analysis was performed using the software RStudio for R language (RStudio Team, 2013). We compared the parasite load distributions between the municipality, environment (peridomicile and sylvatic ecotopes) and *T. cruzi* lineages (TcI and TcII) identified in triatomine vectors using Wilcoxon-Mann-Whitney tests. We applied a Fisher Exact Test for TcI and TcII lineage frequencies between the environment and municipality. Also, we applied an Exact Hypergeometric Test for the 2x2x2 contingency table (Zelterman, 1999) using 'hypergea' package for R (Boenn, 2018) to test the three-way interaction effect between TcI and TcII lineage + environment + municipality. *P*-values ≤ 0.1 were considered statistically significant. We excluded all genotypes or genotype combinations considered rare (N<5).

3. **Results**

3.3. Trypanosome infection in *T. brasiliensis*

One hundred and twenty-four samples tested positive for the 121/122 primers of kDNA-PCR were selected, being 78 (63%) and 46 (37%) *T. brasiliensis* from Marcelino Vieira (MV) and Currais Novos (CN) municipalities, respectively. Regarding the ecotypic distribution, 73 were collected in artificial ecotopes (tile/wood/brick piles and shelters for domestic animals), in addition to 51 samples that were found in rocky outcrops in four distinct sylvatic spots around domiciles (200-300 m). All kDNA-PCR-positive were characterized by FFLB and qPCR assays. However, 7 samples were negative using FFLB, but positive when analyzed by kDNA-PCR and qPCR assays. The other 7 samples were negative for qPCR assays but positive by kDNA-PCR and FFLB assays. Of these, three insects from peridomicile and one from sylvatic in CN did not amplify by FFLB, as well as three from sylvatic ecotope from MV. However, all these samples were amplified by qPCR. Regarding the qPCR assays, six samples collected in peridomestic ecotopes from CN and one from sylvatic ecotope from MV did not have any amplification by qPCR but were genotyped by FFLB (Supplementary material 1).

3.4. Trypanosome genotypes distribution and mixed infections

Out of 124 kDNA-PCR-positive samples of *T. brasiliensis*, trypanosomes of 117 bugs were genotyped using FFLB. *T cruzi* I (~60% - 70/117) was the prevailing genotype, following by TcII (~31% - 36/117). We detected TcIII in ~2% (2/117) of *T. brasiliensis* and ~1% (1/117) was infected by *T. rangeli* genotype A (Tr). As we mentioned above, FFLB technique is not able to discriminate between TcII and TcVI, as also reported for conventional genotyping methods (Hamilton et al., 2011). Because TcVI and TcV have never been reported in the Brazilian northeastern (Herrera et al., 2005; Marcili et al., 2009; Câmara et al., 2010; Araújo et al., 2011; Barbosa-Silva et al., 2016; Ribeiro et al., 2018; Bezerra et al., 2018; Waniek et al., 2020; Honorato et al., 2021), we assumed that TcII/TcVI were indeed TcII. We went to each of these mentioned studies and observed that out of 175 *T. cruzi* genotypes found for Brazilian northeastern, none of them were TcVI (in details in the last paragraph of discussion and Supplementary material 2).

We also detected mixed infections by *T. cruzi* and *T. rangeli* genotype A observed in ~7% (8/117) of the bugs collected. Of these, seven insects were infected by a double trypanosomatid mixture (two insects with TcI + Tr and five with TcI + TcII) and one *T. brasiliensis* was infected by a triple mixture (TcI + TcII + Tr).

3.5. Distribution of trypanosome genotypes between ecotopes

In peridomestic ecotopes, ~86% (60/70) of *T. brasiliensis* insects were infected by TcI. Additionally, ~4% (3/70) were infected by TcII, ~3% (2/70) by TcIII, ~1% (1/70) had Tr and ~6% (4/70) had mixed infections (two specimens infected by TcI + Tr, one specimen by TcI + TcII and one specimen with TcI + TcII + Tr). Regarding the sylvatic ecotope, ~21% (10/47) were infected by TcI, while ~70% (33/47) had TcII and ~9% (4/47) had mixed infection by TcI + TcII. The TcI and TcII frequencies between peridomestic (artificial) and sylvatic (natural) ecotopes were statistically significant (Fisher's Exact Test, $p<0.001$, odds ratio=65.75, df=1).

