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Major Article

Trypanosoma cruzi isolated from a triatomine found in one of the biggest metropolitan areas of Latin America

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ABSTRACT

Introduction: To characterize *Trypanosoma cruzi* (TcI) isolated from a *Panstrongylus megistus* specimen found in one of the biggest metropolitan areas of Latin America, the relationship between the TcI group of *T. cruzi* and the transmission cycle in the urban environment was studied. **Methods:** The *T. cruzi* strain, Pm, was isolated in a culture medium from the evolutionary forms present in the hindgut of a live male specimen of *P. megistus* found in the Jabaquara subway in São Paulo City. The sample from the triatomine showed trypomastigote forms of Trypanosomatidae, which were inoculated in the peritoneum of Balb/c mice. The sample was then inoculated in *Liver Infusion Tryptose* medium and J774 cells for the molecular identification and characterization of the parasite. The Pm strain of *T. cruzi* was identified by isolation in axenic culture medium, and based on the morphology, cell infection, growth kinetics, and molecular characterization. **Results:** After isolation, the protozoan was identified as *T. cruzi.* No parasites were detected in the peripheral blood of the animal, which can be a characteristic inherent to the strain of *T. cruzi* that was isolated. Cell invasion assays were performed in triplicate in the J774 cell line to confirm the invasive ability of the Pm strain and revealed amastigote forms of the parasite within macrophages. **Conclusions:** Our biological and molecular characterizations helped understand parasite-host interactions and their evolutionary history in context of the associations between vectors, ecotopes, hosts, and groups of the parasite.

Keywords: *Trypanosoma cruzi*. *Panstrongylus megistus*. Molecular characterization. Chagas disease.

INTRODUCTION

Triatomine species are vectors of *Trypanosoma cruzi*, the etiological agent of Chagas disease, and so far, 148 Triatomine species have been described[1] [2]. Since the description of *new human trypanosomiasis* in 1909 by Chagas[3], who showed that *T. cruzi* infection in *Panstrongylus megistus*, the protozoan has been considered epidemiologically important owing to its wide distribution; the Atlantic Forest is considered its center of dispersion[4]. According to Galvão et al.[5], *Panstrongylus megistus* species have been found in the States of Alagoas, Bahia, Ceará, Espírito Santo, Goiás, Maranhão, Mato Grosso, Mato Grosso do Sul, Minas Gerais, Pará, Paraíba, Paraná, Pernambuco, Piauí, Rio de Janeiro, Rio Grande do Norte, Rio Grande do Sul, Santa Catarina, São Paulo, and Sergipe.

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Epidemiological surveillance in the State of São Paulo focuses on control actions based on the infestation levels registered in systematic searches in infested areas and residences of people who have reported the presence of triatomines[6]. Vector control in São Paulo has been successful since 1990, particularly for *Triatoma infestans*[7]. However, Leite et al.[8] have identified 109 specimens of *T. infestans* in a peridomestic area in the City of Paulínia, which indicates the possibility of reintroduction of species such as *T. infestans, Triatoma sordida,* and *P. megistus,* which showed *T. cruzi* infection rates of 2.7%, 0.6%, and 6.4%, respectively, from 1968 to 2007[7]. The presence of *T. sordida* and *P. megistus* in the State of São Paulo results from their ability to invade and colonize human habitations[4]. After the control of *T. infestans* infection, *P. megistus* stands out in epidemiological terms as an entomological indicator of home infestation, and studies on the density[5] [9] showed that, in the State of São Paulo, about 1% of the *T. sordida* and 9% of the *P. megistus* species tested positive for *T. cruzi* infection. According to Silva et al.[10], *Panstrongylus megistus* is an important vector for *T. cruzi* infection in the state, with high rates of natural transmission. In addition, given that *P. megistus* is native to the Atlantic Forest where Forattini[11] and De Paula et al.[12] examined specimens with high (8.3%) rates of *T. cruzi* infection, surveillance on this species cannot be ignored.

