Chapter 12

Detection of *Trypanosoma cruzi* by Polymerase Chain Reaction

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Abstract

American Trypanosomiasis (Chagas disease) is an infectious disease caused by the hemoflagellate parasite *Trypanosoma cruzi* which is transmitted by reduviid bugs. *T. cruzi* infection occurs in a broad spectrum of reservoir animals throughout North, Central, and South America and usually evolves into an asymptomatic chronic clinical stage of the disease in which diagnosis is often challenging. This chapter describes the application of polymerase chain reaction (PCR) for the detection of *Trypanosoma cruzi* DNA including protocols for sample preparation, DNA extraction, and target amplification methods.

Key words *Trypanosoma cruzi*, Chagas, Diagnosis, PCR

1 Introduction

American Trypanosomiasis (Chagas disease) continues to be among the most neglected of all tropical diseases, affecting millions of people and posing a serious health and economic setback for most Latin American countries [1, 2]. Estimates from the World Health Organization and the Centers for Disease Control and Prevention indicate that between 8 and 11 million people are infected, and at least 100 million are at risk of infection, by the disease-causing parasite *Trypanosoma cruzi* [1–3]. Traditional diagnosis of Chagas disease relies on demonstration of trypomastigotes in blood, amastigotes in tissues, serological testing, or culture [4]. However, most of these methods have important limitations; for example: circulating Trypomastigotes are usually absent in chronic stages of the disease, cultures and xenodiagnosis are cumbersome, and because most of the available serological tests use epimastigote antigens, these methods usually exhibit a high degree of cross-reactivity [4]. In this context, different PCR

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strategies have emerged as an important method for identification of *Trypanosoma cruzi*. Herein we describe a series of protocols as a combined strategy for the diagnosis of Chagas disease.

2 Materials

2.1 Equipment

1. Centrifuge.
2. Microcentrifuge.
3. Vortex.
4. Water bath.
5. PCR machine (Eppendorf Mastercycler gradient).
6. Agarose gel electrophoresis equipment.
7. Pipets for PCR.
8. Filter tips.
9. Sterilized tubes for master mix preparation (0.5, 1.5 ml), and reaction tubes.

2.2 Reagents

1. Axy Prep Blood Genomic DNA Miniprep Kit (Axygen Biosciences).
2. Phenol–Chloroform–isoamyl alcohol (25:24:1).
3. PCR kit.
4. Primers.
5. Milli-Q water.

3 Methods

3.1 Sample Nature and Storage

3.1.1 Blood Collection

Blood should be collected into ethylenediaminetetraacetic acid (EDTA) tubes, and processed as soon as possible. If not processed immediately, samples must be kept at 4 °C for no longer than 1 week until its use.

For prolonged storing, guanidine buffer (Guanidine–HCl 6 M pH 8.0, 0.2 mM EDTA) should be added to blood samples in a relation 1 to 1 (blood:buffer), and then stored at 4 °C. It has been reported that with Guanidine–HCl, blood could also be kept at ambient temperature for up to 3 months, which may be useful in field sample collection [5]. We have verified that in Guanidine–HCl treated blood samples, *Trypanosoma cruzi* DNA remains non-degraded for months, and we have successfully amplified *Trypanosoma* DNA from samples stored at 4 °C for 1.5 years. However, it should be noted that Guanidine–HCl is a salt which could inhibit PCR amplification and therefore dilutions of extracted DNA may be required for successful amplification.

3.1.2 Mononuclear Cells Purification

In order to attain better yields of DNA from whole blood samples, obtaining the buffy coat before extraction or purifying mononuclear cells is recommended. Both of these methods will enrich the DNA sample, and therefore increase the probabilities of amplifying the parasites DNA. For this purpose, we have used the Hystopaque 1077 solution as follows:

1. Transfer carefully 3 ml of whole blood onto 3 ml of Hystopaque 1077 (at ambient temperature). Centrifuge at 400 × *g* for 30 min at ambient temperature.
2. Carefully aspirate and discard the upper layer, taking extra care not to disturb the opaque interface. Then transfer the mononuclear layer (the opaque interface) to a clean, labeled, 15 ml centrifuge tube.
3. Wash twice the cells, with 10 ml of isotonic phosphate buffered saline solution (PBS) and centrifuge at 1000 × *g* for 10 min each time (at this point the process could be done at 4 °C).
4. Resuspend cell pellet in 1 ml of PBS and transfer to a clean 1.5 ml centrifuge tube. Centrifuge at Vmax for 5 min.
5. At this point you may store the sample at −20 or −70 °C until it is ready to use, or total DNA could be directly extracted.

