

Monitoring *Trypanosoma cruzi* infection in triatomines using PCR in Mato Grosso do Sul, Brazil

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ABSTRACT

Introduction: The aim of the present study was to assess the polymerase chain reaction (PCR) as a method for detecting *Trypanosoma cruzi* infection in triatomines that had been previously determined by microscopic examination in the State of Mato Grosso do Sul, Brazil. **Methods:** In total, 515 specimens were collected. Material from the digestive tract of each triatomine was analyzed for the presence of *T. cruzi* by microscopic examination and PCR using the 121/122 primer set. **Results:** Among the 515 specimens tested, 58 (11.3%) were positive by microscopy and 101 (19.61%) were positive by PCR and there was an association between the results of the techniques ($\chi^2 = 53.354$, $p = 0.001$). The main species of triatomine identified was *T. sordida* (95.5%). **Conclusions:** The use of PCR in entomological surveillance may contribute to a better assessment of the occurrence of *T. cruzi* in triatomine populations.

Keywords: Epidemiology. Triatomine infection monitoring. Polymerase chain reaction. Microscopy examination. *Trypanosoma cruzi*.

INTRODUCTION

Trypanosoma cruzi is a protozoan belonging to the order Kinetoplastida and the Trypanosomatidae family. *T. cruzi* is the causative agent of Chagas disease and is transmitted by triatomines, which are Hemiptera insects of the Reduviidae subfamily that are characterized by hematophagy (both males and females) from juvenile to adult stages¹.

Among the 138 described species of triatomines, only four play a direct role in the epidemiology of the parasite²: *Triatoma brasiliensis* (Neiva, 1911), *Panstrongylus megistus* (Burmeister, 1835), *Triatoma pseudomaculata* (Corrêa and Espínola, 1964) and *Triatoma sordida* (Stal, 1859). In a recent study of Triatominae in the State of Mato Grosso Sul (MS), *T. sordida* was frequently found to be parasitized by flagellate protozoa.³ In the same study, the presence of three major species of triatomines was confirmed in MS: *T. brasiliensis* (Neiva, 1911), *P. megistus* (Burmeister, 1835) and *T. sordida* (Stal, 1859). Infestation rates for domiciliary and peridomestic areas

were only significant for *Triatoma sordida* (9.3% and 86.6%, respectively), whereas *T. brasiliensis* and *P. megistus* exhibited less than 0.2% infestation³.

T. cruzi in triatomines is identified using optical microscopy and material extracted from the digestive tract of the insect. Although this method is inexpensive and widely used, various drawbacks have been reported, particularly in relation to sensitivity and specificity^{4,5}.

The aim of the present study was to evaluate the frequency of infection with *T. cruzi* in triatomines in the State of Mato Grosso do Sul, Brazil using polymerase chain reaction (PCR) and microscopic examination (ME).

METHODS

Study area

Insects were collected in the municipalities of Jaraguari (May to August 2009 and September 2011), Rochedo, Caarapó, Douradina, Antônio João, Dourados, Terenos, São Gabriel do Oeste, Aparecida do Taboado, Paranaíba, Rio Verde de Mato Grosso, Corumbá, Miranda and Aquidauana (August 2011 to November 2012) in the State of Mato Grosso do Sul (**Figure 1**) using the method described by Cominetti et al.

Triatomine identification and microscopic examination

Triatomines were identified using the dichotomous keys proposed by Carcavallo et al. Flagellated protozoa were detected using the method described by Souza.

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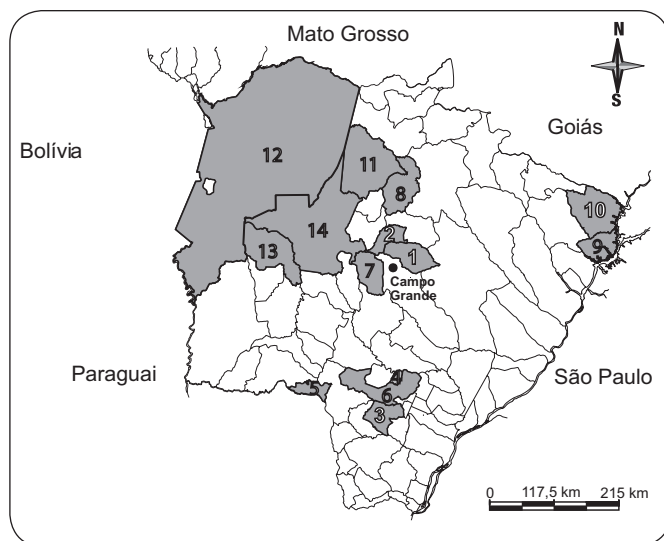


FIGURE 1 - Map of the state of Mato Grosso do Sul, Brazil, highlighting municipalitie s where insects were captured. 1: Jaraguari; 2: Rochedo; 3: Caarapó; 4: Douradina; 5: Antônio João; 6: Dourados; 7: Terenos; 8: São Gabriel do Oeste; 9: Aparecida do Taboado; 10: Paranaíba; 11: Rio Verde de Mato Grosso; 12: Corumbá; 13: Miranda; 14: Aquidauana.