According to Barreto[13], most triatomines retain their primitive wild habits. Although they are often found to be naturally infected, triatomines are rarely or never exposed to humans, and, therefore, play a prominent role in the sylvatic cycle of *T. cruzi*, but have a small epidemiological significance to humans. On the other hand, domiciled or synanthropic species of triatomines live in close contact with humans, making them responsible for the domestic cycle of *T. cruzi* infection; such triatomines are therefore considered important from an epidemiological point of view. Between these two extreme scenarios, there is a series of gradual transitions, as observed in *P. megistus*.

Factors such as species diversity, environmental changes, and residence invasion allow vector-mediated transmission of Chagas disease[14]. For example, in their study in Columbia, Cordovez et al.[15] showed that climatic changes can interfere with the route of vector-mediated transmission of the disease. The domiciliation of triatomines is essentially a dynamic phenomenon and can be explained by the relationship between some species and their natural and artificial ecotopes[13]. The domiciliation of *P. megistus*, which is in line with the adaptability of this species could be the reason for the high rate of *T. cruzi* infection[5]. *Panstrongylus megistus* and small mammals have been found to cohabitate since the beginning of the last century, and the first occurrence of *P. megistus* in natural environments was reported in the State of São Paulo[16]. The presence of *P. megistus* in natural environments represents possible sources of infestation and re-infestation and contributes to the maintenance of the life cycle of *T. cruzi*[17]. *Panstrongylus megistus* was found in the City of São Paulo, which has been known to have a higher rate of infection in triatomines associated with opossums and rodents[18]. Although *P. megistus* can adapt to human habitation and its dependencies, it can also be found in different natural ecotopes participating in the sylvatic cycle of *T. cruzi* owing to its involvement in the transport of the parasite to artificial ecotopes and in the initial infestation or reinfestation of these ecotypes[19]. Forattini et al.[11] found *P. megistus* foci in hollow trees inhabited by bats, rodents, birds and, mainly, opossums. In artificial ecotopes, *P. megistus* feeds on humans, domestic animals (especially dogs and cats), commensal mammals (rats), synanthropic mammals (bats and possums), and birds[20] [21], which results in high infection rates and is also an important link in the domestic transmission cycle of *T. cruzi*[13].

In this study, we report *T. cruzi* infection in a *P. megistus* specimen found in the Jabaquara subway yard in the City of São Paulo. Our finding raises the possibility that this *P. megistus* specimen came from the Fontes do Ipiranga State Park (23°38'55.40"S to 46°37'18.21"O), which is 2km away from the place where the triatomine was found. Our results reemphasize the need for constant surveillance on *T. cruzi* infection in *P. megistus*.

METHODS

Isolation

The *Trypanosoma cruzi* strain, Pm, was isolated in culture medium from the evolutionary forms present in the hindgut of a live male specimen of *P. megistus*. The triatomine was found in the Jabaquara subway, more specifically on the mezzanine hallway, Block H "23°38'41.94 S to 46°38'29.46O" (Office for Logistics and Storage of Products of the Subway, Responsible - Rosane Correa de Oliveira), which is an urban area in the City of São Paulo. The biological material obtained by abdominal compression was diluted in 0.9% saline and observed between the slide and the cover slip under an optical microscope[22]. The sample showed trypomastigote forms of Trypanosomatidae, which were inoculated (0.3mL) in the peritoneum of 23 to 35-day-old Balb/c mice and in *Liver Infusion Tryptose* (LIT) axenic culture medium for the molecular identification and characterization of the parasite. After isolation, the parasite was identified as *T. cruzi*, whose strain was preserved by repeated subculturing in LIT culture medium.

Growth kinetics

The growth dynamics of the epimastigote forms of the Pm strain of *T. cruzi* were studied by inoculating 5×10^6 parasites/mL in 5mL of LIT medium[23]. Triple counting was performed over 10 days on a Neubauer chamber under an optical microscope.