3.1.3 Tissue Samples

For experimental procedures and human postmortem cases, tissue samples can be used for Chagas disease diagnosis. For DNA tissue extraction, fresh samples can be used or otherwise stored in liquid nitrogen or at −80 °C until its use. Paraffin embedded tissue may be used as well for diagnosis using the proper extraction method. The suggested protocol for DNA extraction from tissue samples is as follows [6]:

1. Homogenize organ samples and immediately mix with five volumes of lysis buffer (10 mM Tris–HCl pH 7.6, 10 mM NaCl, 0.5 % SDS, and 300 μg of proteinase K), incubating at 50 °C for about 18 h.
2. A total of 100 μl of phenol–chloroform–isoamyl alcohol (25:24:1) is added and then mix by vortex for 1 min and centrifuge for 4 min at 12,000 × *g*.
3. Transfer the aqueous phase to a clean tube and precipitate the DNA with ethanol and sodium acetate (3 M pH 5.5). Mix by vortex and then centrifuge at 12,000 × *g* for 10 min.
4. The pellet is washed with 70 % ethanol and resuspended in Tris–EDTA solution at pH 8.0 (TE buffer) or milli-Q water and stored at −70 °C until its use.

Diagnostic sensitivity can be enhanced by maximizing the amount of target DNA in the aliquot used for DNA extraction. This can be obtained by mixing blood specimens with guanidine

HCl–EDTA solution which lyses the parasites and releases their genetic content, thus making it possible to detect as little as one parasite in 20 ml of blood [5, 7]. It has also been reported that sensitivity could be enhanced by using blood clot as a primary specimen [8]. In addition, an alternate method consists in concentrating the parasites in the buffy coat fraction prior to DNA extraction [9]; this has been reported to significantly increase the sensitivity in comparison to frozen EDTA–blood and guanidine HCl–EDTA treated blood [10].

In a study developed by Qvarnstrom et al. [11], the authors compared PCR results obtained using buffy coat as primary specimen versus fresh EDTA–blood, and found that analysis using buffy coat allowed earlier detection and increasing levels of circulating parasitic genome material in three cases: two reactivation cases and a post-transplant acute infection. Combining both the buffy coat and whole blood aliquot in parallel can also provide helpful information to ensure test validity and to troubleshoot suspicious false positive PCR results. False positive PCR results obtained can be immediately flagged as suspicious, when the whole blood fraction is positive while the buffy coat is negative, therefore being advantageous to use both sample extraction processes. In such scenario, problem with the quality of the blood specimen, the DNA extraction process, or the PCR accuracy should be assessed.

Although promising, assessment about the advantages and robustness of analyzing both whole blood and buffy coat samples needs to be validated through further large studies. In conclusion, we propose that in reference laboratories with the adequate infrastructure, the use of two or more PCR tests with different performance characteristics combined with the analysis of buffy coat and whole blood can strengthen the use of PCR for accurate diagnosis of Chagas disease.

3.2 DNA Extraction with Phenol–Chloroform–Isoamylic Alcohol

1. Mix 100 µl of blood (mixed with Guanidine–HCl) with 100 µl of phenol–chloroform–isoamylic alcohol (25:24:1) (phenol Tris–EDTA pH 8, USB Corporation, USA).
2. Centrifuge for 3 min at 13,000 rpm, and then add 150 µl of distilled water, vortex, and centrifuge for 3 min at 13,000 rpm.
3. Transfer carefully the aqueous phase and add 200 µl of chloroform, vortex, and centrifuge for 3 min at 13,000 rpm.
4. Transfer the aqueous phase to a clean tube and mix with 40 µg of glycogen (from rabbit liver, Sigma, USA).
5. Precipitate DNA and glycogen with 200 µl of isopropanol, vortex and incubate for 35 min at −20 °C, centrifuge at 13,000 rpm for 15 min.
6. Wash the pellet adding 500 µl of ethanol 70 %, do not vortex, and centrifuge again for 15 min at 13,000 rpm.