3.6. Distribution of *Trypanosoma cruzi* genotypes between municipalities

Overall, TcI infections were the prevailing genotype, comprising ~52% (22/42) and ~64% (48/75) in CN and MV, respectively. Concerning triatomines from CN municipality, ~26% (11/42) of *T. brasiliensis* were infected by TcII, ~5% (2/42) by TcIII, ~2% (1/42) by Tr and ~14% (6/42) were mixed infected (2 samples with TcI and Tr, 3 samples with TcI and TcII, 1 sample with TcI, TcII and Tr). *Trypanosoma rangeli*

was only found in the peridomestic environment of Currais Novos. In MV municipality, we detected lower diversity of DTUs, comprising ~33% (25/75) of the insects infected by TcII, ~3% (2/75) showing mixed infections by TcI and TcII. No significant differences were found for TcI and TcII frequencies between municipalities (Fisher's Exact Test, *p*-value=1, odds ratio=0.97, df=1).

3.7. Distribution of *Trypanosoma cruzi* genotypes between ecotopes within municipalities

Considering peridomestic ecotopes, TcI was more prevalent in both municipalities, comprising ~71% (22/31) in CN and 97% (38/39) in MV, besides mixed infections (~13% in CN). In contrast, TcII was more frequent in sylvatic triatomines, with ~82% (9/11) in CN and ~67% (24/36) in MV. However, we detected lower rates: ~28% (10/36) of single (MV) and mixed (~5%, 2/36 in MV and ~18%, 2/11 in CN) infections by TcI also in sylvatic ecotope, as well as TcII (~7%, 2/31 in CN and ~3%, 1/39 in MV) in peridomicile. Only two (~7%) *T. brasiliensis* from peridomestic ecotopes in CN municipality were infected by TcIII. One specimen (~3%) with Tr was detected only in this municipality also in peridomestic ecotopes, as mentioned before. Mixed infections were observed in peridomestic and sylvatic ecotopes in both municipalities (Figure 1). Although the test correlating TcI and TcII frequencies and municipalities was not statistically significant, when three-way interaction was considered (genotypes x ecotopes x municipality) a significant statistical difference was observed (Exact Hypergeometric Test for 2x2x2 Table, $p<0.001$, odds ratio=2.35). Therefore, TcI and TcII frequencies were not different between municipalities but they differed between ecotopes, regardless of the geographic component.