Cell invasion

The assay for parasite invasion in the J774 cells was performed in triplicate[24]. Cells were cultivated in Roswell Park Memorial Institute (RPMI) medium 1640 supplemented with 20% fetal bovine serum (FBS), streptomycin (100µg/mL), and penicillin (100U/mL) in a humid incubator (5% CO_2). For the invasion assay, 1×10^6 parasites were seeded onto a 24-well plate. After 72 hours, the plate was stained with *Giemsa*. The invasion of the J774 cell line by the Pm strain of *T. cruzi* was observed based on the presence of amastigote forms of the parasite within macrophages.

Molecular characterization of the Pm strain of *Trypanosoma cruzi* and evolutionary placement

After deoxyribonucleic acid (DNA) extraction, polymerase chain reaction was performed using 100ng genomic DNA; 100ng of each primer; 200mM of each deoxynucleotide triphosphate (dNTP); 5µL buffer solution (200mM Tris-HCl, pH 8.4, 500mM KCl, and 1.5mM MgCl2); 2.5U Taq DNA polymerase and deionized with bidistilled water (qsp 50µL). The amplification cycle and the temperatures were defined in accordance with the primers used. DNA fragments amplified using polymerase chain reaction (PCR) were subjected to agarose gel electrophoresis (1.5%) and then to sequencing reactions using the Big Dye Terminator kit (Perkin Elmer) in an automated sequencer[25]. The nucleotide sequences obtained from GenBank (http://www.ncbi.nlm.nih.gov/) were used as reference. The San Agustin strain of *Trypanosoma rangeli* was included as an external group.

Ethical considerations

This study was carried out in accordance with the recommendations of the Ethical Committee for Animal Experimentation. The protocol was approved by the Ethics Committee on Animal Use [*Comissão de Ética no Uso de Animais* (CEUA)] of the

Universidade Estadual Júlio de Mesquita Filho (UNESP) at Araraquara, SP (Permit Number: 13/2012). Three Balb/c mice were studied in a controlled light and temperature environment. After the study, the mice were euthanized by CO_2 and the appropriate disposal of the animals was assured by the Faculdade de Ciências Farmacêuticas de Araraquara.

RESULTS

Isolation of the Pm strain of *Trypanosoma cruzi*

The Pm strain of *T. cruzi* was identified by isolation in axenic culture medium, as well as based on its morphology (**Figures 1A** and **B**), cell infection (**Figure 1C**), growth kinetics (**Figure 2**), and molecular characterization (**Figure 3**). Andrade[26] classified *T. cruzi* strains according to biological parameters such as the morphology of trypomastigotes and the mortality rate of the infected animals. The Pm strain of *T. cruzi* was inoculated in the peritoneum of Balb/c mice. No parasites were detected in the peripheral blood of the animals, which can be a characteristic inherent to the TcI group of *T. cruzi*. To evaluate the invasive ability of the Pm strain, cell invasion assays were performed in triplicate in J774 cells, which revealed amastigote forms of the parasite within macrophages.

Growth kinetics

A growth kinetics study of the Pm strain of *T. cruzi* was conducted in triplicate, and the data are shown in **Figure 2**. The results of this study indicate a growth profile similar that of to the Bolivia strain of *T. cruzi*, which belongs to the TcI group, and different from that of the Y strain of *T. cruzi*, which has a higher ability to multiply and belongs to the TcII group.

Molecular characterization and evolutionary placement

To complete the biological characterization and to identify the group that the Pm strain of *T. cruzi* isolated from the *P. megistus* specimen in this study belongs to, the small subunit ribosomal ribonucleic acid (SSU rRNA) gene was amplified, and its initial placement was studied. The amplification of the V7V8 region of the SSU rRNA gene resulted in products of around 900 bp[25] (**Figure 3**). By using a dendrogram built after alignment of the SSU rRNA gene sequences, it was found that the Pm strain belongs to the discrete typing unit (DTU) TcI of *T. cruzi*.

