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ORIGINAL ARTICLE

Molecular characterization of *Trypanosoma cruzi* DTUs of the triatomine species in a Chagas disease endemic area

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Abstract *Trypanosoma cruzi* is the etiological agent of Chagas disease, a neglected tropical infection with great public health importance. This protozoan has triatomine insects as vector but may also be transmitted through blood transfusion, organ transplants, ingestion of contaminated food, or congenitally. It has a heterogeneous population classified into Discrete Typing Units (DTUs), TcI–TcVI and TcBat. The aim of this study was to molecularly characterize the DTUs of *T. cruzi* in triatomines from a Chagas disease endemic area in Northeastern Brazil. Triatomines were collected and the gut content was microscopically analyzed to investigate the presence of trypanosomatid flagellates. In addition, digestive tracts of some specimens were dissected and molecularly analyzed through PCR for *Trypanosoma* spp. and sequencing. PCR positive samples were further submitted to a multiplex PCR for DTUs of *T. cruzi*. A total of 117 triatomines were collected, 93.16% being in intradomicile and 6.84% in peridomicile environments. Insects were identified as *Panstrongylus lutzi* (37.60%), *Triatoma pseudomaculata* (26.50%), *Triatoma brasiliensis* (23.08%) and *Panstrongylus megistus* (12.82%). The specimens herein analyzed presented infection rates by *T. cruzi* of 5.49% and 12.09% in parasitological and molecular examinations, respectively. Multiplex PCR screening revealed 70.59% of the TcI genotype, detected in all triatomine species identified in this study and 29.41% of the DTU TcIII/TcIV detected in *P. megistus* and *P. lutzi*. *T. cruzi* infect triatomines in intradomicile and peridomicile environments, which brings attention to the risk of human infections and to the importance of the implementation of surveillance and entomological control actions.

Keywords Discrete typing units · Triatominae · Chagas disease vectors · Northeastern Brazil

Introduction

Protozoa of the Trypanosomatidae Family have been causing important diseases of public health concern for a long time. Amongst these parasites, *Trypanosoma cruzi* (Kinetoplastida: Trypanosomatidae) has been identified as the etiological agent of Chagas disease or American trypanosomiasis, a neglected tropical zoonosis still presents in Latin America. Kissing-bugs (Hemiptera: Reduviidae: Triatominae) act as vectors of this protozoan, mainly in developing countries where poor housing conditions of the population facilitate the infestation of insect vectors (Barbosa-Silva et al. 2019). It may also be transmitted through blood transfusion, organ transplant, ingestion of contaminated food, and congenitally. Currently, it is believed that approximately 8 million people are infected worldwide, with over 10,000 deaths every year (WHO 2021).

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It is known that *T. cruzi* has a heterogeneous population with a genetic diversity currently classified into Discrete Typing Units (DTUs), denoted TcI, TcII, TcIII, TcIV, TcV, TcVI and TcBat (Marcili et al. 2009a; Zingales et al. 2009, 2012). In Brazil, all DTUs have been reported in different biomes (Jansen et al. 2018) and isolated from wild and domestic hosts (Marcili et al. 2009b; Bezerra et al. 2014; Jansen et al. 2018), as well as from triatomines recovered from domestic or sylvatic habitats (Cominetti et al. 2014; Martins et al. 2015; Ribeiro et al. 2016; Barbosa-Silva et al. 2016; Bezerra et al. 2018; Jansen et al. 2018; Lima-Oliveira et al. 2020).

TcI has a wide geographical distribution throughout the American continent and different mammalian (e.g., opossums, rodents, primates, and anteaters) and triatomine species [e.g., *Panstrongylus megistus* (Burmeister, 1835), *Triatoma brasiliensis* Neiva, 1911, *Triatoma pseudomaculata* Corrêa & Espínola, 1964, *Rhodnius nasutus* Ståal, 1859, *Rhodnius pictipes* Ståal, 1872] are involved in the sylvatic cycle (Zingales et al. 2012; Zingales 2018). It has also been isolated from domestic dogs (*Canis lupus familiaris*) (Bezerra et al. 2014). Conversely, TcII is predominantly found in southern and central regions of South America, being rarely reported in North America (Zingales 2018). This DTU has been detected especially in bats, primates, rodents, marsupials, coatis (Jansen et al. 2015, 2018); and triatomine vectors (Lilioso et al. 2017; Dario et al. 2018).

