

## Investigation regarding the presence of *Trypanosoma cruzi* in triatomines and humans in rural households in the State of Paraná, southern Brazil

Investigação sobre a presença de *Trypanosoma cruzi* em triatomíneos e humanos em domicílios rurais no Estado do Paraná, sul do Brasil

Investigación sobre la presencia de *Trypanosoma cruzi* en triatominos y humanos en hogares rurales en el Estado de Paraná, sur de Brasil

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### Abstract

*Trypanosoma cruzi*, the etiologic agent of Chagas disease (CD), is transmitted by hematophagous insects belonging to the subfamily Triatominae. After elimination of *Triatoma infestans*, the infestation of human dwellings by secondary species of vectors continues to pose a risk of transmission of the parasite. Our aim was to investigate the *T. cruzi* presence in triatomines and humans in rural households in the State of Paraná, southern Brazil. The capture of the insects was carried out by technicians of the municipalities after residents reported the outbreak. Five residents and 27 triatomines captured in four municipalities in the North and Midwest of the state were evaluated. The research of *T. cruzi* was carried out using parasitological, serological, and molecular techniques, in human blood, excreta, intestinal contents and insect macerate. *Panstrongylus megistus*, *P. geniculatus* and *Triatoma* sp. were identified. Ten specimens of *P. megistus* were captured in a house in Mandaguari with five residents and presented an infection rate of 70% for *T. cruzi* like. All residents tested negative for *T. cruzi* infection. Another 15 *P. megistus* were captured in the peridomicile in Janiópolis and had 100.0% positivity. The only adult specimen of *P. geniculatus* captured in the intradomicile in Amaporã, as well as the nymph of *Triatoma* in the peridomicile in Paiçandu, were negative. The

finding of *P. megistus* naturally infected by *T. cruzi* in households in rural area of Paraná demonstrates a potential risk of vector transmission of CD in these regions.

**Keywords:** Chagas disease; Epidemiological surveillance; Southern Brazil.

### Resumo

*Trypanosoma cruzi*, agente etiológico da doença de Chagas (DC), é transmitido por insetos hematófagos pertencentes a subfamília Triatominae. Após eliminação do *Triatoma infestans*, a infestação de habitações humanas por espécies secundárias de vetores continua a oferecer risco de transmissão do parasito. Nosso objetivo foi investigar a presença de *T. cruzi* em triatomíneos e humanos em domicílios da área rural do Estado do Paraná, sul do Brasil. A captura dos insetos foi realizada por técnicos dos municípios após denúncia de foco por moradores. Foram avaliados cinco moradores e 27 triatomíneos capturados em quatro municípios do Norte e Centro-Oeste do estado. A pesquisa de *T. cruzi* foi realizada por meio de técnicas parasitológicas, sorológicas e moleculares, em sangue humano, excretas, conteúdo intestinal e macerado dos insetos. Foram identificados *Panstrongylus megistus* (25 exemplares), *P. geniculatus* (1) e *Triatoma* (1). Dez exemplares de *P. megistus* foram capturados em uma residência em Mandaguari com cinco moradores e apresentaram taxa de infecção de 70% para *T. tipo cruzi*. Todos os moradores apresentaram resultados negativos para infecção pelo *T. cruzi*. Outros 15 *P. megistus* foram capturados no peridomicílio em Janiópolis e apresentaram 100.0% de positividade. O único exemplar adulto de *P. geniculatus* capturado no intradomicílio em Amaporã, assim como a ninfa de *Triatoma* no peridomicílio de Paiçandu, estavam negativos. Apesar dos resultados negativos dos moradores, o encontro de *P. megistus* naturalmente infectado por *T. cruzi* em domicílios da área rural do Paraná demonstra risco potencial de transmissão vetorial da DC nessas regiões.

**Palavras-chave:** Doença de Chagas; Vigilância epidemiológica; Sul do Brasil.

### Resumen

*Trypanosoma cruzi*, el agente etiológico de la enfermedad de Chagas (EC), es transmitido por insectos hematófagos pertenecientes a la subfamilia Triatominae. Después de la eliminación de *Triatoma infestans*, la infestación de viviendas humanas por especies secundarias sigue planteando un riesgo de transmisión del parásito. Nuestro objetivo fue investigar la presencia de *T. cruzi* en triatominos y humanos en hogares rurales en el estado de Paraná, sur de Brasil. La captura de insectos fue realizada por técnicos de los municipios después de los residentes reportaron el brote. Se evaluaron cinco residentes y 27 triatominos capturados en cuatro municipios del Norte y Medio Oeste del estado. La investigación de *T. cruzi* se realizó mediante técnicas parasitológicas, serológicas y moleculares, en sangre humana, excrementos, contenido intestinal y macerado de insectos. Se identificaron *Panstrongylus megistus* (25 especímenes), *P. geniculatus* (1) y *Triatoma* (1). Diez especímenes de *P. megistus* fueron capturados en una casa en Mandaguari con cinco residentes y mostraron una tasa de infección de 70% para *T. tipo cruzi*. Todos los residentes resultaron negativos para la infección por *T. cruzi*. Otros 15 *P. megistus* fueron capturados en los peridomicilio de Janiópolis y mostraron 100.0% de positividad. El único espécimen adulto de *P. geniculatus* capturado en interior en Amaporã, así como la ninfa de *Triatoma* en el peridomicilo de Paiçandu, fueron negativos. A pesar de los resultados negativos de los residentes, el hallazgo de *P. megistus* naturalmente infectado por *T. cruzi* en hogares en el Paraná rural demuestra un riesgo potencial de transmisión vectorial de EC en estas regiones.