7. Discard the ethanol and allow the pellet to dry for 10 min at 37 °C. Resuspend the pellet in 50 µl of TE buffer or sterile milli-Q water. DNA should be stored at −20 °C until its use.

The organic extraction method is a conventional technique for DNA extraction. First, the cells are lysed using a detergent which disrupts the cellular structure to create a lysate, which is followed by cell debris removal through centrifugation. Subsequently proteins are denatured using a protease, which together with lipids, carbohydrates, and other cell debris is later removed through extraction of the aqueous phase with the organic mixture of phenol, chloroform, and isoamylic alcohol [12]. The organic solvents precipitate the proteins leaving behind the polynucleotides (DNA and RNA), and the protein precipitate is then separated by centrifugation. In the last step, purified DNA is usually recovered by precipitation using ethanol or isopropanol (a property of ethanol precipitation is that it precipitates only polymeric nucleic acids and leaves behind short chain and monomeric nucleic acid components including the ribonucleotides from RNase treatment). The precipitated DNA is commonly resuspended in TE buffer or double distilled water, for further use in PCR amplification process [13]. The limitations of this method are that it uses hazardous organic solvents and is relatively time-consuming. Moreover, residual phenol or chloroform may be present in the extracted DNA, which can limit its use in downstream application such as PCR amplification. The process also generates toxic waste that must be disposed with care and in accordance with hazardous waste guidelines. In addition, although it generates a good yield of DNA, this technique is almost impossible to automate, making it unsuitable for high-throughput applications [14].

3.3 DNA Extraction Using a Commercial Kit

DNA is extracted from blood using the Axy Prep Blood Genomic DNA Miniprep Kit (Axygen Biosciences). The protocol is described subsequently, but for more details and troubleshooting, please refer to the kit handbook.

1. Add 500 µl of Buffer AP1 to a 1.5 ml microfuge tube.
2. Add 250 µl of anti-coagulated whole blood and mix by vortexing at top speed for 10 s (*see* **Note 1**).
3. Add 100 µl of Buffer AP2 and mix by vortexing at top speed for 10 s. Centrifuge at 12,000 × *g* for 10 min at ambient temperature to pellet cellular debris.
4. Place a Miniprep column into a 2 ml Microcentrifuge tube. Pipette the clarified supernatant obtained from **step 4** into the Miniprep column. Centrifuge at 12,000 × *g* for 1 min.
5. Discard the filtrate from the 2 ml Microfuge tube. Place the Miniprep column back into the 2 ml microfuge tube. Add 700

µl of Buffer W2 to the Miniprep column and allow it to stand at room temperature for 2 min. Centrifuge at 12,000 × *g* for 1 min (*see* **Note 2**).

6. Discard the filtrate from the 2 ml Microfuge tube. Place the Miniprep column back into the 2 ml microfuge tube. Add 800 µl of Buffer W2 to the Miniprep column and centrifuge at 12,000 × *g* for 1 min.

   Optional Step: Discard the filtrate from the 2 ml Microfuge tube. Place the Miniprep column back into the 2 ml Microfuge tube. Add 500 µl of Buffer W2 to the Miniprep column and centrifuge at 12,000 × *g* for 1 min.

7. Discard the filtrate from the 2 ml Microfuge tube. Place the Miniprep column back into the 2 ml Microfuge tube. Centrifuge at 12,000 × *g* for 1 min.

8. Place the Miniprep column into a 1.5 ml Microfuge tube. Add 100 µl of Buffer TE or milli-Q water. Allow to stand at room temperature for 1 min. Centrifuge at 12,000 × *g* for 1 min to elute the genomic DNA (*see* **Note 3**).