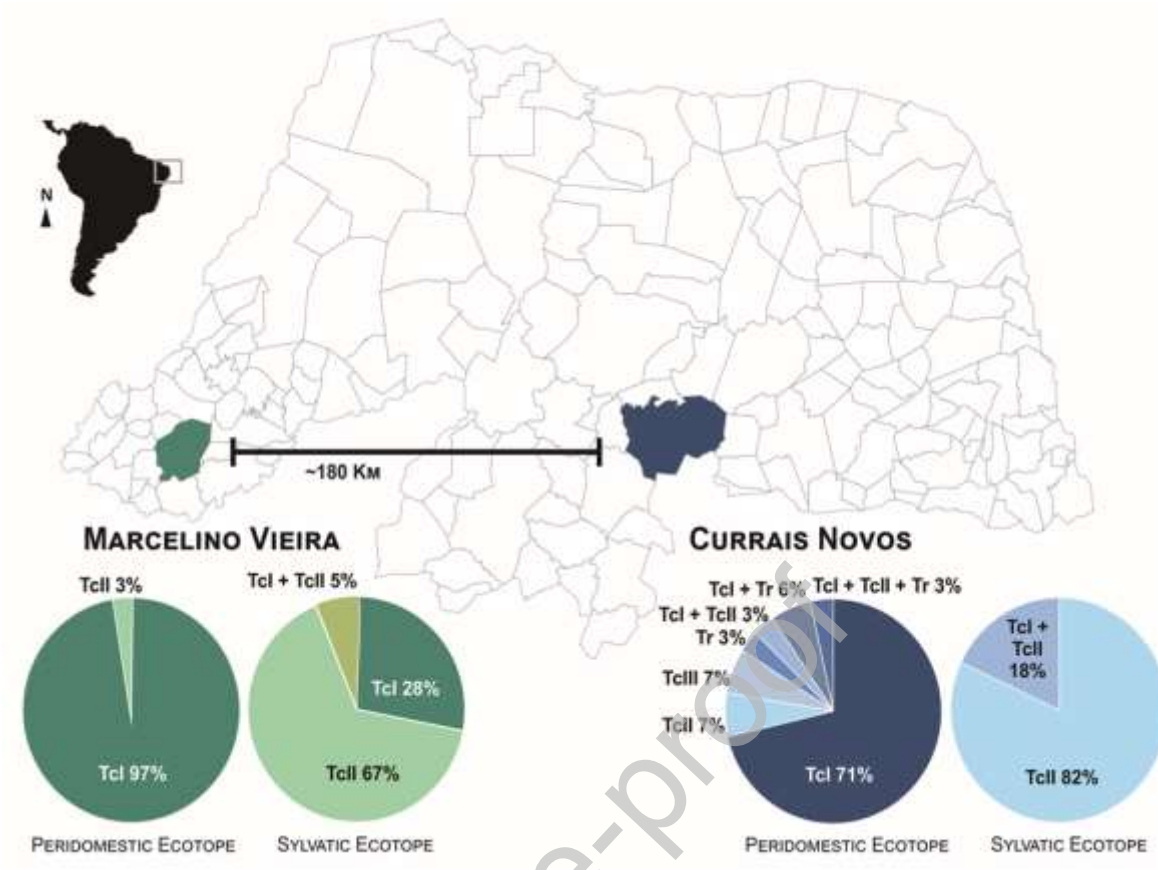

Figure 1: Trypanosomes' genotypes distribution per ecotopes observed in 117 *Triatoma brasiliensis* collected in Currais Novos (N = 42) and Marcelino Vieira (N = 75) municipalities. TcI = *T. cruzi* DTU I; TcII = *T. cruzi* DTU II; TcIII = *T. cruzi* DTU III; Tr = *T. rangeli* genotype A.

3.8. Quantification of trypanosome loads in infected *Triatoma brasiliensis*

In this study, the dynamic range for the parasite load quantification in triatomine intestine samples observed was from 10^6 to 1 parasite equivalents and from 3 to 0.0003 intestine unit equivalents. The observed dynamic extension provided linear quantification in at least 6-log range, for both *T. cruzi* and triatomines, allowing an accurate normalization of parasite load. The linearity coefficients (R^2) were 0.99 for both targets. Also, PCR efficiencies were 97.93% for *T. cruzi* satDNA target and 79.60% for the triatomine 12S ribosomal subunit target, confirming the improved performance of the assay (Figure 2).

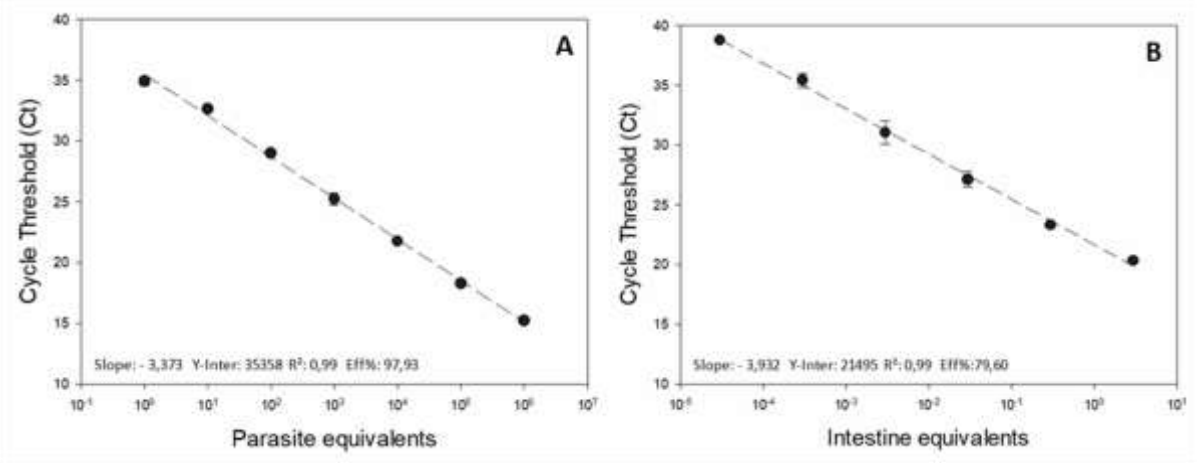

Figure 2: Dynamic range for *Trypanosoma cruzi* and triatomine intestine unit quantification by real-time qPCR. (A) Detection of *T. cruzi* DNA was linear from 10^6 to 1 parasite equivalents; (B) Detection of triatomine DNA was linear from 3 to 0.0003 intestine unit equivalents. Slope, R^2 and amplification efficiency (Eff) are indicated in the chart.