**Palabras clave:** La enfermedad de Chagas; Vigilancia Epidemiológica; Sur de Brasil.

## 1. Introduction

*Trypanosoma cruzi*, hemoflagellate protozoan responsible for Chagas disease (Chagas, 1909) affects approximately 7 million people worldwide, mainly in Latin America, where the main route of transmission to humans is associated with the presence of the insect vector (WHO, 2021).

Triatomines are hematophagous insects, belonging to the subfamily Triatominae (Reduviidae, Hemiptera) which has 153 described species and are distributed in 18 genera and five tribes. Among the species, 68 are part of the Brazilian fauna, with potential to transmit *T. cruzi*, being *Triatoma infestans* Klug, 1834, *T. brasiliensis* Neiva, 1911, *T. pseudomaculata* Corrêa & Espínola, 1964, *T. sordida* Stål, 1859, *Rhodnius prolixus* Stål, 1859, *R. neglectus* Lent, 1954, *Panstrongylus geniculatus* Latreille, 1811, and *P. megistus* Burmeister, 1835, the vectors of greatest epidemiological importance in the country (Ravazi et al., 2017; Ministério da Saúde, 2019; Nascimento et al., 2019; Vivas et al., 2021).

The occurrence of triatomines in intra and peridomicile environments has been recorded in rural areas of municipalities in different Brazilian states. In the Northeast region of Brazil, the presence of the species *T. brasiliensis*, *T. pseudomaculata*, *P. megistus* and *P. lutzii* Neiva & Pinto, 1923, was verified in Ceará, with an infection rate for *T. cruzi* of

4.77% (Candido et al., 2019), and in 8.4% of the homes in Pernambuco (Farias et al., 2019). These data demonstrate that continuous entomological surveillance, especially in rural areas, is important to assess the potential risk of vector transmission of Chagas disease.

The State of Paraná, located in southern Brazil, was considered free of vector transmission of *T. cruzi* by *T. infestans* in 2005 (Silveira e Dias, 2011). Since then, five other native species have been found in human dwellings in 39.9% of the municipalities and in 47.8% of them there were insects positive for *T. cruzi* (Ferro e Silva et al., 2018).

In Brazil, the vector control measures implemented by entomological surveillance involve the use of residual insecticides in areas with infestation by triatomines, and were effective in eliminating *T. infestans* (Dias, 2007). The confirmation of *T. cruzi* infection in the triatomines captured in these locations is given by the simple observation of the parasite through light microscopy analysis of the material from the insect's digestive tract. Molecular and genetic evaluation of *T. cruzi*, so important for understanding the epidemiology and pathophysiology of Chagas disease, is generally not performed.

Based on studies that used several molecular markers, *T. cruzi* has been classified into six discrete typing units (DTUs), from TcI to TcVI (Zingales et al., 2009, 2012). In Paraná, both TcI and TcII have been detected in household triatomines, in pure and mixed infections (Spitzner et al. 2007). However, more than 90% of the dozens of *T. cruzi* strains isolated from chronic patients in the state were classified as TcII (Abolis et al., 2011). These data demonstrate the complexity of the transmission cycles of *T. cruzi* and that its genetic heterogeneity must be taken into account in clinical and epidemiological studies on Chagas disease.

In this context, this study aimed to identify the species of triatomines captured in the intra and peridomicile, verify the presence of natural infection of these insects by *T. cruzi* and perform parasitological, serological, and molecular tests on residents from locations where these triatomines were captured, in the rural area of municipalities in the North and Midwest of the State of Paraná, southern Brazil.

## 2. Methodology

### *Study design*

An exploratory and cross-sectional study was carried out from 2016 to 2018, based on the occurrence and active search of triatomine insects captured in the peri and intradomicile of households (Barbosa et al., 2012).