DISCUSSION

Chagas disease has become a zoonosis since triatomines were brought from their wild ecotopes to human environments[12]. The presence of residual foci of *T. infestans* in some Brazilian states and the large number of native species of potential vectors of Chagas disease (*Panstrongylus megistus*, *Triatoma brasiliensis*, *Triatoma pseudomaculata* and *T. sordida*) found in homes are risk factors for the transmission of the disease[4] [27].

This work reports the finding of a live male specimen of *P. megistus* containing potential reservoirs of *T. cruzi* in November 2011 in the Jabaquara subway yard located in the urban area of the City of São Paulo (**Figure 4**) next to the State Park (**Table 1**). In 2003 and 2007, Laboratory-Fauna (Municipality of São Paulo, Municipal Health Secretariat-Responsible - Rosane Correa de Oliveira) received three specimens of *P. megistus* from areas next to the State Park, none of which tested positive for *T. cruzi*. In 2004, 2005, and 2007, another three negative specimens were found about 20km from the State Park (**Table 1**), suggesting different centers of dispersion for the species. The molecular characterization (**Figure 3**) performed in this study showed that the Pm strain of *T. cruzi* belongs to group I-TcI. Therefore, we then focused on the TcI group of *T. cruzi* and its transmission cycle in the urban environment. Previously, Alvarado-Otegui et al.[28] identified the TcI and TcIII groups of *T. cruzi* in 22.7% of the studied mammals using xenodiagnosis and polymerase chain reaction in

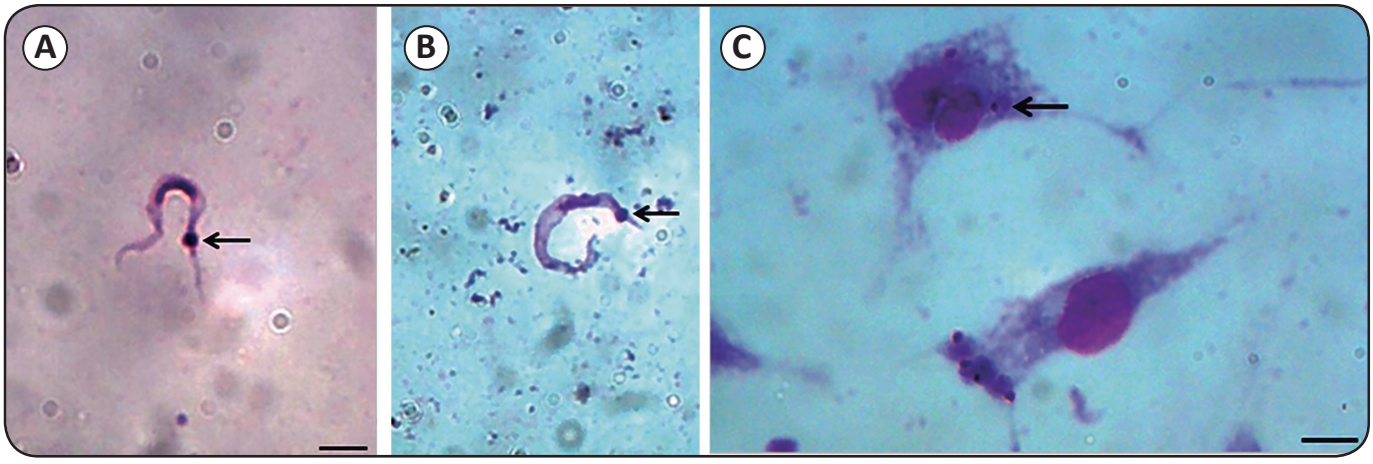


FIGURE 1 - Identification of a *Trypanosoma cruzi* strain isolated from *Panstrongylus megistus*. A, B: Trypomastigote forms of the Pm strain of *Trypanosoma cruzi* observed in axenic culture medium and in the intestinal content of the *Panstrongylus megistus* specimen. C: Arrows show the kinetoplast of the parasite. Amastigote forms of *Trypanosoma cruzi* in the J774 cell line after 72 hours of infection. Bars represent 10μm.