TcIII is geographically distributed from northeastern Venezuela to Argentina, being predominantly associated with the sylvatic cycle (Zingales 2018). This DTU is commonly found in terrestrial and fossorial ecotopes, having armadillos of the genera *Dasypus*, *Chaetophractus* and *Euphractus* acting as main reservoirs (Zingales et al. 2012; Zingales 2018), but they can also be found in marsupials (e.g., *Didelphis* spp., *Monodelphis* spp.), rodents (e.g., *Galea spixii*), and humans (Abolis et al. 2011; Zingales et al. 2012; Martins et al. 2015). Similarly, TcIV is mostly related with the sylvatic cycle, being reported in North and South America. The main hosts for this DTU in South America are wild primates and coatis (*Nasua nasua*), whereas raccoons act as important reservoirs in North America (Zingales 2018).

TcV and TcVI are rare in the wild cycle and data about their host range is scant. In fact, some reports have been performed in mammalian hosts of the genera *Dasypus, Euphractus* and *Octodon* (Zingales et al. 2012). In Brazil, TcVI has been isolated in *T. brasiliensis* (Lima-Oliveira et al. 2020), and both DTUs (TcV and TcVI) have been associated with cardiomyopathy and mega syndromes in humans (Zingales et al. 2012). Lastly, TcBat has been isolated from *Myotis* spp., *Noctilio* sp., and humans (Marcili et al. 2009a; Ramírez et al. 2014), and it was also detected in *Triatoma sordida* (Ståal, 1859) (Cominetti et al. 2014).

Despite of all efforts of Brazilian Health Service, Chagas disease is still a real trouble for indigenous populations living in endemic areas. In some regions the domiciliation of triatomine species increase the risk of human and animal infection as it has been observed with the increase of reports of *T. cruzi* infection in dogs over the last five years. Therefore, investigations on naturally infected *T. cruzi* vectors, as well as the genotypic characterization of this protozoan contribute to the understanding of the eco-epidemiology of Chagas disease, facilitating decisions on preventive measures to reduce the risk for human and animal infections. The aim of this study was to detect different DTUs of *T. cruzi* in triatomines from a Chagas disease endemic area in Northeastern Brazil.

Methods

Study area

The study was performed in the state of Pernambuco, Northeastern region of Brazil (Fig. 1). The area is comprised of 21 municipalities belonging to the micro region of Garanhuns (Latitude 8°53′27″ South and Longitude 36°29′48″ West), and it is featured by a semi-arid climate with average annual temperature of 22 °C (ranging from 17 °C to 30 °C), rainfall mean of 147 mm (ranging from 25 to 295 mm) and air relative humidity of 90%.

All rural communities in this region have similar landscapes, with residences located near forest fragments and the presence of native palm trees. Dogs, cats and chickens are frequently reported in these domiciles.

Triatomine collection and morphological identification

From July 2018 to June 2019, triatomine specimens were actively collected with the aid of tweezers in intradomicile (e.g., bed frames, stored objects, boxes, walls, pictures stuck on walls) and peridomicile (e.g., chicken coops, pigpens, cattle sheds, piles of tiles, wood and bricks) areas that could serve as natural shelter for these insects. Two operators performed each sampling between 8 and 12 am for a period of 30 min. Afterwards, samples were placed in plastic vials and transported to laboratory for morphological identification (Lent and Wygodzinsky 1979). Information about vector species, life stage, site of capture, and municipality were recorded.