We successfully obtained 117 parasite load quantification in *T. brasiliensis*. Of this, 110 were also genotyped. It was observed a wide range of distribution of parasite load in *T. brasiliensis*, varying from 3.94 to 7.66 x 10^6 *T. cruzi* per insect. The observed median value for parasite load was 2.24 x 10^5. We considered the *p*-value significant at ≤0.1 in face of this remarkable variation in the parasite load among insects (see Supplementary file 1). In general, most of the *T. cruzi* load in the present study was between 10^4 and 10^6 parasites per intestine (Figure 3A), and no samples were below 1 parasite per insect.

3.9. Trypanosome load according to the parasite genotype

The qPCR assays quantified 66/110 *T. brasiliensis* infected by TcI, 36/110 infected by TcII, 2/110 infected by TcIII, 5/110 infected by TcI and TcII and 1/110 infected by TcI, TcII and Tr. Even though the general parasitic load was between 10^4 and 10^6, for TcI a substantial part of the sampling had values below 10^5 whereas the boxplots for TcII had their lowest edge at 10^5 (Figure 3B.1). Therefore, a significant difference was observed for parasite load between *T. cruzi* TcI and TcII lineages (Mann-Whitney Test, U=969, *p*=0.100). The lowest parasite load was 3.94 and 7.1 for TcI and TcII respectively, and the highest was 2,221,050 and 7,660,315. The observed median values for parasite loads were 149,077 and 299,504 for TcI and TcII, respectively (Figure 3B.1).

3.10. Trypanosome load between ecotopes

A total of 67/117 insects from peridomicile and 50/117 sylvatic ecotopes, respectively were successfully quantified as parasite load. No significant difference was observed for TcI and TcII load between ecotopes (Mann-Whitney Test, U = 1180, p = 0.50) (Figure 3B.2).

3.11. Trypanosome load between municipalities

The qPCR assays successfully quantified 40/117 *T. brasiliensis* from CN and 77/117 from MV. There was no significant difference observed for TcI and TcII load between municipalities (Mann-Whitney Test, U=1080, p=0.75) (Figure 3B.3)