a rural area in Argentina, confirming the presence of TcI in natural wild environments. The invasive ability of *T. cruzi* was observed in the J774 cell line with amastigote forms of the parasite found within macrophages (**Figure 1C**)[23]. Complementing the results of the biological analysis, the molecular characterization of the Pm strain showed that it belongs to *DTU*-TcI (**Figure 3**), which led us to hypothesize on the origin of the sample, given that the TcI group is characteristic of wild environments. Understanding the *T. cruzi-DTU* groups can lead to the elucidation of the epidemiological implications and to the complete understanding of the biology of the parasite[29].

The decrease in the frequency of collection of specimens in the state of São Paulo is probably due to the captures carried out during 1984-1988 and 1992-1993 and the control actions taken along the way[30]. Martins et al.[27] performed searches in two experimental captive sites installed in Marília, São Paulo, for a year and four months, but without successful colonization of wild triatomines. However, the capture of species infected by *T. cruzi* in the urban environment is a reason for concern,

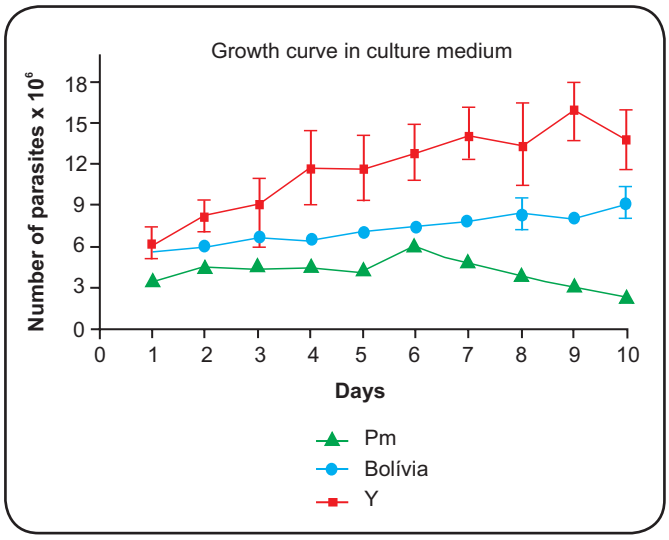
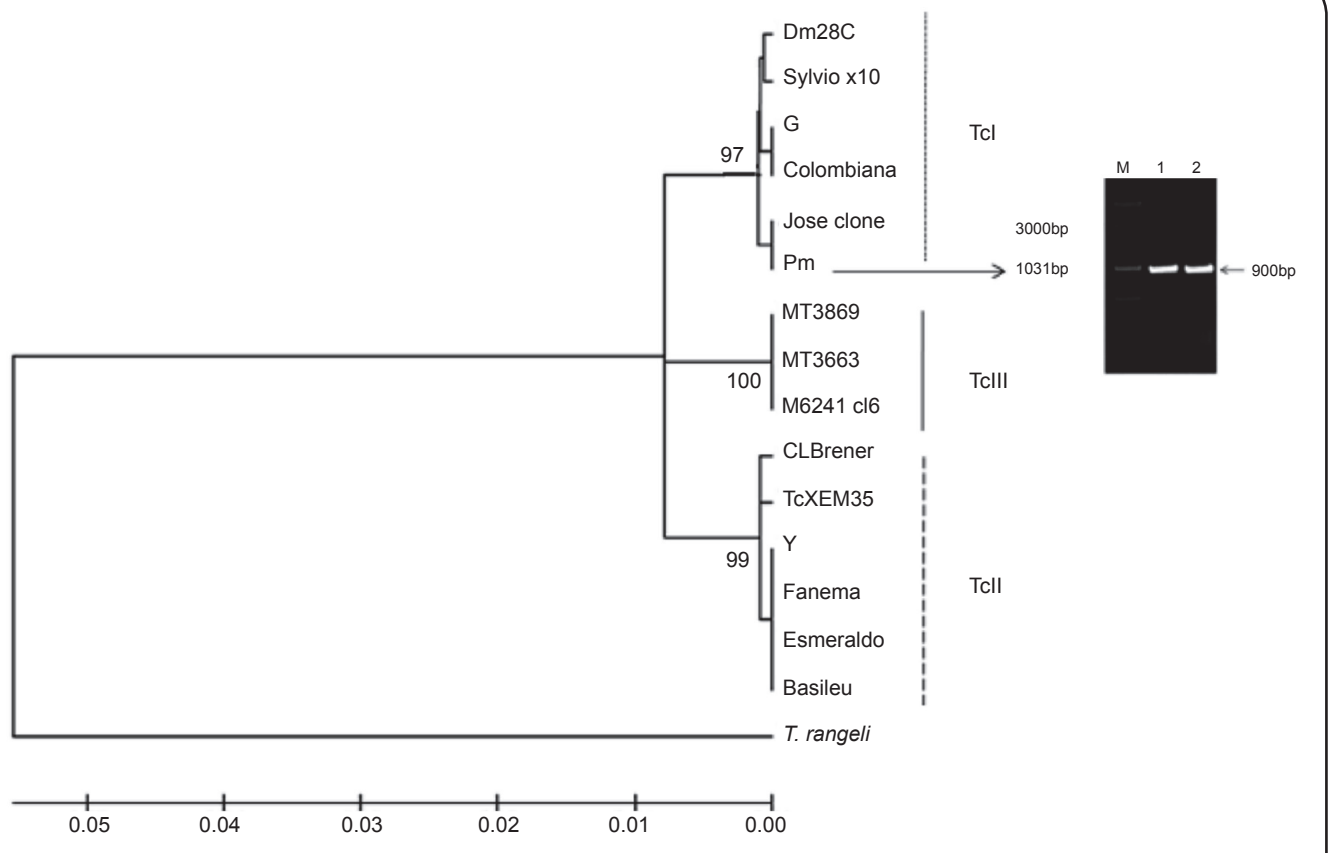