The concentration and quality of DNA should be measured spectrophotometrically at 260/280 nm in triplicate.

3.4 Comparison of DNA Isolation Methods

In a study developed by Ramírez et al. in 2009 [15], the authors compared the two proposed DNA isolation methods based on the efficiency of PCR amplification using the kinetoplast DNA (kDNA) and satellite DNA (stDNA) genomic regions when testing 100 positive samples that were ELISA, IIF, and TESA-blot positive, as well as ten negative control samples. Results of PCR efficiency amplification showed that the phenol–chloroform extraction method was 17 % more sensitive than the AquaPure Genomic DNA blood/tissue kit from Bio-Rad for the stDNA PCR, and 13 % more sensitive for the kDNA PCR. These results indicate that the differences between the two extraction methods using both PCR detection targets were statistically significant, and all the negative controls revealed an absence of amplification by both PCR methods. A statistically significant difference was observed when evaluating the DNA concentration, as the final DNA concentration obtained by the phenol–chloroform method was much higher than that obtained with the commercial kit. Therefore, as for Ramírez et al. [15] study and our experience in Chagas disease diagnosis, the phenol–chloroform DNA extraction method is the ideal method for *T. cruzi* DNA detection in blood or tissue samples. However, in a study performed by Schijman et al. [16] they determined that commercial DNA extraction kits offer better specificity than solvent extraction protocols.

3.5 PCR for Detection of Kinetoplast DNA from Trypanosoma cruzi

The most widely used PCR assays used for diagnostic purposes target either the kDNA, also called the minicircle, or a nuclear mini-satellite region designated TCZ. Both of these targets are present in multiple copies in the parasite genome, which increases the sensitivity of detection [17].

*T. cruzi*mitochondrial genome represents about 20 % of the parasite's total DNA and is organized as a concatenated network where thousands of small circular molecules, the minicircles, are found tightly interlocked, representing 95 % of the parasite's kDNA content. These molecules have characteristics that make them ideal targets for PCR detection, considering that they are present in an elevated number of copies (about 10,000) and that each minicircle contains four regions of highly conserved DNA sequences found in all strains and representative isolates of different *T. cruzi* lineages [18, 19].

PCR strategies using kDNA as an amplification target employ oligonucleotides designed for the conserved minicircle regions. As first described, the primers 121, 122 [20] amplify a 330 bp fragment of the minicircle kDNA which is the minicircle variable region [5, 21]. This approach has proven to be highly specific as it allows the successful detection of different *T. cruzi* strains, and also sensitive because it does not recognize other kinetoplastids [17, 22–25]. Studies using PCR assays targeting multicopy kDNA minicircles reported 100 % sensitivity in chronic Chagas disease patients, highlighting that an excess of human DNA does not interfere with the selective parasite DNA amplification process [26]. The sensitivity of the kDNA-PCR protocol is about 5 fg of total *T. cruzi* DNA in the reaction tube, equivalent to one parasite in 10 ml of blood [7, 27].

3.5.1 kDNA-PCR Protocol

1. An amplification reaction in a total volume of 25 µl, containing 250 ng of sample DNA, 1× *Taq* polymerase buffer, 3 mM of $MgCl_2$, 250 µM dNTP mix, 1 U of Go *Taq* polymerase, 0.25 µM of each forward and reverse primers (121: 5′·AAATAATG TACGGGKGAGATGCATGA·3′ and 122: 5′·GGTTCGATT GGGGTTGGTGTAATATA·3′ respectively) and a quantity of Milli-Q water sufficient to complete the final volume.
2. Suggested thermal profile:

	94.0 °C 3 min
5 cycles of:	94.0 °C 1 min
	68.0 °C 1 min
	72.0 °C 1 min

35 cycles of:	94.0 °C	45 s
	64.0 °C	45 s
	72.0 °C	45 s
	72.0 °C	10 min

3. An amplification reaction without DNA template is included in each PCR round as a negative control to exclude false positives resulting from contamination of reagents. A *T. cruzi* positive sample from a patient and genomic DNA from *T. cruzi* strain Y are included as positive controls.
4. The presence of an amplification product (kDNA 330 bp) is analyzed in a 2 % agarose gel, detected with ethidium bromide (Fig. 1).