DNA extraction and PCR

Genomic deoxyribonucleic acid (DNA) was extracted from the insects as described by Westenberger et al. The integrity of the DNA sample was determined using 0.8% agarose gel electrophoresis, staining with ethidium bromide (0.5µg/ml) and visualization under ultraviolet light. Additionally, one glass slide containing material from the digestive tract of the insects was analyzed. After the fixed material was scraped from the slide, DNA extraction was performed as described above.

The following primers described by Wincker et al¹⁰ were used for molecular identification of *T. cruzi*: 121 (5'-AAATAATGTACGGG (T/G) GAGATGCATGA-3') and 122 (5'-GGTTCGATTGGGGTTGGTGTAAATATA-3'). This set of primers allows the amplification of 330 bp of kDNA from *T. cruzi*¹⁰. The amplification scheme utilized was previously described by Schijman et al. Under natural conditions, *T. cruzi* and *Trypanosoma rangeli* frequently co-infect triatomines. Therefore, the samples were also subjected to PCR for *T. rangeli* using the primers TrF3 (5'-CCCCATACAAAACACCCTT-3') and TrR8 (5'-TGGAATGACGGTGCGGCGAC-3'), which target a conserved subtelomeric region in *T. rangeli* (SubTr, GenBank accession number: AF426020). The amplification profile utilized was previously described by Chiurillo et al. All amplification reactions were performed on an Eppendorf AG 22331 thermocycler. Control DNA (*T. cruzi* as positive and *T. rangeli* as negative) was kindly donated by Dr. Marta M.G. Teixeira (Universidade de São Paulo, Brazil). Furthermore, ultrapure water was used as an additional negative control.

The reactions were performed in a final volume of 25µl containing 1X PCR buffer [10mM Tris-HCl (pH 8.3), 50mM KCl], 1.5mM MgCl₂, 0.2mM dNTP mix, 5pmol of each primer, 1U of *Taq* DNA polymerase (Platinum®, Invitrogen) and 20ng of genomic DNA.

The amplification products were visualized under ultraviolet light after electrophoresis on an agarose gel (2%) and staining with ethidium bromide.

Statistical analysis

The results were compared using the chi-squared test (χ^2) with a significance level of 5%. Statistical analysis was performed using MedCalc 12.4.0.0.

RESULTS

In total, 515 samples were analyzed. Among the samples, 58 (11.3%) were positive for flagellated protozoa as determined by optical microscopy, and 101 (19.6%) were positive for *T. cruzi* as determined by PCR.

The main species of triatomine identified was *T. sordida*, which represented approximately 95.5% of the specimens collected, thus confirming the findings of Almeida et al. The frequency of *T. cruzi* infection in this species was 10.7% when assessed by microscopy and 18.1% when assessed by PCR.

Association was found between the results obtained by the techniques ($\chi^2 = 53.354$, $p = 0.001$) (Table 1) and the number of positive samples by PCR was higher than by ME.

PCR was the only technique that has found *T. cruzi* in triatomines in the municipalities of Rochedo, Corumbá, Aquidauana and Terenos (Table 2), since ME only confirms the cases in which the parasite is visible in the test, and the PCR also identifies the parasite in samples where this was not seen.

Reactions using primers for *T. rangeli* (data not shown) produced no overlapping data, thereby confirming that the amplicons were specific to *T. cruzi*.

TABLE 1 - Comparison of the results obtained by PCR and microscopy in triatomines from the municipalities of Mato Grosso do Sul, Brazil.

ME	PCR		total
	positive	negative	
Positive	58	0	58
Negative	43	414	457
Total	101	414	515

ME: microscopic examination; PCR: polymerase chain reaction.

DISCUSSION

In Brazil, measures to control triatomine populations are enacted after the detection of insect outbreaks² and involve the use of insecticides in the infested areas. This practice has been effective for many years^{13,14}. Microscopic examination of material from the digestive tract of the insects is routinely performed to monitor the distribution of flagellate protozoa (possibly *T. cruzi*). However, microscopy does not permit

TABLE 2 - Triatomines positive for *T. cruzi* by PCR and microscopy in municipalities of the State of Mato Grosso do Sul, Brazil.