FIGURE 2 - Growth kinetics of the Pm strain of *Trypanosoma cruzi* in Liver Infusion Tryptose culture medium. Pm: strain of *T. cruzi* TcI; **Bolívia:** strain of *T. cruzi* TcI; **Y:** strain of *T. cruzi* TcII.



Sequences from 14 *T. cruzi* isolates included in the phylogenetic analysis from GenBank with acession number in bold: Dm28c **AF245382.1**; Sylviox10 **AF303659.1**; G **AF239981.1**; Colombiana **AF239980.1**; Jose clone **AY785573.1**; CL Brener **AF245383.1**; TcXEM35 **AY785567.1**; Y **AF301912.1**; Famema **AY785584.1**; Esmeraldo **AY785564.1**; Basileu **AY785562.1**; MT3869 **AF303660.1**; MT3663 **AF288660.1**; M6241cl6 **AY785578.1**; and *Trypanosoma rangeli* **AJ012417.1**.

SSU rRNA: small subunit ribosomal ribonucleic acid; **TcI/TcII/TcIII:** discrete typing units *DTUs;* electrophoresis in 1.5% agarose gel stained with GelRed®; **M:** molecular weight marker; **Lane 1 and 2:** Pm strain of *T. cruzi;* **bp:** base pair; The bars represent the expected changes per site.

