

Comparison of conventional serology and PCR methods for the routine diagnosis of *Trypanosoma cruzi* infection

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ABSTRACT

Introduction: *Trypanosoma cruzi*, a flagellated protozoan, is the etiologic agent of Chagas disease, and it is estimated that approximately 5 million people in Brazil are infected with this parasite. This work aimed to compare the current diagnostic methods for Chagas disease, including conventional serological (IFAT and ELISA) and molecular techniques (PCR), to introduce PCR as an auxiliary technique. **Methods:** A total of 106 chagasic patients were evaluated: 88 from endemic areas of Paraná, 6 from São Paulo, 3 from Minas Gerais, 3 from Rio Grande do Sul, 1 from Bahia and 5 from the Santa Catarina *T. cruzi* outbreak. The samples were analyzed by conventional serological methods (IFAT, ELISA), hemoculture and PCR to confirm Chagas disease. **Results:** When IFAT was used to determine antibody levels, the sensitivity was 81.7% for patients with the cardiac form of the disease and 100% for the other clinical forms. In contrast, ELISA showed 84% sensitivity and 100% specificity. The use of serological and molecular techniques and their implications for the diagnosis of Chagas disease in non-endemic area are discussed. **Conclusions:** PCR constitutes an excellent support methodology for the laboratory diagnosis of Chagas disease due to its high sensitivity and specificity.

Keywords: Chagas disease. Inconclusive diagnosis. Routine diagnosis.

INTRODUCTION

Chagas disease is an anthroponosis caused by *Trypanosoma (Schizotrypanum) cruzi* (Chagas, 1909). It is estimated that 16 to 18 million people are infected with the parasite in 19 Latin American countries, and that approximately 50,000 individuals will die annually due to Chagas disease¹. In Brazil, approximately 5 million people are infected with *T. cruzi*, with 60% residing in urban areas, similar to other countries^{2,3}. Despite a significant decrease in the incidence of *T. cruzi* infection in humans, numerous chronic chagasic patients still receive medical attention and are treated in such reference centers as the University Hospital of the *Universidade Federal do Paraná*⁴. Asymptomatic *T. cruzi* carriers with positive serology and blood donors with inconclusive serology are often

identified in blood banks. Considering that these individuals do not present clinical signs and receive no definitive diagnosis, it is possible that such individuals carry antibodies against another Trypanosomatidae, including *T. rangeli*.

The World Health Organization recommends two methods that use different principles and different antigens to determine a serological diagnosis of *T. cruzi* infection⁵. The indirect immunofluorescence antibody test (IFAT) and the enzyme-linked immunosorbent assay (ELISA) are widely used due to the difficulty of directly visualizing the parasite in chronic patients⁶. However, serological cross-reactivity may reduce the sensitivity of these tests in regions where other Kinetoplastida (*Leishmania* spp. and *T. rangeli*) parasites are present⁷. Molecular diagnostic methods have enabled the more precise identification of pathogenic agents; indeed, such molecular techniques as the polymerase chain reaction (PCR) have improved diagnostic specificity and the ability to identify the type of parasite circulating in a specific area^{6,8-10}. With regard to Chagas disease, however, PCR has presented highly variable levels of sensitivity. The reasons for this include variations in the sample volume and sample preservation, the methods used for DNA extraction, the primers selected, the quality of the reagents, the thermocycling conditions and the intermittent presence and quantity of circulating parasites¹¹.

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Regardless, PCR is more sensitive than parasitological methods¹² and can be introduced as an auxiliary technique for diagnosing Chagas disease in patients with inconclusive serology. In this work, serology, hemoculture and PCR techniques were simultaneously performed in an attempt to verify the presence of *T. cruzi* in patients with the chronic, acute and indeterminate forms of disease. *Trypanosoma rangeli* was also investigated in patients with positive serology who were negative by PCR for *T. cruzi*.

METHODS

Study population

All the individuals enrolled in the study signed a term of informed consent and filled out an epidemiological questionnaire.

One hundred and six patients from 10 to 89 (average 55.32) years of age were enrolled in this study. Eighty eight out of these came from different endemic areas of Chagas disease of the State of Paraná, Brazil and also five patients from an outbreak in Santa Catarina, six from São Paulo, three from Minas Gerais, three from Rio Grande do Sul and one from Bahia. We included 15 non-infected individuals as negative controls.