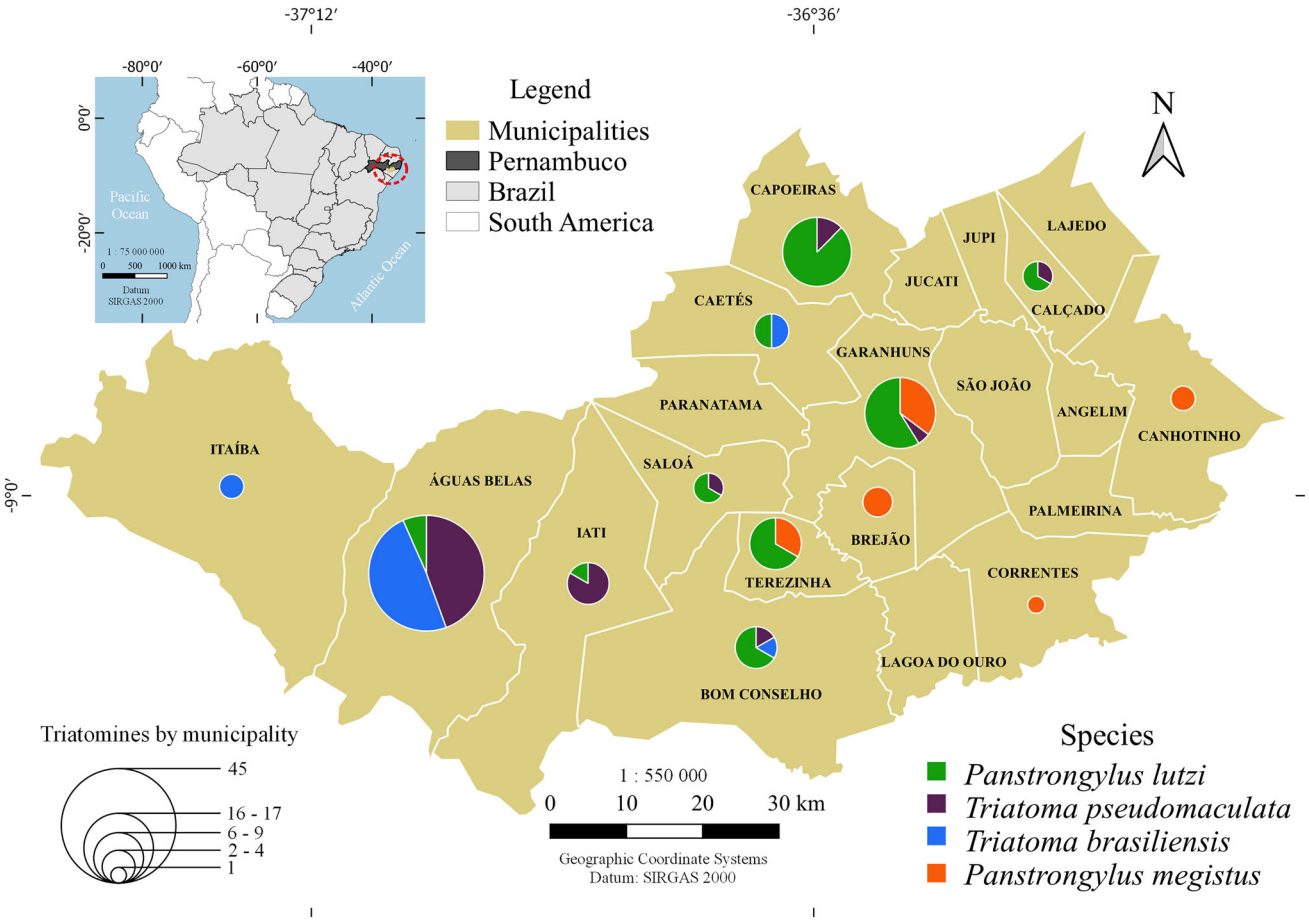

Fig. 1 Study area and distribution of species of triatomines collected in the microregion of Garanhuns, state of Pernambuco, Brazil

Detection of *T. cruzi* infection in triatomines

Microscopic examination

The direct parasitological detection was made by abdominal compression of each insect in 50µL saline solution (0.9% NaCl), that was then examined in an optical microscopic at 400× magnification to investigate the presence of trypanosomatid flagellates.

Molecular analysis

Triatomines were individually dissected and digestive tracts were separated for genomic DNA extraction, which was performed following a previously described protocol (Ramos et al. 2015).

DNA samples were individually tested for *Trypanosoma* spp. through Polymerase Chain Reaction (PCR) using the primers 18ST nF2 (5′-CAACGATGACACCCATG AATTGGGGA-3′) and 18ST nR3 (5′-TGCGCGACCAAT AATTGCAATAC-3′), which amplify a product of 700-800 bp of the 18S rRNA gene (Geysen et al. 2003). Positive (DNA of *T. cruzi* from a triatomine) and negative (DNA of an uninfected triatomine) controls were used in all reactions. PCR products were subjected to electrophoresis in 1.5% agarose gel, stained with GelRed™ (Biotium) and visualized under an UV transilluminator. The amplified products were purified using ExoSAP-IT® (Thermo Fisher Scientific), according to manufacturer's instructions, and sequenced in both directions in an automatic sequencer ABI 3130 Genetic Analyzer (Applied Biosystems), using the Sanger's method (Sanger et al. 1977). DNA sequences were compared with sequences from the GenBank database using the BLASTn search tool (Altschul et al. 1990).