### *Ethical aspects*

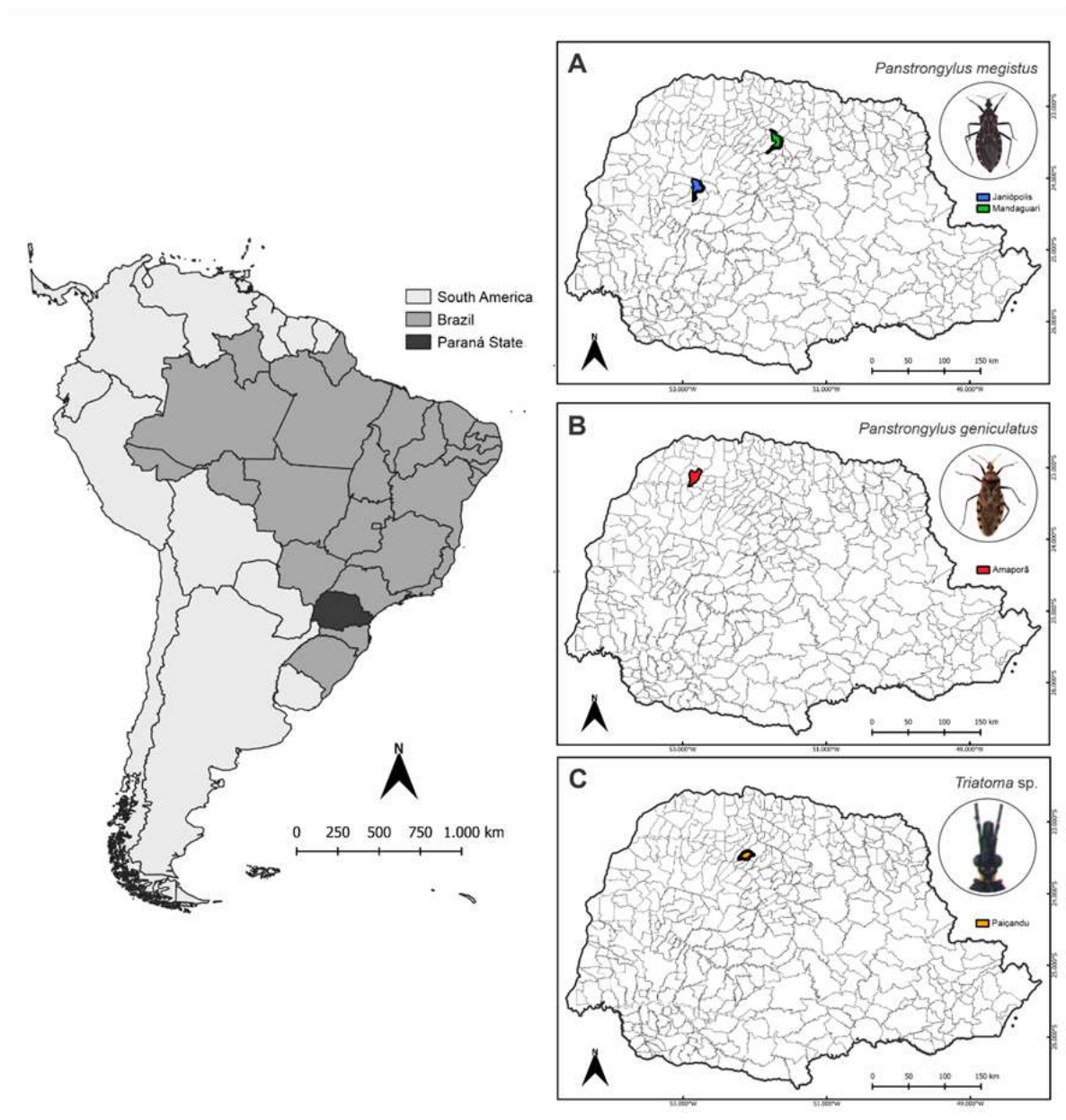
The study was approved by the Standing Committee on Ethics in Research with Human Beings of the State University of Maringá (UEM), registration N°. 2,627,127 (27/04/2018), for collection of data, blood, capture of triatomines present in the intra and peridomicile, isolation of *T. cruzi* and referral for treatment of patients reactive for *T. cruzi*.

### *Study area*

The State of Paraná is located in the southern region of Brazil with an area of 199,307.945 km<sup>2</sup>, with an estimated population of 11,433,957 inhabitants and a demographic density of 52.40 inhabitants / km<sup>2</sup> distributed in 399 municipalities (IBGE, 2019). It has 97.8% of its territorial area included in the Atlantic Forest biome. These municipalities include Amaporã, Mandaguari and Paiçandu, located in the North (IBGE, 2019), and Janiópolis, located in the Midwest of the state (IBGE, 2017) (Figure 1). These municipalities have medium to high climate and landscape suitability for the occurrence of the vector and, consequently, a higher risk of vector transmission of *T. cruzi* (Ferro e Silva et al., 2018).

**Figure 1.** Map showing the municipalities where triatomines were captured in the State of Paraná. A. *Panstrongylus megistus* captured in the municipalities of Janiópolis and Mandaguari; B. *Panstrongylus geniculatus* captured in Amaporã; C. *Triatoma* sp. captured in Paçandu. Triatomine images adapted from Jurberg et al. (2014), Chaves (2019) e Peixoto et al. (2020).

Source:



Authors.

### Identification of the insects

Localities with the presence of foci of triatomines were reported by residents and the active search for insects was carried out by the technical staff of the Department of Environmental Health Surveillance (DVAS), Division of Vector-Borne Disease Control, of the Municipal Health Secretariats and by the researchers (Barbosa et al., 2012).

The insects were captured in rural households in the four municipalities. From a total of 27 insects captured, ten were identified by the DVAS technicians as being of the species *P. megistus*, and the remaining 17 were identified by the researchers, using the Dichotomous Keys for genus and species described by Galvão and Dale (2014) and Justi e Galvão

(2017). Of the latter, 15 were *P. megistus*, one was *P. geniculatus* and one was *Triatoma* sp. It was not possible to identify the species of this last specimen as it was heavily damaged.