3.6 PCR for Detection of Satellite DNA from Trypanosoma cruzi

T. cruzi satellite DNA (Sat-DNA) is present in 120,000 copies in the parasite genome, is a 195-bp repeated sequence, and it represents 10 % of the parasites' total DNA, which makes it a highly sensitive target [28, 29]. To establish the sensitivity of sat-DNA

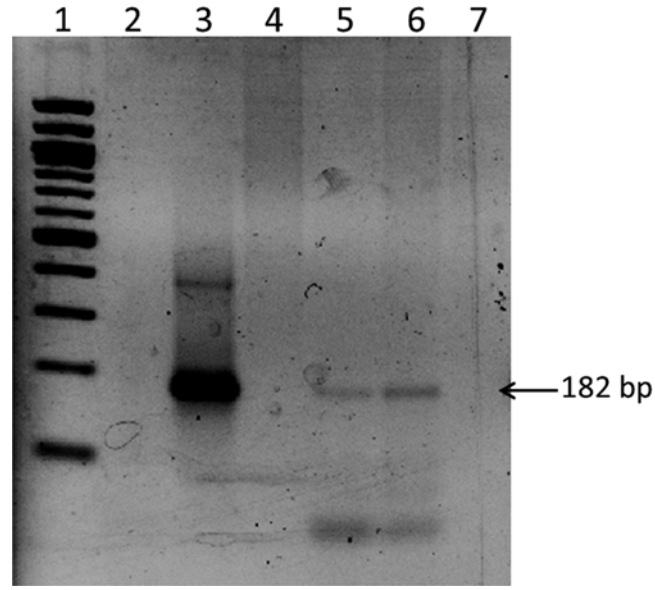

Fig. 1 Polymerase chain reaction (PCR) amplification for the kinetoplast DNA gene (kDNA) of *Trypanosoma cruzi*. Amplification bands are visualized on a 2 % agarose gel stained with ethidium bromide. *Lanes*: (*1*) 100 bp DNA ladder, each lane corresponds to 100 base pairs, (*2*) Negative control without DNA, (*3*) Positive control with *T. cruzi* genomic DNA, (*4*) DNA from an infected dog post-treatment, (*5*) DNA from an infected dog, (*6*) DNA from an infected dog, (*7*) DNA from a non-infected dog. All DNA samples from dogs were extracted from whole blood

amplification primed by TCZ1 and TCZ2 primers, genomic DNA from various sources and templates were used (Y, Tulahuén, Corpus Christi, and Sylvio X-10/4 isolates) and obtained from patients in widely separated geographical areas (Brazil, Chile, and Texas) yielding a 188 bp amplification band; thus suggesting that a fragment size is universally present in these parasites. Moreover, TCZ1 and TCZ2 are highly specific, because they do not amplify DNA of closely related species, as *Leishmania spp.* (*L. mexicana*, *L. major*, *L. braziliensis*, *L. donovani*) which overlap with *T. cruzi* in some endemic areas, as well as the African trypanosomes (*T. brucei brucei*, *T. brucei rhodesiense*, *T. brucei gambiense,* and *T. congolense*) which are also phylogenetically related to *T. cruzi* [30]. Additionally, mammalian hosts of *T. cruzi* such as mice or humans do not have DNA sequences that are amplified to any significant degree with these primers [29]. Sat-DNA PCR tests showed high specificity and sensitivity values of 0.05–0.5 parasites/ml, whereas specific kDNA tests detected 5×10^{-3} parasites/ml [16]. Studies conducted in infected monkeys and in patients using serum samples to amplify Sat-DNA revealed a high sensitivity and specificity in the detection of DNA from *T. cruzi* at any stage of the disease [31].