Venous blood (30mL) was collected using a vacutainer system (Becton Dickinson) for the following assays: a) 5mL for serology; b) 5mL in ethylenediaminetetraacetic acid (EDTA) for DNA extraction and PCR; c) 20mL in EDTA for hemoculture. All the patients were surveyed in different medical specialties at the UPFR University Hospital.

Serological assays

The IFAT assays were performed using IFI-Imunocruzi®, Fluoline M® and Fluoline G® kits (bioMérieux) in accordance with the manufacturer's instructions. Each serum sample was diluted (1/20-1/2560) in phosphate-buffered saline (PBS), pH 7.2 and incubated for 30min at 37°C with *T. cruzi* epimastigotes pre-adsorbed onto immunofluorescence glass slides. The unbound immunoglobulins were removed by washing the slides twice with PBS, followed by incubation with fluorescein-labeled anti-human IgG or IgM conjugates at 37°C for 30min; the unbound conjugates were removed by washing with PBS. The slides were mounted with buffered glycerin (pH 9.5) and were observed under a fluorescence microscope at 400x magnification (Olympus BH2). Positive and negative sera were used as controls. The cutoff for IFI was 1/40.

ELISA was performed using the commercial kit Chagatek® (bioMérieux) in accordance with the manufacturer's recommendations. Briefly, sera were diluted 1/20 in phosphate-buffered saline-Tween 20 (0.05%) containing 1% bovine serum albumin. A 200µL aliquot of diluted serum was added to the wells of a microtiter plate and incubated for 30min at 37°C. After 6 washes in 0.1M PBS to remove the unbound immunoglobulin, the samples were incubated at 37°C with a 1:10 dilution of peroxidase-labeled an anti-human IgG conjugate. Following another washing step, the microplates

were detected with the addition H₂O₂ in 50mM citrate buffer (pH 3.2) and 0.01mM TMB in 0.1N HCl. After a 20min incubation at room temperature, the reaction was stopped by the addition of 2N sulfuric acid. The absorbance of the samples was measured at 450nm using a microplate reader. The mean absorbance of the negative controls plus 0.100 was used for determining the cutoff, in accordance with the manufacturer's instructions. A borderline ELISA result was defined as reactivity within 2 standard deviations of the control.

Hemoculture

Hemoculture was performed as proposed by Kopp⁴. Briefly, 20mL of heparinized venous blood was collected from each individual in a vacutainer system and centrifuged at 600 x g for 30min at 4°C. The plasma was collected and stored at -20°C. The buffy coat was resuspended in 1mL of physiological serum at 0.9% and distributed into 10 tubes containing CCS medium. The cultures were maintained at 28°C and examined weekly over a three-month period. The positive cultures were divided into two aliquots: the first was grown in LIT medium supplemented with 10% fetal bovine serum for DNA extraction of the parasite, and the remaining aliquot was cryopreserved in liquid nitrogen (-196°C).

DNA extraction

DNA was extracted from two sources: from the epimastigotes of positive cultures and from the patients' blood. The parasites obtained from cultures were washed twice with PBS, and the resulting pellet was stored at -20°C until use. DNA extraction was performed using the protocol proposed by Sambrook et al.¹³, and the resulting the DNA was stored at -70°C until use.

DNA extractions from the patients' blood and artificially contaminated blood were achieved by the BOOM method using a Nuclisens® kit (bioMérieux, France). Briefly, 10µL of buffy coat was resuspended in 900µL of lysis buffer (guanidine thiocyanate buffer solution, 5mol/L) and maintained at 4°C overnight. Then, 50µL of silica gel was added; following homogenization, the mixture was centrifuged for 3min at 10,000 x g. The supernatant was discarded, and the silica containing the immobilized DNA was subjected to successive washing steps to remove any contaminants. The DNA was eluted from the silica and stored at -20°C until use.

PCR technique

To perform the specificity and sensitivity tests, authenticated negative samples were contaminated with parasites from cultures in LIT medium. The *T. cruzi*, *T. rangeli*, *Leishmania (Viannia) braziliensis* and *L. (Leishmania) amazonensis* strains were used to determine specificity. The positive controls consisted of 20ng of *T. cruzi* or *T. rangeli* DNA, and the negative controls consisted of a reaction mixture without DNA.