PCR positive samples were further submitted to a multiplex PCR, based on the non-transcribed spacer of the miniexon gene (Fernandes et al. 2001). For this, the set of primers TC1 (5′-ACACTTTCTGTGGCGCTGATCG-3′), TC2 (5′-TTGCTCGCACACTCGGCTG-CAT-3′) and TC3 (5′-CCGCGWACAACCCCTMATAAAAATG-3′) from the intergenic region of *T. cruzi* miniexon, and a common oligonucleotide downstream from the most conserved part of the miniexon gene Me (5′-TACCAATATAG

TACAGAAACTG-3′) were used. These primers amplify products with 200 bp (TcI), 250 bp (TcII/ TcV/TcVI) and 150 bp (TcIII/TcIV) (Fernandes et al. 2001; Aliaga et al. 2011). Positive (DNA of *T. cruzi* from a triatomine) and negative (DNA of an uninfected triatomine) controls were used in all reactions, which were also subjected to electrophoresis as previously described, but in 3% agarose gel.

Results

Out of 117 triatomines, 93.16% (109/117) were collected in intradomicile areas and 6.84% (8/117) in peridomicile areas. All insect samples were classified as adults and identified as *Panstrongylus lutzi* (37.60%; 44/117), *Triatoma pseudomaculata* (26.50%; 31/117), *Triatoma brasiliensis* (23.08%; 27/117) and *Panstrongylus megistus* (12.82%, 15/117) from 57.14% (12/21) of municipalities of the study area. The direct parasitological detection rate was 9.40% (11/117). The infection rate observed in triatomines was 13.33% (2/15) for *P. megistus*, 12.90% (4/31) for *T. pseudomaculata*, 7.41% (2/27) for *T. brasiliensis* and 6.82% (3/44) for *P. lutzi* (Neiva and Pinto 1923).

The digestive tracts of 91 specimens (34 *P. lutzi*, 30 *T. pseudomaculata*, 15 *T. brasiliensis* and 12 *P. megistus*) were molecularly analyzed because in some samples (n = 26) the material was insufficient to perform both analyses. The microscopical examination detected an overall infection rate by *T. cruzi* of 5.49% (5/91), whereas *Trypanosoma* spp. DNA was detected in 26.37% (24/91) of the samples. In particular, 26.47% (9/34) were detected in *P. lutzi*, 20.00% (6/30) in *T. pseudomaculata*, 13.33% (2/15) in *T. brasiliensis* and 58.33% (7/12) in *P. megistus*. Homologies varying from 96.5% to 99.8% were detected with *T. cruzi* sequences available at the GenBank database. The molecular assessment revealed an infection rate by *T. cruzi* of 12.09% (11/91), being 8.82% (3/34) in *P. lutzi*, 10.00% (3/30) in *T. pseudomaculata* and 41.67% (5/12) in *P. megistus*. The DNA sequences herein obtained were deposited in the GenBank under the accession numbers: MN721297, MN721298, MN721299, MN721300, MN721302, MN721303, MN721304, MN721305, MN721306, MN721308, MN721309. It is worth mentioning that the difference between the number of positive samples for PCR (24) and the number of sequences deposited in GenBank (11), was due to the quality of the sequences obtained.

Molecular typing for the miniexon gene of *T. cruzi* was performed for 17 samples, in which *Trypanosoma* spp. DNA was detected. Twelve samples were classified as TcI and the remaining five were classified as DTU TcIII/TcIV (Table 1). All samples of TcI were obtained from triatomines captured from the intradomicile environment. Most of the samples that amplified for TCIII primer (DTU TcIII/TcIV) were triatomines from intradomicile and only one sample from peridomicile environment.