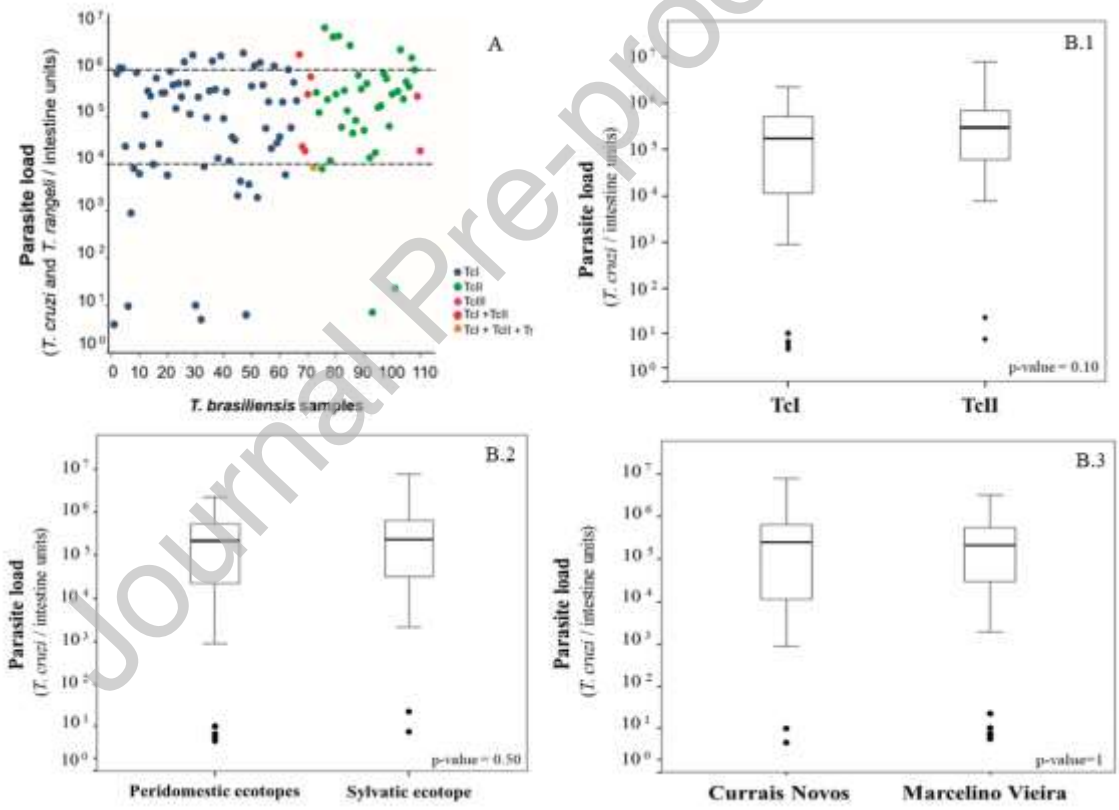

Figure 3: A) Distribution of parasite load (y-axis) per trypanosomes' genotypes in 110 *Triatoma brasiliensis* specimens (x-axis) collected in Currais Novos and Marcelino Vieira municipalities. The dashed line in the graphic represents the distribution of most samples ranging from 10^4 to 10^6 parasites/intestine units. B) Boxplots of TcI and TcII parasite load analyses, considering three variables: B.1) Distribution between TcI and TcII lineages; B.2) Distribution between environment (peridomicile and sylvatic ecotopes); B.3) Distributions between municipalities (Currais Novos and Marcelino Vieira). **p*-value ≤ 0.1 (Mann-Whitney Rank Sum test).

4. Discussion

Northeastern Brazil, one of the poorest and underdeveloped regions in the country, occupies a prominent position in the epidemiological context of ChD. It is the second region in the ranking of infected individuals and all counties located in the semi-arid region are at risk of vectorial transmission of *T. cruzi* (Silveira and Rezende, 1994). In this context, *T. brasiliensis* is considered the most important ChD vector in the region (Costa et al., 2003). The present study employed a molecular epidemiological approach in two areas from RN state, Brazil, where *T. brasiliensis* is a hazard. Both spots present similar eco-epidemiological scenarios with high *T. cruzi* infection rates in *T. brasiliensis* (peri)domestic and sylvatic populations. However, one of these municipalities (Marcelino Vieira) occupies a prominent place, since it was recently (2017) marked by an acute ChD outbreak (Lilioso et al., 2017; Vargas et al., 2018; Monsalve-Lara et al., 2021). This study was pioneer by correlating the *T. cruzi* genotypes with parasitic loads and by employing these approaches (*T. cruzi* genotyping and quantification) to a large sample size distributed across varied geographic and ecotypic scales for field-caught insects. Additionally, we worked also in an area of a recent ChD outbreak, collecting insects in the precise site where it occurred – which adds an epidemiological meaning to the study.

In general, *T. brasiliensis* maintains a high parasitic load in wild and peridomestic environments in both municipalities investigated in this study. The most relevant findings were: i) a clear segregation of DTUs (TcI and TcII) by ecotopes; ii) TcI is the predominant lineage in the study and the peridomestic ecotopes, while TcII prevailed in the sylvatic ecotope; iii) peridomestic and sylvatic cycles of *T. cruzi* transmission overlapped in both areas; iv) TcIII was scarce detected only in two insects from peridomicile; v) *T. rangeli* of lineage A mixed with *T. cruzi* were also found, but rarely detected in *T. brasiliensis*; v) *T. brasiliensis* had overall high *T. cruzi* load (details are provided below), but TcII has twice the mean parasite load of TcI; and vi) there was no significant difference in *T. cruzi* load either per ecotopes or per municipality.

In this study, the distribution of *T. cruzi* load ranged between 10^4 to 10^6 parasites per intestine (*in natura*), with some specimens infected with almost 10^7 *T. cruzi*/intestine units. The only study using this *T. cruzi* qPCR quantification approach in six triatomines species (*T. brasiliensis*, *T. pseudomaculata*, *T. sordida*, *T. wygodizinskyi*, *T. vitticeps* and *P. lutzi*) from three Brazilian biomes revealed a median of 10^3 parasites/intestine units (Moreira et al., 2017). The authors showed that the

parasite load for *T. brasiliensis* collected in Ceará, Brazil did not exceed 10^5 *T. cruzi*/intestine. Just to draw a parallel, to test the oral infection in an experimental study, Silva-dos-Santos et al. (2017) standardized used 10^6 trypomastigotes in 50 μL of parasite suspension orally applied to mice to ensure the oral infection in an experimental study.