PCR amplification of the repetitive nuclear sequence using primers TCZ1 and TCZ2

Amplification reactions were performed in a final volume of 25µL containing 10mM Tris-HCl (pH 8.3), 1.5mM MgCl₂, 50mM KCl, 0.01% gelatin, 200µM each dNTP, 25pmol each primer and 0.625 units *Taq* DNA polymerase (Invitrogen,

USA). Different amounts of DNA extracted from the patients' blood (2, 4, and 8 μ L) or 20ng of culture DNA was then added. The cycle amplification was performed using a Thermo Hybaid thermal cycler with the following conditions: 5min at 94°C (initial denaturation), 35 cycles of 1min at 95°C, 1min at 65°C and 1min at 72°C and a final extension of 5min at 72°C. The primer sequences used were TCZ1, 5'CGAGCTCTTGCCACACGGGTGCT3', and TCZ2, 5'CCTCCAAGCAGC GGATAGTTCAGG3'.

PCR amplification of the P542 repetitive sequence of *T. rangeli* using primers R1 and R2

Amplification reactions were performed in a final volume of 25 μ L containing 10mM Tris-HCl (pH 8.3), 1.0mM MgCl₂, 50mM KCl, 0.01% gelatin, 100 μ M each dNTP, 12.5 pmol each primer, 1U *Taq* DNA polymerase and 4 μ L of DNA extracted from the patients' blood or 20ng of culture DNA. The cycling conditions used were as follows: 5min at 94°C (initial denaturation); 2 cycles of 1min at 94°C, 1min at 65°C and 1min at 72°C; 10 cycles of 1min at 94°C, 1min from 65°C to 55°C, with a decrease of 1°C in each cycle, and 1min at 72°C; 18 cycles of 1min at 94°C, 1min at 55°C and 1min at 72°C and a final extension of 5min at 72°C.

The primer sequences used were R1 (F), 5'CGC GGCTCGCACTGCACCTC3', and R2, (R) 5'GGC GCATCCACCGAGCACTG3'.

For all the reactions, the amplified products were subjected to 1.6% agarose gel electrophoresis, stained with ethidium bromide, visualized under UV light and digitally recorded.

Statistical analysis

The degree of agreement between the serology and PCR results was determined by calculating the Kappa (*k*) values with 95% confidence intervals. The analysis of agreement test was analyzed using web-based software developed by *Laboratório de Epidemiologia e Estatística* (FAFESP), which is available online at <http://lee.dante.br/pesquisa/kappa/kappaupload.html>. Kappa values of 0.01 indicated no concordance, values between 0.1 and 0.4 indicated weak concordance, values between 0.41 and 0.60 indicated clear concordance, values between 0.61 and 0.80 indicated strong concordance, and values between 0.81 and 1.00 indicated nearly complete concordance.

Ethical considerations

The study was approved by the Research Ethics Committees of the Federal University of Paraná (1161.009/2006-01), by the *Universidade Positivo* and by CONEP, MS (Register in CONEP: 11163 and Register in: Doc: CAAE: 0105.0.094.000-10 Protocol no. 114/2010).

RESULTS

Serological diagnosis

The comparative results of the two serological methods and PCR for the diagnosis of Chagas disease are shown in **Table 1**. For the IFAT technique, 94 of 106 patients presented positive antibodies, with titers varying from 1/40 to 1/2,560. Two patients (1.60%) presented inconclusive serology (titer 1/20). Four patients from the 2005 outbreak of acute Chagas disease in Santa Catarina State also presented positive IgM serology, with titers of 1/40, 1/80 and 1/640. All the negative controls were below the cutoff level.

Using the ELISA approach, 89 of 106 patients presented positive IgG antibodies. Ten (9.43%) patients presented borderline absorbance and were considered inconclusive cases; the samples with inconclusive serology were all blood bank donors. All the patients that were positive by IFAT were also positive by ELISA, except for the patients with acute disease. Two patients who presented IgM titers of 1/40 and 1/80 had no detectable antibodies by ELISA. All the negative controls were below the cutoff.

Hemoculture

Of the 106 hemocultures conducted using the buffy coat from patients presenting positive serology, eleven (10.4%) were positive. These cases permitted the isolation of five *T. cruzi* strains. Ten cultures were isolated from chronic patients and one from an acute case.