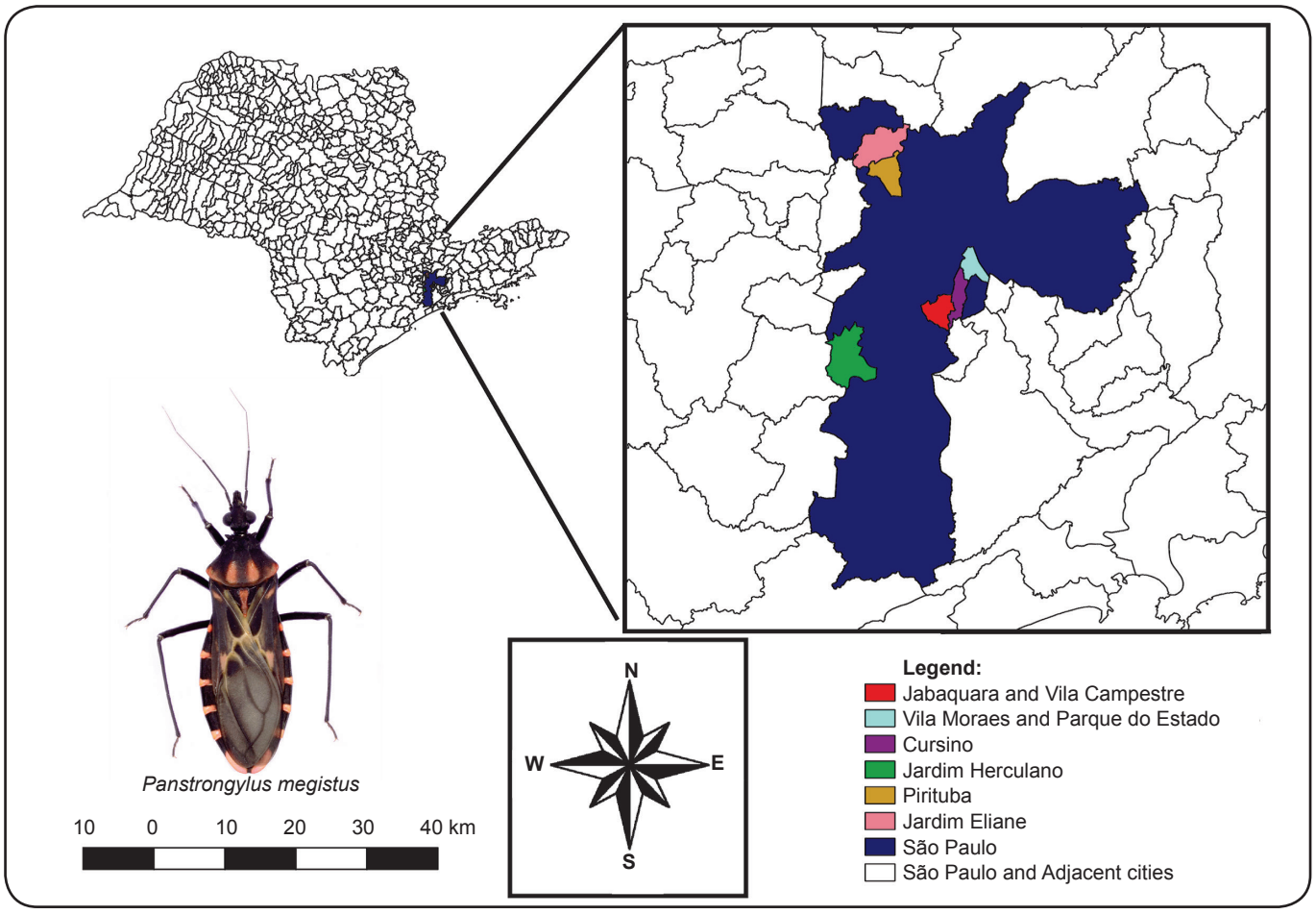


FIGURE 4 - Map showing the municipalities surveyed in the State of São Paulo, Brazil. Triatomine photo by André Teco.

TABLE 1 - Records of *Panstrongylus megistus* received by Laboratory-Fauna since 2003.