Discussion

This study revealed for the first time the presence of *T. cruzi* DTUs TcI and TcIII/TcIV group in triatomine species collected from a Chagas disease endemic area in Northeastern Brazil (state of Pernambuco), with predominance of the genotype TcI strain detected exclusively in samples collected in intradomicile environment. The genotyping of *T. cruzi* based on a single genetic target had been considered a limitation of the research due to the potential influence of genetic exchange (Zingales et al. 2012). Even though, data herein presented are important and contributes to the epidemiological knowledge of *T. cruzi* genotypes in Brazil.

Different triatomine species (i.e., *P. lutzi, T. pseudomaculata*, *T. brasiliensis* and *P. megistus*), most of them captured in intradomicile areas, were evaluated in this study. This data is supported by the results obtained in a recent research in the Northeastern region of Brazil, which demonstrated that 94.5% of the triatomine specimens were captured in indoor environments (Silva et al. 2019). In general, species of both genera herein detected (i.e., *Panstrongylus* and *Triatoma*) are found in burrows, tree cavities, terrestrial rocky habitats and rodent lairs (Gaunt and Miles 2000). However, they may search for refuge or food sources in artificial environments close to domestic animal shelters, increasing the risk of human and animal infection (Ribeiro et al. 2014; Barbosa-Silva et al. 2019).

In previous study conducted in the same area, *P. lutzi* was also the most frequent species (Silva et al. 2012). This insect has a promiscuous feeding behavior using domestic and synanthropic animals, as well as human as source of blood (Silva et al. 2017). Additionally, it presents high infection rates by *T. cruzi*, demonstrating its epidemiological importance in endemic areas for CD (Silva et al. 2012). On the other hand, *T. pseudomaculata* is found predominantly in tree trunks, and feed mainly on blood of birds. Another important species herein detected, *T. brasiliensis,* has been considered the main vector of *T. cruzi* in Northeastern Brazil. Although, this species had been predominantly associated with rodents (e.g., *Galea spixii* and *Kerodon rupestris*) (Lilioso et al. 2020; Ferreira et al. 2020), it has been proved that they may use a wide variety of blood food sources (e.g., bird, skunk, dog, goat and human) (Silva et al. 2017). The area of study is an important producer of milk in Brazil. Accordingly, the expansion of areas of bovine rearing increase the deforestation, which may be associated reduction of natural

Table 1 Triatomines examined by species and infected with different DTUs of *Trypanosoma cruzi*

Triatomine species	DTU TcI		DTU TcIII/TcIV	
	Positive/Total examined	RF (%)	Positive/Total examined	RF (%)
P. megistus	4/6	66.67	2/6	33.33
P. lutzi	2/5	40.00	3/5	60.00
T. pseudomaculata	5/5	100.00	0/5	0.00
T. brasiliensis	1/1	100.00	0/1	0.00
Total	12/17	70.59	5/17	29.41

DTU: Discrete Typing Units / RF: Relative frequency

habitats of vectors, resulting in an intense invasion of domiciles (Parente et al. 2017; Santos et al. 2020).

The specimens herein analyzed presented infection rates by *T. cruzi* of 5.49% and 12.09% at microscopic and molecular examinations, respectively. Microscopical studies performed in other endemic areas demonstrated lower infection rates in the states of Rio Grande do Norte (2.5%) (Barbosa-Silva, 2019), Ceará (1.4%) (Fidalgo et al. 2018) and Piauí (0.8%) (Gurgel-Gonçalves et al. 2010). Similarly, a molecular investigation performed in the state of Bahia showed an infection rate of 10% (Ribeiro-Junior et al. 2019), also lower than what was observed in the present study. The higher infection rate herein detected in the molecular analyses was an expected finding, since the sensitivity of PCR is very high when compared to microscopic techniques (Dworak et al. 2017). The microscopical analysis cannot be disregarded as it is pivotal in differentiating the stages of protozoan development and consequently the metacyclogenesis rate, which is an important feature related to the dispersion ability of the parasite. Accordingly, the combination of microscopical analysis and molecular tools is advisable to increase accuracy in diagnosis and avoid false negative results (Dworak et al. 2017; Herrera et al. 2021).