Validation and sensitivity of PCR for *Trypanosoma cruzi* and *Trypanosoma rangeli*

The sensitivity of PCR for the primer pair TCZ1/TCZ2 was determined using negative blood that was infected with the epimastigotes of the reference strain. As expected, amplification of the 188-bp genetic fragment occurred at all dilutions.

TABLE 1 - Samples and results of different laboratory diagnoses.

Group	Number of samples	Positive samples			
		hemoculture	IFI	ELISA	PCR
Cardiac	60	6	49	46	44
Digestive	29	3	29	29	28
Indeterminate	12	1	12	12	4
Acute	5	1	4	2	5
Negative control	10	0	0	0	0
Total	106	11	94	89	81

IFI: indirect immunofluorescence; ELISA: enzyme-linked immunosorbent assay; PCR: polymerase chain reaction.

Therefore, the technique was sensitive enough to detect a single parasite in 10mL of blood. The specificity analysis showed that TCZ1/TCZ2 amplified DNA from *T. cruzi* (strain 152 Y and isolate HC45), *T. rangeli*, *L. braziliensis* and *L. amazonensis*. Therefore, we conclude that this primer pair permits the amplification of PCR fragments from parasites belonging to the order Kinetoplastida (**Figures 1A and 1B**).

Evaluation of the sensitivity of primer pair R1/R2 showed that it was able to detect DNA of a single parasite. Amplifications were conducted with cultures of various trypanosomatids (*T. rangeli*, *T. cruzi* and *Leishmania*) to determine specificity.

As expected, the *T. rangeli* cultures were positive when using primer pair R1/R2, whereas the *T. cruzi* cultures were all negative. Only one culture of *L. (L.) amazonensis* was positive using these primers, but the amplification signal was weak, showing 90% specificity (**Figures 2A and 2B**).

Patient evaluation using the PCR technique

When using the primer pair TCZ1/TCZ2, the expected 188-bp band (**Figure 3**) was detected in 81 of the 106 Chagas patients. Sixty-nine of these patients presented positive PCR and serology, and nine showed inconclusive or positive serology in only one of the serological assays. Two of the seronegative patients were positive by PCR.

None of the individuals with inconclusive serology presented the expected *T. rangeli* band when their blood was subjected to

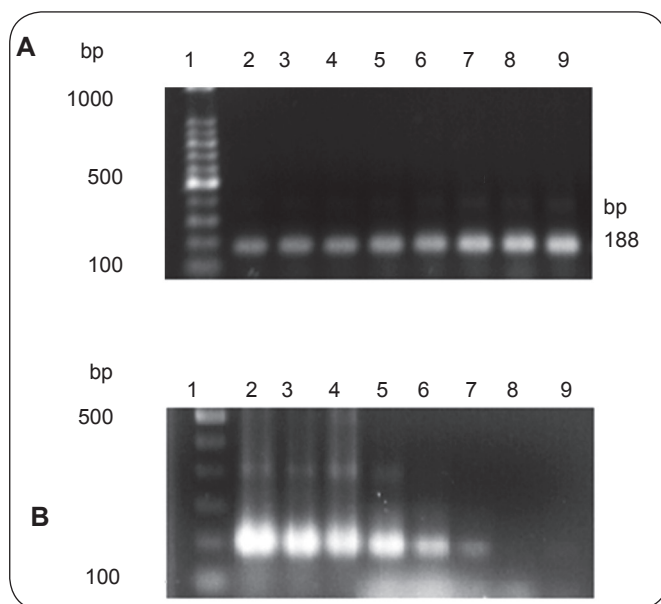


FIGURE 1 - A: Evaluation of the sensitivity of the primer pair TCZ1/TCZ2 for the PCR amplification of *Trypanosoma cruzi* DNA. Lane 1: 100-bp molecular weight standard; Lanes 2 through 9: PCR amplification of *T. cruzi* DNA isolated from 1, 5, 10, 50, 100, 150, 200, 250, 300 and 600 parasites. Electrophoresis was performed using a 1.6% agarose gel. B: Specificity test of the primer pair TCZ1/TCZ2 for the PCR amplification of *T. cruzi* DNA. Lane 1: 100-bp molecular weight standard; Lane 2: reference strain of *T. cruzi* (152 Y); Lane 3: *T. cruzi* isolated from humans (HC 45); Lane 4: *T. rangeli* (strain CHOACHI); Lane 5: *T. rangeli* (SC 58); Lane 6, *L. braziliensis* (CUR 22); Lane 7: *L. amazonensis* (LEM 690); Lane 8: negative control (water); Lane 9: negative control (human blood negative for *T. cruzi*). Electrophoresis was performed using a 1.6% agarose gel. bp: base pairs.