#### *Analysis of excreta and intestinal content*

Ten specimens of *P. megistus* were captured in the peridomicile and interior of a residence with five adult residents, in the municipality of Mandaguari. The material obtained by abdominal compression of all insects was submitted to fresh examination (FE) by the DVAS technicians, who provided a report containing the data regarding the species identification and the infection rate for *T. cruzi* like. However, the insects sent to our UEM Chagas Disease Laboratory were not stored in a way that would allow further molecular analysis of the intestinal content (IC) in the present study. Thus, for this focus, only residents were submitted to additional laboratory evaluation to verify the possibility of infection by *T. cruzi*.

Another 15 specimens of *P. megistus* were captured in the peridomicile area, in the municipality of Janiópolis and sent to our laboratory by the DVAS technicians (**Table 2**). Of these, five (one female and four 3rd instar nymphs) arrived alive. The excreta analysis of these insects was performed by abdominal compression and subsequent examination of this material. The dissection with removal of the rectal ampoule and obtaining of IC was performed. FE of excreta and IC was performed according to the Brener's (1962) method with modifications and part of this material was used to perform xenoculture (XC) and stored for further DNA analysis. An aliquot of 5 µL of the excreta pool was subjected to FE under optical microscopy at 400× magnification, to search for flagellated protozoa. For XC, an aliquot of 250 µL of the IC was inoculated into 2.5 mL of LIT (Liver infusion tryptose) culture medium, with and without antibiotic (6.6 mg/mL of penicillin) (Bronfen et al., 1989; Bisugo et al., 1998). Finally, the remaining IC was added to 500 µL of 70 °GL ethyl alcohol and stored at -20 °C until DNA extraction (Sá et al., 2013; Sá et al., 2016).

The remaining 10 specimens of *P. megistus* from Janiópolis were received without life and were stored in bottles containing a 70 °GL ethyl alcohol solution, not allowing the performance of FE and XC. The material previously obtained by abdominal compression of these insects was submitted to microscopic analysis by the DVAS technicians. Thus, it was not possible to remove the rectal ampoule for insect dissection and, for these specimens, DNA extraction was performed with the macerate of the entire insect (Sá et al., 2013).

The only specimen of *P. geniculatus* was an adult male found and captured alive by the resident in the intradomicile in Amaporã (**Table 2**). For this specimen, it was possible to obtain an IC sample through dissection and to perform the parasitological tests (FE and XC) mentioned above, in addition to storing a part of the material in ethyl alcohol 70 °GL for subsequent DNA extraction.

A 5th instar nymph of the genus *Triatoma* was captured in Paiçandu, District of Água Boa. However, due to the state in which it was found, it was not possible to identify the insect at the species level, nor was it possible to collect the IC. Thus, the extraction of *T. cruzi* DNA of this specimen was also performed with the insect entirely macerated.

#### **Conventional polymerase chain reaction (cPCR)**

*T. cruzi* DNA was extracted from the 17 samples of biological material obtained (six IC and 11 macerated), using the conventional phenol/chloroform method with precipitation in 70 °GL ethanol, as described by Macedo et al. (1992). After extraction, the DNA was diluted in Low-TE buffer and stored at -20 °C until its use.

In cPCR, two tests using different molecular markers were used. The first test was performed to identify or confirm the presence of *T. cruzi* in the insect by amplifying, in 35 cycles, the 330-base pair (bp) fragment of *T. cruzi* kinetoplast minicircle DNA (kDNA), using primers 121 and 122. The products of this reaction were revealed by silver staining in a 4.5% polyacrylamide gel, as described by Miyamoto et al. (2006). The second test was performed to identify the *T. cruzi* DTU in the sample. The 24Sa rDNA marker, primers D71 and D72 were used, and amplicons were revealed in a 6% polyacrylamide gel

stained with silver (Souto et al., 1996; Sá et al., 2016). The size of PCR products in bp of *T. cruzi* DTUs for 24Sa rDNA is 110 (TcI, TcIII, and TcV), 120 (TcIV), and 125 (TcII and TcVI) (Zingales et al. 2012).

### ***Parasitological and serological evaluations in humans***

The residents (n=5) of the house in Mandaguari where the triatomines were found, including engorged insects captured in the bed, in which the sheet and pillowcase had blood stains, signed the Informed Consent Term authorizing blood collection and analysis. Thirty milliliters of blood were collected from each of the five residents, 15 days after the insects were found, for the diagnosis of *T. cruzi* infection through different laboratory methods. Two Vacutainer® tubes with clot activator gel were used to obtain serum (10 mL), which was used to investigate anti-*T. cruzi* IgG using Indirect Immunofluorescence (IFI) and Enzyme Linked Immuno Sorbent Assay (ELISA) techniques. Whole blood was also stored in five Vacutainer® tubes with EDTA-K3 (20 mL) for parasitological analysis (fresh examination, thin layer smear stained by Giemsa, and blood culture), and molecular analysis (conventional PCR and real-time PCR).