The TcI genotype was predominant in all species of triatomines herein identified. It is known that this DTU is commonly found in these invertebrates (Brenière et al. 2016), being highly prevalent in anthropic environments (Lima-Oliveira et al. 2020). In Brazil, TcI has been isolated from *P. megistus* (Ribeiro et al. 2016), *Triatoma petrochiae* Pinto and Barreto, 1925 (Lima-Oliveira et al. 2020), *T. brasiliensis* (Bezerra et al. 2018; Costa et al. 2018; Lilioso et al. 2017; Lima-Oliveira et al. 2020), *Triatoma vitticeps* (Stål, 1859) (Dario et al. 2018), *Triatoma sordida* (Cominetti et al. 2014), *T. pseudomaculata*, *Rhodnius nasutus* (Brito et al. 2008) and *Rhodnius pictipes* (Xavier et al. 2014). Though detected only in single infections in this study, this genotype has been found in mixed infections with TcIV in triatomine species collected across United States, suggesting that the vectors take blood meal from different hosts species, or from a single vertebrate host species co-infected with distinct DTUs (Curtis-Robles et al. 2018). In Brazil, TcI co-infections have been reported in *T. brasiliensis* (TcI + TcII/VI and TcI + *Trypanosoma rangeli* genotype A) (Lima-Oliveira et al. 2020); in açaí samples (TcI + TcIII + TcV + TcVI) (Ferreira et al. 2018) and cardiac tissue from a fatal case of acute oral Chagas disease (TcI + TcII + TcIII + TcIV + *Trypanosoma dionisii*) (Dario et al. 2016). In experimental conditions, TcI presented the highest rate of infection in macrophages, followed by TcII and TcIII (Ribeiro et al. 2018). This demonstrates the complexity of the Chagas disease pathogenesis and the influence of the heterogeneity of different *T. cruzi* strains in the physiopathology of the disease (Ribeiro et al. 2018). Moreover, TcI has been isolated in humans presenting different clinical evolution of Chagas disease (i.e., asymptomatic, severe cardiomyopathy, and in chronic and fatal acute infections) (Abolis et al. 2011; Ramírez et al. 2010; Santana et al. 2014; Oliveira et al. 2017; Calvopina et al. 2020).

Five specimens of *Panstrongylus* spp. amplified for the primer TCIII, which is specific for DTUs (TcIII and TcIV), related to the sylvatic cycle (Zingales 2018). TcIII has already been isolated from *P. lutzi* and from a chronically infected human in Brazil (Abolis et al. 2011). Although predominant in the sylvatic cycle, TcIV has already been isolated in triatomines from the domestic cycle and in humans in Venezuela (Carrasco et al. 2012) and Brazil (Monteiro et al. 2012).

Overall, the genetic diversity of *T. cruzi* is underestimated (Jansen et al. 2020) and the real importance of this knowledge has been neglected in endemic regions of Latin America. In fact, the molecular characterization of these strains is pivotal to better understand the eco-epidemiology of the infection (Brenière et al. 2016). This approach provides important information on host-parasite interactions (Ribeiro et al. 2018). However, the association of these DTUs with vertebrate hosts and different biological cycles should be carefully interpreted (Jansen et al. 2020), since it has not been possible to unequivocally associate *T. cruzi*

genotypes with any biological response variable (biome and environment) or host species (Jansen et al. 2020).

In the state of Pernambuco, Brazil, in a previous study, blood samples from patients with chronic Chagas disease presented TcII and TcVI (Rodrigues-dos-Santos et al. 2018), however, to the best of our knowledge, this is the first molecular analysis of infections and genotyping of *T. cruzi* in triatomines in the studied area, demonstrating a higher infection rate compared to the technique (microscopic examination) commonly used by the National Program of Control of Chagas Disease (PNCDCh). The detection of positive vectors inside or close to human dwellings suggests that people living in this Chagas disease endemic area have potential risks of becoming infected by *T. cruzi*. Additionally, it is an alert for the need of implementing preventive measures such as entomological surveillance to reduce the risk of human and animal infection.

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Author's contribution Conceptualization: TRMS, RANR, Methodology: TRMS, TGR, CAdNR, TARFL, Formal analysis and investigation: TRMS, TGR, CAdNR, Writing—original draft preparation: TRMS, RANR, Writing—review and editing: TRMS, TGR, CAdNR, AS, TARFL, LCA, RANR, GAdC.

Declarations

Conflicts of interest The authors declare that they have no conflict of interest.

Ethics The Ethics Committee on Animal Experimentation of the Federal Rural University of Pernambuco approved all procedures herein performed (approval number 12/2019).

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