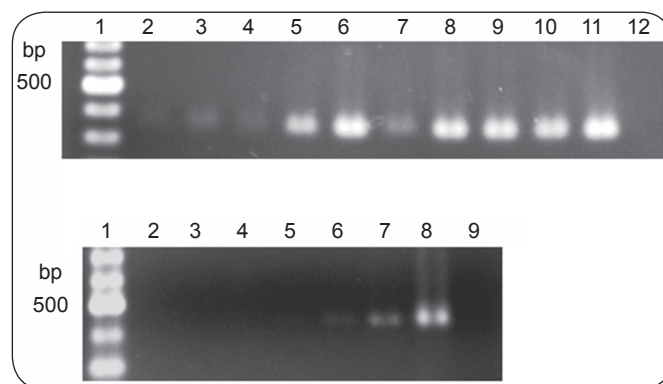


FIGURE 2 - A: Sensitivity test of the primer pair R1/R2. Lane 1: 100-bp molecular weight standard; Lanes 2 through 11: *Trypanosoma rangeli* DNA from 1, 5, 10, 50, 100, 150, 200, 250, 300 and 670 parasites; Lane 12: negative control (water). B: Specificity test of the primer pair R1/R2. Lane 1: 100-bp molecular weight standard; Lane 2: *T. cruzi* (HC 43); Lane 3: *T. cruzi* (HC 10); Lane 4, *T. cruzi* (reference strain 152 Y); Lane 5: *Leishmania braziliensis* (CUR 22); Lane 6: *L. amazonensis* (LEM 690); Lane 7: *T. rangeli* (SC 58 strain); Lane 8: *T. rangeli* (CHOACHI strain); Lane 9: negative control. The expected fragment was 450 bp. bp: base pairs.

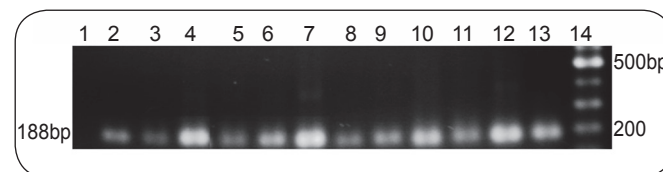


FIGURE 3 - PCR using the primer pair TCZ1/TCZ2 and blood from patients positive for Chagas disease. 1: negative control; 2: acute patient; 3: chronic patient; 4: patient with megaesophagus; 5: asymptomatic patient; 6-8: cardiopathy; 9-10: patient with megaesophagus; 11: patient with cardiopathy; 12: patient with megaesophagus; 13: patient with cardiopathy; 14: 100-bp molecular weight standard. bp: base pairs.

PCR using primer pair R1/R2. None of the negative controls showed amplification fragments specific for *T. cruzi*.

Comparison of molecular and serological methods

Data from the 106 patients were considered to determine the sensitivity and specificity of the laboratory methods in question (**Table 1**).

The sensitivity of IFAT was 81.7% for the cardiac form of the disease and 100% for patients with the digestive and indeterminate clinical forms. For patients from epidemic areas presenting acute disease, 80% were diagnosed using IFAT IgM measurement. The ELISA technique showed 100% sensitivity for patients with the digestive and indeterminate forms of the disease; the sensitivity was 76.7% for patients with the cardiac form. PCR using primer pair TCZ1/TCZ2 showed 76.4% (81/106) positivity for the cardiac patients. A specificity of 100% was observed for all the tests.

The calculated Kappa value with a 95% confidence interval was $k = 0.27$ (0.093; 0.439) for IFAT and PCR, $k = 0.23$ (0.05; 0.42) for ELISA and PCR and $k = 0.80$ (0.615; 0.988) for IFAT and ELISA.