Serological analysis were performed at the Clinical Analysis Teaching and Research Laboratory (LEPAC) at UEM. Parasitological and molecular analysis were performed at the Chagas Disease Laboratory at the same institution (Gomes et al., 1998, 1999).

Of the whole blood samples placed in Vacutainer® tubes with EDTA-K3, a 5 µL aliquot of each patient was used to perform the fresh blood examination, in duplicate, being read in optical microscope with a magnification of 400× (Brenner, 1962). Then, 10 µL aliquots were used to make thin layer smears stained by Giemsa, in duplicate. Examination by optical microscopy was performed along the entire length of the smear with an immersion objective (1000×).

Afterwards, 5 mL of blood from the same sample were inoculated into 15 mL of LIT culture medium to perform the blood culture. This test was also performed in duplicate and the cultures were examined every two weeks for 120 days, according to previously established protocols (Chiari et al., 1989; Gomes et al., 1999).

### ***Real-time polymerase chain reaction (qPCR)***

The whole blood samples of the residents were used for DNA extraction using the PureLink® Genomic DNA Mini Kit (Invitrogen®), according to the manufacturer's instructions, in order to eliminate contaminants interfering with the qPCR reaction (Caldas et al., 2012; Duffy et al., 2013). The qPCR was performed using the QuantiNova SYBR Green PCR kit (Quiagen) and Rotor-Gene 5 plex thermocycling equipment (Quiagen), with TCZ-F and TCZ-R primers (Valdez et al., 2012). The samples were amplified in the LightCycler® 480 under the following conditions: initial denaturation of 95 °C for 10 seconds, 35 cycles of amplification at 95 °C for 15 seconds and 60 °C for 10 seconds. At the end of each run, melting curve analysis was performed from 65 °C to 97 °C in order to monitor primary dimers or the formation of non-specific products. A standard curve was established using purified *T. cruzi* DNA; serial dilutions ranging from 100 to 0.001 ng of DNA were introduced into the wells of the reaction plate in triplicate and the standard curve was generated using LightCycler®96 software (Cummings & Tarleton, 2003).

### ***Statistical analysis***

The data obtained were entered into a Microsoft Excel 2016 spreadsheet and statistically analyzed using the BioEstat 5.0 and GraphPad Prism 8.3.1 softwares. The chi-square test or G test was used to verify possible associations between the proportions. The significance level used was 5%.

### 3. Results

#### *Species, developmental stages, and insect capture site*

During the investigation period, from 2016 to 2018, 27 insects belonging to three different species of triatomines were captured in the intra and peridomicile of the rural area of four municipalities located in the North and Midwest regions of the State of Paraná. *P. megistus* (n=25) was the species with the highest occurrence, corresponding to 92.6% of the captured insects. Of these, 10 specimens were captured in the intradomicile of a residence located in the municipality of Mandaguari, corresponding to 8 adult insects (five females and three males) and two 5th instar nymphs (**Table 1**). The other 15 specimens (two females, two males, four 3rd instar nymphs and seven insects in the nymphal stage) were captured in an abandoned house wooden in Janiópolis. The only specimen of the species *P. geniculatus*, a male adult, was found by the resident in the intradomicile of a residence in Amaporã, and a 5th instar nymph of the genus *Triatoma* sp. was found in the peridomicile in the district of Água Boa, Municipality of Paiçandu (Table 2).

#### *Parasitological and molecular analysis of triatomines*

The FE technique was performed by the DVAS technicians using the material obtained by abdominal compression of the 25 specimens of *P. megistus* captured. According to the report provided, 7/10 (70%) specimens captured in Mandaguari were positive for *T. cruzi* like (Table 1). Positivity varied significantly ( $p < 0.0001$ ) according to the developmental stage of the insect, and the adult females had a higher infection rate (80.0%) (Table 1).

**Table 1.** Results of the fresh examination (positivity for *Trypanosoma cruzi* like)<sup>a</sup> of the excreta of *Panstrongylus megistus* captured in the intradomicile of a residence in the rural area of the Municipality of Mandaguari, Paraná, in 2018.

Developmental stage	Sex	Number of positive	Total of examined	%
Adult	Female	4	5	80.0
Adult	Male	2	3	66.7
5th instar nymph	-	1	2	50.0
Total		7	10	70.0

<sup>a</sup>Provided by the Department of Environmental Health Surveillance, Division of Vector-Borne Disease Control. Source: Authors.

According to the report provided by the DVAS technicians, all 15 specimens (100%) of *P. megistus* captured in Janiópolis had positive FE for *T. cruzi* like (Table 2). The excreta of five of these insects (one adult female and four third instar nymphs), which were received alive in our laboratory, were re-examined, confirming the infection by morphologically indistinguishable trypanosomes of *T. cruzi*. However, the amount of excreta from these specimens was insufficient for molecular analysis and only the IC of the 15 insects was used to perform the cPCR.

All 15 specimens of *P. megistus* from Janiópolis had a positive cPCR result, as demonstrated by the presence of the 330-bp band of the mini-circle of *T. cruzi* kDNA, confirming the FE results (Table 2).