The transmission of *T. cruzi* can regularly occur in three epidemiological cycles: domestic, peridomestic and sylvatic; where the protozoan circulates among several mammals' hosts/reservoirs, triatomine species and human hosts. *Trypanosoma cruzi* comprises a set of genotypes (DTUs) displaying different levels of pathogenicity and eco-epidemiological complexity (Monteiro et al., 2010; Dumonteil et al., 2018). Therefore, evaluation of *T. cruzi* DTUs circulation is crucial to understand transmission cycles, vector species involved, geographic distribution and clinical manifestation to some extend (Ramírez et al., 2010; Guhl and Ramírez, 2011; Zingales et al., 2012). Herein, we found TcI as the prevailing DTU in both municipalities, mainly in peridomestic ecotope, whereas TcII was mostly observed in the sylvatic ecotope. Infections by TcII in *T. brasiliensis* collected in wild (sylvatic) environments were already reported in RN state (Câmara et al., 2010; Barbosa-Silva et al., 2016). Although a significant difference was not observed in the distribution of TcI and TcII between municipalities, the analysis including municipalities + ecotopes brought the difference in the distribution to a significant value, corroborating the high effect of ecotopes in these genotypes' distribution. Low infection prevalence of TcI in the sylvatic ecotope and of TcII in the peridomestic ecotopes in both investigated areas were detected here but it was evidenced that no genotypes were ecotypically exclusive. In RN state, Câmara et al. (2013) reported an overlap of TcII-related wild and domestic TcII cycles in *T. brasiliensis*. The higher prevalence of TcII in the sylvatic environment may be explained by the higher diversity of hosts, as shown by Lilioso et al. (2020).

Our results indicate that peridomestic and sylvatic transmission cycles of *T. cruzi* overlapped in both areas, probably by the presence of small synanthropic animals, such as rodents (Sarquis et al., 2006; Almeida et al., 2016) that could mediate the exchange of *T. cruzi* lineages between environments (Cardinal et al., 2008). In both sylvatic and peridomestic habitats, this triatomine is associated with sylvatic and synanthropic animals – likely links for the parasite circulation between environments (Valença-Barbosa et al., 2014a, 2014b). The gene flow between sylvatic, peridomestic and domestic populations of *T. brasiliensis* has been shown in RN (Almeida et al.,

2016) – which poses a challenge for vector control activities since sylvatic foci are uncontrollable and can also explain some exchange of *T. cruzi* genotypes between ecotopes.

Native rodents, such as *Galea spixii* and *Kerodon rupestris* (Rodentia: Caviidae) are widely distributed throughout the Caatinga biome and are well-known hosts/reservoirs of *T. cruzi* TcI and TcII. These small synanthropic mammals occupy natural ecotopes that are available in the studied area, usually close to domiciles. (Herrera et al., 2005; Roque et al., 2005; Sarquis et al., 2010; Valença-Barbosa et al., 2015). Moreover, the most frequent food source observed in *T. brasiliensis* from peridomicile of the ChD outbreak area in MV was the cavy *G. spixii* (Valença-Barbosa et al., 2014b; Lilioso et al., 2020), which reinforces our hypothesis that native and synanthropic rodents are a crucial link between the wild and domestic environments. The marsupial *Didelphis* spp. (opossum) was previously reported infected by TcI and TcII in northeastern Brazil, and are easily adapted to peridomestic areas, where *T. brasiliensis* forms dense colonies, consequently, maintain the *T. cruzi* transmission cycle in the peridomicile (Herrera et al., 2005; Jansen et al., 2020). Opossums were also found by Lilioso et al. (2020) as food source for infected *T. brasiliensis* populations in RN state but in lower prevalence than cavies.

The TcIII genotype was only found in peridomestic ecotopes in CN municipality, outside the outbreak area. This genotype has been usually associated with sylvatic cycles predominantly associated with armadillos (Morocoima et al., 2012) and occasionally associated with dogs (Brenière et al., 2016). However, it has been an important agent of CD, such as in 26 human cases in Brazil, Paraguay and Venezuela (Brenière et al., 2016). This DTU was previously reported in *T. brasiliensis* in this state (Câmara et al., 2013), and also in the marsupial *Monodelphis domestica* (Didelphimorphia: Didelphidae) in the state of Ceará in northeast Brazil (Bezerra et al., 2014).