DISCUSSION

The World Health Organization has recently recognized Parana State in southern Brazil as an intradomiciliary transmission-free zone for *T. cruzi*. However, the parasite has been circulating among humans in chronic cases¹⁴, and a sylvatic cycle with risks of occasional transmission has been registered¹⁵. Routine diagnostic techniques in reference laboratories, such as the Central Laboratories (LACENs), are necessary to assist chronic patients or those with an undetermined diagnosis. The techniques used in this work aimed to determine safer means to confirm or invalidate a diagnosis of Chagas disease.

The IFAT technique was sensitive and specific, and ELISA produced inconclusive results more frequently than IFAT. This finding can be explained by the fact ELISA is a qualitative rather than a quantitative test. Nonetheless, serological tests are the gold standard, even for the diagnosis of chronic *T. cruzi* infection, due to their high sensitivity and specificity and because antibody levels remain elevated even decades after infection¹⁶⁻¹⁹.

In this study, hemoculture presented only 10.4% positivity, and the positivity rate is known to vary by geographical region and the stage of illness. While, Luz et al.²⁰ observed 90% positivity in untreated chronic chagasic patients and Fernandes et al.²¹ observed 76% positivity among children infected with *T. cruzi* from the state of Rio Grande do Sul. Indeed, parasitological methods usually present low sensitivity during the chronic phase of Chagas disease^{12,22,23}. *Trypanosoma cruzi* parasitemia is highly variable, and this biological feature can affect the results of parasitological methods.

The positivity rate was 76.4% using primer pair TCZ1/TCZ2 to detect *T. cruzi* DNA by PCR (81/106). According to the clinical classification, the sensitivity of PCR varied from 33.3% to 96.5% (Table 1). Diagnosis was confirmed by PCR in five patients presenting inconclusive serology by ELISA but compatible epidemiology (living in an endemic area), the result was negative by PCR in three cases with inconclusive serology and with no accompanying epidemiology. Different rates of PCR sensitivity have been reported in the literature. Steindel et al.²⁴ demonstrated 100% positivity by PCR for 19 patients from an outbreak of acute Chagas disease in Navegantes, Santa Catarina State. Fernandes et al.²³ showed PCR positivity of 86.7% in a study performed with 240 asymptomatic chronic chagasic patients from the state of Rio Grande do Sul.

The primer pair TCZ1/TCZ2 amplifies a contiguous 188-bp segment of tandem, repetitive DNA sequences, i.e., on the same chromosome. This characteristic allows for an 'all or nothing' phenomenon when only a fraction of the extracted DNA is analyzed²⁵. For this reason, all the samples presenting a negative PCR result were repeated twice using a higher DNA concentration, which resulted in a 50% increase in positivity. Picka et al.²⁶ reported that PCR was the technique most likely to resolve inconclusive serology for chagasic infections. PCR also constitutes an excellent tool for *T. cruzi* detection among transplant or immunosuppressed patients, as antibodies are

often absent in these cases. Because *T. cruzi* takes advantage of suppressed immunity, PCR is the laboratory method of choice to detect circulating parasites, without exacerbating Chagas disease in organ transplant patients.

Recently, an international survey¹¹ evaluated PCR methods for the detection of *T. cruzi*, showing large variations in accuracy and a lack of quality controls worldwide among the 48 reviewed PCR studies. This international collaborative study was launched by expert PCR laboratories from 16 countries from which the four best performance tests were evaluated. The results indicated the limitations of PCR for the diagnosis of patients with chronic disease, and the authors¹¹ recommended PCR only for post-treatment follow-up, the diagnosis of congenital disease in newborns, post-organ transplantation control, AIDS patients and oral transmission patients.

Importantly, chronic patients with negative serology were positive by conventional PCR in our study. Nevertheless, our analysis used fresh blood samples instead of samples that had been stored at 4°C for two years, as was performed by Schijman et al.¹¹.

Public health service reference laboratories (LACENs) are responsible for the diagnosis of infectious diseases, and, as recommended by the Ministry of Health²⁷, two or more methodologies must be available to confirm a diagnosis of Chagas disease. As these laboratories regularly receive many samples awaiting diagnosis confirmations, a standardized PCR method would be very useful.

Our data show that PCR constitutes an auxiliary methodology for the laboratory diagnosis of individuals rejected by blood banks due either to inconclusive serology for *T. cruzi* or when one of the gold standard serology techniques is negative. None of the patients who were positive by serology and negative by PCR for *T. cruzi* (TCZ1/2) were positive for *T. rangeli*.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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