The number of parasitic forms (PF) observed in the FE of the five specimens received alive ranged from 1,400 to 11,000 PF/0.1 mL among the 3rd instar nymphs, and was 33,500 PF/0.1 mL for the adult female (Data not shown). It was not

possible to carry out the isolation and growth of protozoa by xenoculture from these samples due to strong fungal contamination.

**Table 2.** Results of fresh examination (FE), xenoculture (XC), conventional polymerase chain reaction (cPCR) and discrete typing units (DTUs) of *Trypanosoma cruzi* obtained from excreta, intestinal contents, and macerates of triatomine insects captured in the intra and peridomicile of rural areas in municipalities in the Midwest and North regions of Paraná, between 2016 to 2018.

Sample	Species	Developmental stage	Municipality	FE	XC	cPCR	DTU <sup>a</sup>
PMJ1 <sup>b</sup>	<i>P. megistus</i>	Adult female	Janiópolis	+	-	+	ND
PMJ2 <sup>b</sup>	<i>P. megistus</i>	3rd nymph	Janiópolis	+	-	+	TcI/TcIII/TcV
PMJ3 <sup>b</sup>	<i>P. megistus</i>	3rd nymph	Janiópolis	+	-	+	TcI/TcIII/TcV
PMJ4 <sup>b</sup>	<i>P. megistus</i>	3rd nymph	Janiópolis	+	-	+	ND
PMJ5 <sup>b</sup>	<i>P. megistus</i>	3rd nymph	Janiópolis	+	-	+	ND
PMJ6	<i>P. megistus</i>	Adult female	Janiópolis	+	NR	+	ND
PMJ7	<i>P. megistus</i>	Adult male	Janiópolis	+	NR	+	ND
PMJ8	<i>P. megistus</i>	Adult male	Janiópolis	+	NR	+	ND
PMJ9	<i>P. megistus</i>	Nymphal stage	Janiópolis	+	NR	+	ND
PMJ10	<i>P. megistus</i>	Nymphal stage	Janiópolis	+	NR	+	ND
PMJ11	<i>P. megistus</i>	Nymphal stage	Janiópolis	+	NR	+	ND
PMJ12	<i>P. megistus</i>	Nymphal stage	Janiópolis	+	NR	+	TcI/TcIII/TcV
PMJ13	<i>P. megistus</i>	Nymphal stage	Janiópolis	+	NR	+	TcI/TcIII/TcV
PMJ14	<i>P. megistus</i>	Nymphal stage	Janiópolis	+	NR	+	TcI/TcIII/TcV
PMJ15	<i>P. megistus</i>	Nymphal stage	Janiópolis	+	NR	+	ND
PGA1 <sup>b</sup>	<i>P. geniculatus</i>	Adult male	Amaporã	-	-	-	ND
TAB <sup>c</sup>	<i>Triatoma</i> sp.	5th nymph	Paçandu	NR	NR	-	ND

<sup>a</sup> Determined by PCR of the *24Sa* rDNA gene; PMJ = *P. megistus* from Janiópolis; <sup>b</sup> live insects at the time of exams; PGA = *P. geniculatus* from Amaporã; TAB = *Triatoma* from Agua Boa District; NR = not performed; ND = not determined. Source: Authors.

The IC sample obtained from the specimen of *P. geniculatus* found in Amaporã presented negative results in the parasitological analysis (FE and XC) and in the cPCR, as well as the macerate sample of the specimen of *Triatoma* sp. from the District of Água Boa, Paçandu, which was also negative in the cPCR (Table 2). These results made it impossible to genotype the DTU of *T. cruzi*, as well as for the samples of the 10 specimens from Mandaguari.

The biological material used in the genotyping of the DTU of 17 samples of *P. megistus* (15), *P. geniculatus* (1) and *Triatoma* (1), was the IC for six samples and macerated for 11 samples. Of the 15 *P. megistus* samples, PCR genotyping of the *24Sa* rDNA gene from *T. cruzi* was achieved in 2/5 (40%) of the samples of IC from the specimens received alive and in 3/10 (30%) of the samples of total macerate of the insect (Table 2). DNA fragments with 110 bp, characteristic of TcI/TcIII/TcV, were detected, indicating the presence of these DTUs of *T. cruzi* in these triatomines (Table 2).



#### **Laboratory diagnosis of the residents**

Five residents, three men aged between 28 and 85 years and two women aged 57 and 76 years, from the residence located in the rural area of the Municipality of Mandaguari, where ten specimens of *P. megistus* were captured in its interior, agreed to participate in this research and agreed to attend LEPAC, in Maringá, to collect blood. Despite the high infection rate for *T. cruzi* like observed in the insects captured in the intradomicile (70%) and the strong clues that they had bitten and sucked the blood of the residents, the parasitological, molecular, and serological tests performed in these individuals were negative for infection by *T. cruzi*.

#### **4. Discussion**

Even though it is considered a neglected disease, CD should attract more attention from public health services, since worsening of the disease can cause work incapacity for activities that require physical effort, and disability, and these aggravations impact both on public health services and in socioeconomic activities (da Silva et al., 2014; WHO, 2021).