Trypanosoma rangeli was detected in the intestine of *T. brasiliensis* collected in peridomestic environment in CN municipality, appearing also mixed with *T. cruzi*. Previous studies demonstrated that different genotypes of both *T. cruzi* and *T. rangeli* may coexist in the same vector and a single host, including humans, monkeys, bats, rodents, marsupials and xenarthrans (Maia da Silva et al., 2004, 2007; Espinosa-Álvarez et al., 2018). *Trypanosma rangeli* is common in *Rhodnius* species (which included *R. nasutus* that occurred in the caatinga) and is prevalent from the northwestern region of

South America, being spread from Brazilian Amazonia to Central America. Despite being considered non-pathogenic to humans, this trypanosome is of epidemiological importance due to false-positive in microscopical and serological *T. cruzi* diagnosis (Guhl et al., 1985; Zuniga et al., 2007). The finding of *T. rangeli* in the digestive tract of a *Triatoma* species is worth emphasizing because according to Dario et al. (2021), this parasite is common for *Rhodnius*, also sometimes infecting *Panstrongylus* species. Additionally, we must stress that this parasite was found in peridomestic *T. brasiliensis* populations, which indicates it is circulating physically close to humans.

We here observed that TcII had a higher parasitic load than TcI and that this first genotype was more frequently found in the sylvatic environment. It is essential to point out that the parasite development and; consequently, their parasite load are inherent to the conditions of each vector or mammalian host (e.g. gut bacterial microbiota) (Azambuja et al., 2005; Buarque et al., 2016). The TcI genotype was prevailing in peridomestic ecotopes but also found in the sylvatic. The lower values of parasitic load for this genotype may be a result of a more ancient co-evolution and enhanced adaptation with *T. brasiliensis*– which brings up a (possible) biological explanation for the difference found. However, additional features that may drive the difference in parasitic load must still be investigated.

The ChD outbreak was reported in the MV municipality, probably by oral transmission through the consumption of cane juice from the same source (Vargas et al., 2018). Although no statistical difference was observed for comparison between municipalities, it is important to highlight that 79% of *T. brasiliensis* collected in sugar cane mill involved in the ChD outbreak had high parasitic loads, ranging from 10^5 to 10^6, and 95% of the insects harboured TcI (Supplementary material 1). According to Roque et al. (2008), the TcI genotype was the one prevalent in the three distinct areas of ChD outbreaks in the states of Santa Catarina, Pará e Ceará states.

Additionally, the similarity of the epidemiological scenarios from both areas points to the possibility of new outbreaks. The high rates of triatomine infestation (Lilioso et al.; 2017) and the close association among *T. brasiliensis*, *T. cruzi* and cavies (Lilioso et al. 2020) may be related to the high parasite load in *T. brasiliensis* here observed and also to the ChD outbreak. However, it is important to highlight that occurrence of ChD is directly associated with how the population occupies and explores the environment where they live since the transmission of this disease is influenced by political, economic, sociocultural, environmental and historical factors. Educational

programs may change the way residents deal with spaces surrounding homes, together with rodent control (e.g., by limiting the availability of suitable refuges, such as large timber or tile/brick piles around houses) to avoid peridomestic infestation foci and, consequently, protecting people (Valença-Barbosa et al., 2014; Monsalve-Lara et al. 2021).

Finally, the higher genetic diversity of trypanosomes detected here may be related to the technique (FFLB) employed, which genotypes the parasites directly from the insect gut (Hamilton et al., 2011). It is widely known that the classic way to genotype the parasite – by isolation and *T. cruzi* cultivation – may lead to the selection of some trypanosome species (*T. rangeli* is often lost in cultures when mixed with Tc) and *T. cruzi* DTUs (Lima et al., 1995; Brenière et al., 2016).