Neighborhood	Address	Date	Distance from the State Park
Vila Moraes	1013, Eduardo Ferreira França Street	January 8, 2003	1.6km
Vila Eliane	5, Carlos Jose Spezio Street	March 12, 2004	25km
Jardim Herculano	57, Principal Street	February 15, 2005	23km
Cursino	1103, Jean da La Herta Street	January 4, 2007	2.2km
Jabaquara	215, Luís Rocha Miranda Avenue	March 6, 2007	2.7km
Pirituba	3750, Turística do Jaraguá Road	November 28, 2007	24km
Vila Campestre	134, Francisco de Paula Quintanilha Ribeiro Avenue	November 29, 2011	2km

and it is recommended that cities perform continuous entomological surveillance to detect behavioral changes in the species[4] [5].

Carvalho et al.[31] reported the presence of 72 specimens of *Rhodnius neglectus* in the central area of the City of Monte Alto in São Paulo, which is 374km away from São Paulo City. Their work revealed colonies of *R. neglectus* in palm trees located in the core of the city. The *R. neglectus* specimens found were not carriers of *T. cruzi*, but finding such triatomines in an urban area is an alert for the invasion of species into human environments and the possible transmission of Chagas disease as soon as the epidemiological cycle is completed. This report of *R. neglectus* in palm trees in the central area of Monte Alto was preceded by the study of Rodrigues et al.[32] in Araçatuba, SP wich demonstrates the importance of the palm trees as a natural ecotope of triatomines, specially the *Rhodinus* genre. This finding can be an ecological indicator of the area with risk for Chagas disease.

As mentioned before, *P. megistus* continues to be an important vector of *T. cruzi* in Brazil. The fact that six specimens were found within a period of four years and another one carrying *T. cruzi* was found in the Jabaquara subway yard in November 2011 indicates the need for continuous education on sanitary measures and surveillance services.

The finding of *P. megistus* in an urban area reported in our study raises two possibilities. First, the triatomine has moved from the State Park, which is around 2km from the Jabaquara subway yard. The natural infection by *T. cruzi* can be related to the adaptation between mammals and triatomines living together in forest fragments such as the State Park[33]. Second, *P. megistus* species have been carried by birds that frequent the subway yard. The specimen found may have invaded the office through an open window or may have been transported along with some material coming from different parts of the country. The area does not provide any apparently conducive conditions for triatomine colonization, but the finding of seven specimens indicates the need for continuous surveillance.

Moreover, in this study, of *T. cruzi* was isolated from the *P. megistus* specimen and was characterized as belonging to the *DTU*-TcI group (**Figure 3**). Biological and molecular characterization helps understand parasite-host interactions[34] and their evolutionary history, as demonstrated by a work published by Zingales et al.[29], which discusses associations between vectors, ecotopes, hosts, and the six *DTU*. The authors point out the importance of the relation between *T. cruzi* groups and the ecoepidemiology of Chagas disease, as well as that of understanding the molecular and biological relationship between different strains of the parasite. In this regard, the current study can be helpful for research on the Pm strain of *T. cruzi* isolated from *P. megistus* based on morphology, cell invasion, growth kinetics in culture medium, and molecular characterization.

In conclusion, in this study, we isolated *T. cruzi* from a *P. megistus* found in São Paulo. After isolation, the protozoan was characterized as belonging to the TcI group. Our finding raises the possibility that this *P. megistus* specimen came from the Fontes do Ipiranga State Park, which is 2km away from the place where the triatomine was found. Our results reemphasize the need for constant surveillance on *T. cruzi* infection in *P. megistus* as highlight the importance to search other triatomines specimens that adapted to the human environment and represent epidemiological importance during the transmission of *T. cruzi.* Furthermore, Chagas disease can be controlled through the interruption of the mechanism of transmission, improvement of habitation, education, basic sanitation and treatment. However, in order to have a successful control it is essential to prevent the contact between the vector and the human. Therefore, the maintenance of the epidemiological cycle of the disease would be avoided.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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