Epidemiological surveillance actions employed by government agencies, such as early detection and treatment of acute cases, health education for exposed populations, improvements in housing and its annexes, and chemical control of domiciled vectors, aim at reducing the vectorial transmission of *T. cruzi* (Dias, 2007; Coura and Dias, 2009; Silveira and Dias, 2011; Dias et al., 2016). These prophylactic measures proved to be efficient, so much so that, in 2006, the Pan American Health Organization (PAHO) granted to Brazil the Certification of Interruption of Transmission of Chagas disease by the main domiciled vector, *T. infestans* (Dias, 2007; Silveira and Dias, 2011; Dias et al., 2016).

However, this certification triggered a decline in vector control actions. As a result, secondary species considered wild began to present epidemiological relevance for CD, as currently occurs with the species *T. brasiliensis*, *T. pseudomaculata*, *T. sordida*, *Rhodnius prolixus*, *R. neglectus*, *P. geniculatus* and *P. megistus* (Dias, Ramos, Gontijo, Alejandro, et al., 2016).

This scenario is evidenced in the present study, where 27 specimens of triatomines of three distinct species, *P. megistus*, *P. geniculatus* and *Triatoma* sp. were captured in four municipalities in the State of Paraná. Between 2016 and 2018, the origin of the triatomines captured in the state was the Midwest region, in the Municipality of Janiópolis, with 55.56% (15/27) of the captured specimens, and North, in the municipalities of Mandaguari, Amaporã and Paiçandu, with 44.44% (12/27), with a predominance of the species *P. megistus*, with 92.6% (25/27). Of the total number of triatomines captured, 51.85% were nymphs and 48.15% were adults, with 40.74% in the intradomicile and 59.26%, in the peridomicile. The finding of juvenile stages infected with *T. cruzi* in the intradomicile is associated with a higher risk of transmission of the infection to the residents due to the shorter time between blood meals (Cordero-Montoya et al., 2019).

These results corroborate another study by our research group, which used Ecological Niche Models to predict areas at risk for vector transmission of *T. cruzi* in Paraná (Ferro e Silva et al., 2018), carried out in the period of 2007 to 2013. In this study, *P. megistus* was the most captured species (73%; n = 1,943), followed by *P. geniculatus* (15.4%; 411), *Rhodnius neglectus* (6.0%; 159), *T. sordida* (4.5%; 119) and *R. prolixus* (1.1%; 30). Of the total, 71.9% were captured in the intradomicile and the infection rate by *T. cruzi* was 19.7% of the examined insects.

Until then, *P. megistus* and *P. geniculatus* were considered secondary species of vectors, which began to occupy artificial niches through invasion and infestation of human residences (Cominetti et al., 2013). Among these two species, *P. megistus* stands out as it is currently considered one of the main CD vectors in Brazil. Its presence has been reported in 22/27 (81.48%) Brazilian states, including the State of Paraná, where higher prevalence has been recorded in the North and Northwest regions of the state (Jurberg et al., 2014; Ferro e Silva et al., 2018; Mendes-Sousa et al., 2019).

Among the parasitological techniques used by the researchers in this study, only with the FE it was possible to verify the presence of flagellates in the IC of the examined triatomines, in addition to the excreta. With XC, although it has the advantage of obtaining an isolate of the protozoan, if it is positive, contamination by fungi and bacteria of the insect is a frequent problem. Among the molecular techniques used, cPCR-*kDNA* showed the highest sensitivity, being able to detect the presence of *T. cruzi* DNA in the IC in 100% (15/15) of the analyzed triatomines (*P. megistus* from Janiópolis). With the PCR of the *24Sa rDNA* gene, it was possible to show that TcI/TcIII/TcV was the DTU present in 5/15 (33.3%) of the samples, both from IC (2/5-40%) and from insect macerate (3/10- 30%), making it possible to identify the genetic lineage of *T. cruzi* present in the vector. The lower sensitivity of rDNA PCR may be related to the number of parasitic forms present in the IC and its genetic target compared to the 330 bp *T. cruzi* marker detected by cPCR-*kDNA* (Souto et al., 1996; Gomes et al., 1998; Junqueira et al., 2005). Even though FE is the parasitological technique routinely used to monitor the presence of *T. cruzi* in triatomines by the DVAS technicians, it does not allow the precise identification of the species, as other trypanosomatids could be morphologically similar to *T. cruzi*, which may generate false-positive results (Cominetti et al., 2013). Therefore, the use of molecular techniques are important tools to detect and identify the presence of *T. cruzi* in these insects, thus reducing the occurrence of false-positive results.

In this study, specimens of *P. megistus* captured in two municipalities of Paraná (Mandaguari and Janiópolis) showed a high rate of infection by *T. cruzi* (70.0 to 100.0%). These rates are higher than those recorded by Ferro e Silva et al. (2018), who detected 24.7% positivity for *T. cruzi* in *P. megistus* naturally infected and captured throughout the state. On the other hand, they corroborate other results of the literature (Coura and Borges-Pereira, 2012) in which an average infection rate of 87.9% is observed in *P. megistus* experimentally infected with different strains of *T. cruzi*.