As said in the methods, FFLB technique is unable to distinguish TcII from TcVI. But a large survey in the literature (Marcili et al., 2009; Câmara et al., 2010; Araújo et al., 2011; Barbosa-Silva et al., 2016; Ribeiro et al., 2018; Waniek et al., 2020; Honorato et al., 2021) indicated that TcVI is absent in Northeastern Brazil (Supplementary material 2). Of the 175 *T. cruzi* isolates already genotyped (Supplementary material 2, Table 1) with techniques that are able to detect all lineages, none of than ever found TcVI. Of the total, 77 isolates were TcII (60 harboured by members of *T. brasiliensis* species complex; see Dale et al., 2018) and 13 isolates of TcII were found in Rio Grande do Norte (Supplementary material 2, Table 2). The same set of information was separated for TcI in this complementary material (Supplementary material 2, Table 3) to have a parameter of comparison. To sum, we do not discard the possibility of occurrence of TcVI in the studied region, but consider what we surveyed, if TcVI exists in Northeastern Brazil this genotype is rare.

5. Concluding remarks

We presented here the most comprehensive study on *T. cruzi* genotyping combined with parasitic load, in field-collected *T. brasiliensis*. Our findings showed a clear segregation of DTUs according to ecotopes, with TcI predominance in peridomestic ecotopes, suggesting that this lineage may be responsible for ChD outbreak. The genotype TcI was the main one detected in *T. brasiliensis* collected in the possible site that gave origin to the ChD outbreak in Marcelino Vieira, RN (where the sugar cane mill was located). Nevertheless, TcII was also detected in *T. brasiliensis* from peridomicile in this site; although this genotype was prevailing in insects collected in sylvatic environments. Of high epidemiological importance, *T. brasiliensis* infected

with TcII exhibit a higher parasitic load than those infected by TcI. No genotypes were ectopically exclusive, suggesting the overlap of *T. cruzi* transmission cycles in both areas studied in RN, likely mediated by vector or host´s movements between environments. High levels of *T. cruzi* loads were found in the two investigated areas, suggesting a relevant risk of *T. cruzi* transmission by *T. brasiliensis* that invade human dwellings and eventually new outbreaks everywhere this insect is abundant. Therefore, the multidimensional approach used here allowed us to access diverse, yet crucial elements for better understanding the epidemiological scenarios involving ChD in the Brazilian semiarid Northeastern region.

Acknowledgments

We thank the Platform of Real Time PCR RPT09A from Fiocruz, by the use of the equipment and support; the technicians of Funasa for essential help in the field; in special to Dr. Lúcia Maria Abrantes Aguiar (Secretaria de Estado da Saúde Pública do Rio Grande do Norte, Natal, Brasil) for kindly arranging for all fieldwork. Financial support was provided by the São Paulo Research Foundation (FAPESP process numbers 2017-19420-0, 2016/09176-9), National Council for Scientific and Technological CNPq (434260/2018-5) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001. JG Vergara received PhD fellowships from CNPq (165756/2018-7), OC Moreira and CEA are CNPq Research Productivity Granted (308411/2017-0; 306357/2019-4) and OC is also granted by FAPERJ (JCNE- E-26/203.031/2018).

Abbreviations

ChD: Chagas disease; RN: Rio Grande do Norte state; PCR: Polymerase chain reaction; kDNA: kinetoplast DNA; km: kilometers; FFLB: Fluorescent fragment length barcoding; qPCR: Real-Time quantitative PCR; DTUs: different discreet typing units; TcI: *Trypanosoma cruzi* DTU I; TcII: *Trypanosoma cruzi* DTU II; TcIII: *Trypanosoma cruzi* DTU III; Tr: *Trypanosoma rangeli* genotype A; CN: Currais Novos municipality; MV: Marcelino Vieira municipality; DUs: Dwelling's unities.

Supplementary material 1: Details of each sample analyzed: ID, Municipality, environment, ecotope, insect´s evolutionary stage, Trypanosome genotype and parasite load.

Supplementary material 2: Table 1: Survey of studies that genotyped Trypanosoma cruzi with a method able to discriminate TcII of TcVI infecting different hosts in the Northeastern Brazil; Table 2: Survey of TcII found for the Northeastern Brazil (for details, see sheet "N_Iso_Total"); Table 3: Survey of TcI found for the Northeastern Brazil (for details, see sheet "N_Iso_Total").

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Conceived and designed the experiments: CVB; CEA, OCM and MMGT. Performed the experiments: CVB; MCV; PFA; ABV and JGVM. Field collection: ML. Statistical analyses: MVNA and JN. Facilities and technical support: DCM and FRG. Statistical analyses: MVNA and JN. Data analyses: CVB, OCM, MMGT and CEA. Writing - original draft: CVB. Writing – review and editing: CEA; DCM; FRG; OCM and MMGT.

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