City	Species	Positive		
		n*	ME	PCR
Jaraguari	<i>T. sordida</i>	260	45	76
Rochedo	<i>T. sordida</i>	64	-	3
Caarapó	<i>P. megistus</i>	1	1	1
	<i>P. geniculatus</i>	4	-	1
Douradina	<i>T. sordida</i>	1	-	-
Miranda	<i>T. sordida</i>	7	-	-
Terenos	<i>T. sordida</i>	48	-	1
Rio Verde de Mato Grosso	<i>T. sordida</i>	17	-	-
Aparecida do Taboado	<i>T. sordida</i>	54	10	10
São Gabriel do Oeste	<i>T. matogrossensis</i>	8	-	-
Paranaíba	<i>T. sordida</i>	22	-	-
Dourados	<i>P. megistus</i>	1	**	1
Antônio João	<i>T. sordida</i>	1	-	-
Aquidauana	<i>T. matogrossensis</i>	9	2	5
	<i>T. sordida</i>	12	-	2
	<i>Rhodnius</i> sp.	1	-	-
Carumbá	<i>T. sordida</i>	5	-	1
Total		515	58	101

*number of triatomines captured; ** inconclusive; ME: microscopic examination; PCR: polymerase chain reaction; *P.*: *Panstrongylus*; *T.*: *Triatoma*.

the accurate identification of *T. cruzi* because other species of trypanosomes are morphologically indistinguishable from *T. cruzi*, thereby generating false-positive results. Furthermore, microscopy does not detect the presence of the flagellates in triatomine as PCR, evidenced in the present study.

Advantages of PCR over ME with respect to sensitivity have been reported for *T. cruzi*⁴, *Acanthamoeba* spp.¹⁵, *Plasmodium* spp.¹⁶, *Babesia* spp.¹⁷, *Trypanosoma evansi*¹⁸ and *Leishmania* spp.¹⁹. Advantages related to specificity have also been reported²⁰. Furthermore, PCR is more efficient than ME for the detection of *T. cruzi* in *Triatoma infestans* feeding on patients with Chagas diseases, with a difference of 46% between PCR and ME²⁰. Previous studies have reported differences of 34.9% (*Rhodnius prolixus*), 14.5% (*Triatoma dimidiata*)²¹ and 24.6% (*T. infestans*)⁵ between PCR and ME. In the present study, the difference between the techniques (with respect to the frequency of positive results) was 8.4%. Although this value is lower than that of previous studies, PCR still exhibited highest number of triatomines infected by *T. cruzi* than ME.

A combination of PCR and optical microscopy to monitor *T. cruzi* in triatomines may ensure better results in terms of the presence of the parasite, as shown by the results in the municipalities of Rochedo, Dourados, Corumbá, Aquidauana and Terenos. However, considering the true sensitivity and specificity of microscopic examination, questions may arise

regarding the current distribution of flagellated protozoa among triatomines, e.g., what is the true distribution of the parasite among triatomines?

In the present study, the proportion of false-negative results in microscopic examination was approximately 8.2%. Considering this issue and taking the data published by Almeida et al. regarding the frequency of triatomines positive for *T. cruzi* by ME in the State of Mato Grosso do Sul as an example, the number of insects positive for the parasite should be 724 (8.2%) rather than 15 (0.2%) found by Almeida et al. Similar or higher values have been reported in other studies comparing these techniques^{4,5,20,21}, which demonstrates that the PCR results may be different from the results reported by the current official surveys. Additionally, results obtained from first- and second-stage nymphs using ME are underestimated because of the difficulty in obtaining feces during these stages. This difficulty does not exist when PCR is performed⁵.

In the municipality of Dourados, ME was performed using fixed material on a microscope slide that was previously classified as inconclusive for *T. cruzi*. Inconclusive results in ME may be caused by parasite deformation during preparation on the microscope slide, which hinders the identification of the protozoan. In the present study, the identity of the flagellated protozoa found by microscopy was confirmed by PCR, which demonstrates the effectiveness of this tool, particularly with respect to controversial points that may arise during the identification process. Other studies have shown that PCR may detect the DNA of parasites on microscope slides even years after preparation^{22,23}.

False-positive results are a potential problem with PCR when used for identification of *T. cruzi*²⁴. To reduce the possibility of false-positive results, a negative control was used in the present study. In addition, the different steps of the technique were performed in different rooms.

Although the epidemiological importance of ME is clear in terms of disease control, the use of sensitive techniques, such as PCR, can increase the accuracy of the epidemiological investigation and enable the optimal allocation of financial resources. Furthermore, PCR may be used in epidemiological surveys of other agents of public health importance without significant additional implementation costs.

The frequency of capture of *T. sordida* was higher than that of the other triatomines, which confirms the results of previous entomological surveys in which this species was frequently found^{3,14,25,26}. Although previous studies have reported a low rate of *T. cruzi* infection^{2,3,25}, the present study suggests that these values may be underestimated and that PCR is essential as a tool for detection in official surveys.

Clearly, one detection technique does not exclude the other. The utilization of ME and PCR in combination contributes greater efficacy in identifying outbreaks of the parasite and also a more accurate mapping of its distribution.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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