3.6.1 Sat-DNA PCR Protocol

1. An amplification reaction in a total volume of 25 µl, containing 250 ng of sample DNA, 1× *Taq* polymerase buffer, 3 mM of $MgCl_2$, 250 µM dNTP mix, 1 U of *Taq* polymerase, 0.5 µM of each forward and reverse primers (TCZ1: 5′·GCTCTTGCCCACAMGGGTGC·3′ TCZ2: 5′·CCAAGCAGCGGATAGTTCAGG·3′ respectively), and a quantity of Milli-Q water sufficient to complete the final volume.
2. Suggested thermal profile:

	98.0 °C 10 min
40 cycles of:	98.0 °C 45 s
	55.5 °C 1 min
	72.0 °C 1 min
	72.0 °C 15 min

3. An amplification reaction without DNA template is included in each PCR round as a negative control to exclude false positives resulting from contamination of reagents. A *T. cruzi* positive sample from a patient and genomic DNA from *T. cruzi* strain Y are included as positive controls.
4. The presence of an amplification product (Sat-DNA 182 bp) is analyzed in a 2 % agarose gel, detected with ethidium bromide (Fig. 2).

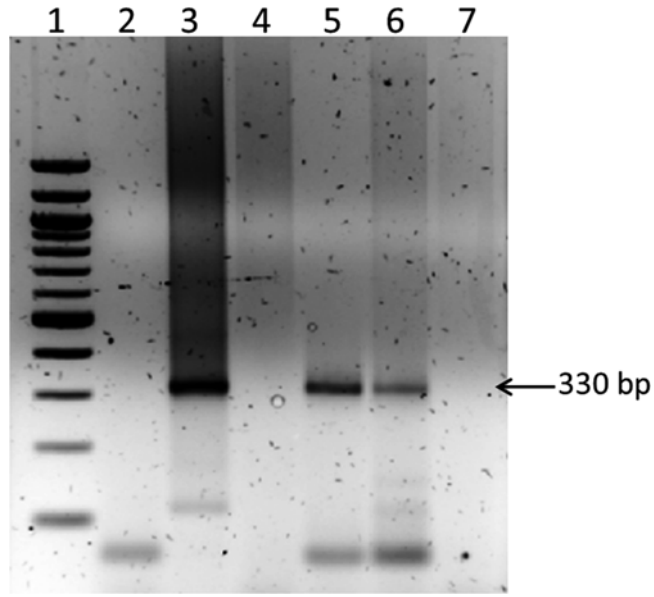

Fig. 2 Polymerase chain reaction (PCR) amplification for the Satellite DNA gene (Sat-DNA) of *Trypanosoma cruzi*. Amplification bands are visualized on a 2 % agarose gel stained with ethidium bromide. *Lanes*: (*1*) 100 bp DNA ladder, each lane corresponds to 100 base pairs, (*2*) Negative control without DNA, (*3*) Positive control with *T. cruzi* genomic DNA, (*4*) DNA from an infected dog post-treatment, (*5*) DNA from an infected dog, (*6*) DNA from an infected dog, (*7*) DNA from a non-infected dog. All DNA samples from dogs were extracted from whole blood

3.7 PCR for Detection of Tc24 Gene from Trypanosoma cruzi

In a study by Guevara et al. [32] the authors experimentally infected mice with *T. cruzi* trypomastigotes and followed up on blood samples periodically, as the mice were sacrificed at 4, 15, 20, 28, and 120 days post-infection (PI). DNA was isolated from blood and a variety of different tissues (heart, liver, kidney, duodenum, ileum, spleen, colon, and skeletal muscle). Though parasites were detected by PCR (Tc24-based PCR) in all tissues at different times, the highest number of parasitism and the better resolved PCR bands were most clearly observed at day 28th PI, with skeletal muscle and heart showing the highest intensity of PCR amplified products, thus suggesting the presence of large numbers of parasites. These results were confirmed by histological analysis for the presence of amastigotes nests. Although parasites were present in other organs, they were mostly localized within macrophages and no extensive tissue damage was observed in comparison with heart and skeletal muscle. Based on the abovementioned findings and the results obtained by us using this same protocol, we have observed that the Tc24-based PCR assay is more sensitive than the *T. cruzi* kDNA amplification based method, making it more suitable for detection on tissue samples. In addition, studies by Taibi et al. [33] propose that the Tc24-based PCR assay is a suitable method for making a differential diagnosis between *T. cruzi* and *T. rangeli*.