The PCR genotyping of *T. cruzi* rDNA, using both the IC and the macerate of triatomines captured in Janiópolis, showed that the TcI/TcIII/TcV genetic lineages of the protozoan was present in 33.3% (5/15) of the samples in which the amplification occurred. The DTU TcI is the one with the greatest dispersion in the Americas, being associated with the wild and domestic transmission cycles, being also isolated from different genera of wild mammal animals that are reservoirs of the parasite, such as those belonging to the orders Marsupialia, Rodentia, Primate, Chiroptera, Xenarthra, Carnivora and Artiodactyl. DTU TcI is associated with Chagas cardiomyopathy, with the highest incidence of infection in humans occurring in northern South America and Central America (Spitzner et al., 2007; Abolis et al., 2011; Zingales et al., 2012). TcI has also been isolated from *P. megistus* and *T. sordida*, in pure and mixed infections with TcII, and from the synanthropic marsupial *Didelphis albiventris*, naturally infected and from the North and Northwest regions of Paraná. However, the presence of TcI has not yet been recorded in autochthonous patients with CD in the southern region of Brazil, where this state is located. TcIII is associated with the wild cycle, it has been isolated from *P. geniculatus*, and cases in humans are rarely reported. TcV, on the other hand, is associated with the domestic cycle and is rarely found in the wild cycle and they are not fully known (Spitzner et al., 2007; Abolis et al., 2011; Zingales et al., 2012).

CD acquired by vectorial transmission has an incubation period that can vary from 4 to 15 days and the individuals infected may present signs and symptoms ranging from nonspecific (fever, subcutaneous edema, enlarged lymph nodes and hepatosplenomegaly) to specific ones, such as meningoencephalitis, severe myocarditis and death (Dias et al., 2016; Souza et al., 2016). Residents of the house in the rural area of Mandaguari, where the colony of *P. megistus* was found in the intradomicile, did not report, or showed any signs or symptoms suggestive of CD during the period investigated. In addition, these individuals had normal and/or non-reactive parameters in the parasitological, serological, and molecular diagnostic tests performed. According to Dias et al. (2016) and to the Ministry of Health of Brazil (2013, 2017), during the period of immunological window in the acute phase, the presence of immunoglobulin of the IgM class is observed. However, most serological tests available in primary health care detect immunoglobulin of the IgG class, *i.e.*, they are not the most indicated

for the diagnosis of the recent acute phase (Brasil, 2013, 2017). This was the case of the serological tests performed on these individuals. Nevertheless, the result of non-reactive serology was confirmed by using molecular techniques (cPCR and real-time PCR), showing that, based on the results of all analysis performed on these individuals, they were actually negative or non-reactive. The literature also shows that the chance of a person becoming infected after the bite of a triatomine known to be infected with *T. cruzi* is about 25% (Rassi and Junior, 2013; Dias et al., 2016).

On the other hand, a recent investigation carried out by our group regarding the clinical and epidemiological profile of patients with CD showed that of the 270 patients from the Center-North of Paraná evaluated, 64% were women, 60% were aged  $\geq 65$  years and 91% were infected by vector transmission during childhood in Paraná. About 2/3 of them had cardiac and/or digestive signs and symptoms and did not received etiological treatment at the time of diagnosis (Gasparim et al., 2018).

The prevention and control measures determined by the Ministry of Health of Brazil, in locations where suspicious insects were reported/found include orientation to the population, housing improvements, sanitation and application of residual insecticides in locations with infestation, in addition to the sustainable management of the environment and serological inquiry in suspected cases. These strategies are the responsibility of the DVAS of each Brazilian municipality (Brasil, 2017). At the Mandaguari residence, the control measures carried out included active search, orientation to the residents, serological inquiry, and the use of residual insecticides by DVAS technicians to eliminate the infestation of insects.

## 5. Conclusion

Even after the elimination of *T. infestans*, considered the main vector of CD in the past, the presence of vectors domiciled in artificial ecotopes in our country makes it difficult to reduce vector transmission of *T. cruzi*, which may trigger a new epidemiological perspective of CD in endemic regions with controlled transmission, as is the case of the State of Paraná, and non-endemic regions.

The current study shows that the finding of *P. megistus* naturally infected by *T. cruzi* in households in rural Paraná may pose a risk of infection for residents by the TcI/TcIII/TcV genotypes, which can be found in some species of triatomines.

In addition to intensifying vector control actions, as in the residual foci of *T. infestans* in Rio Grande do Sul and Bahia, and secondary species, as in the case of *P. megistus* captured in the State of Paraná, used by entomological surveillance. These actions have a direct impact on the vector transmission of *T. cruzi* to humans, and, consequently, on the other forms of transmission.

It is expected that in future studies it will possible to determine the DTUs of *T. cruzi* circulating in the State of Parana, through genotyping techniques from samples of intestinal content obtained from live triatomine insects or even from macerates of dead insects captured by the Department of Environmental Health Surveillance (DVAS), Division of Vector-Borne Disease Control, of the Municipal Health Secretariats.

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