3.7.1 Tc-24 PCR Protocol

Detection of the DNA sequence encoding the Tc-24 protein of *T. cruzi* was used for the identification of parasites in blood and tissue samples. Amplification of Tc-24 sequence was performed using one set of primers for a first PCR as reported by Ouaissi et al. [34].

1. The first PCR mix was prepared to a final volume of 50 µl as follows: 5 µl of 10× Taq polymerase reaction buffer, 2 µl of 2.5 mM dNTP's solution, 3 µl of 50 mM $MgCl_2$, 1 µl of DNA sample (100 ng), and 7 µl of each pair of 3 µM oligonucleotides (sense primer 5′ · GACGGCAAGAACGCCAAGGAC · 3′ and antisense primer 5′ · TCACGCGCTCTCCGGCACGTTG TC · 3′), 1 U of Taq DNA polymerase and water sufficient to complete the final volume.
2. To improve the specificity, a nested PCR was performed designing an internal primer sequence of Tc-24. This reaction was performed as follows: 2 µl of the first PCR amplification product and 7 µl of each pair of 3 µM oligonucleotides (sense 5′ · AAGAAGTTCGACAAGAACGA · 3′ and antisense 5′ · AAACTCGTCGAACGTCACGG · 3′) in a 50 µl reaction.
3. The suggested thermal profile:

	94.0 °C 5 min
35 cycles of:	94.0 °C 1 min
	62.0 °C 1 min
	72.0 °C 2 min
25 cycles (nested PCR):	94.0 °C 1 min
	50.0 °C 1 min
	72.0 °C 1 min
	72.0 °C 7 min

4. PCR product of 550 bp was detected in 2 % agarose gel electrophoresis with ethidium bromide (Fig. 3).

3.8 Utility of PCR Strategies for Diagnosis of Chagas Disease

The application of PCR techniques from blood or tissue samples has raised new diagnostic and prognostic possibilities. Research on the use of a PCR technique based on the 330 bp kDNA and the 195 bp nuclear DNA satellite (Sat-PCR) repetitive sequences allows early diagnosis of *T. cruzi* infection recurrence in heart transplantation (HTx) patients [35]. Indeed, PCR enabled the detection of parasite DNA about 41 days prior to plasma parasite detection by conventional methods. Long-term follow-up of ten consecutive *T. cruzi* infected cases showed kDNA-PCR positive findings around 60 days prior to clinical reactivation. Additionally, PCR has been a potentially useful tool for diagnosis of *T. cruzi* infection in newborn to infected mothers, which is essential for the rapid administration of anti-parasitic drugs. Virreira et al. [36]

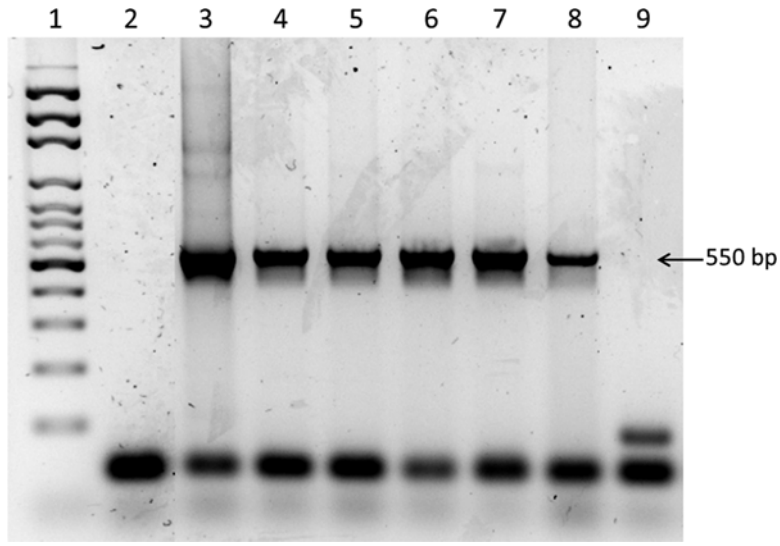

Fig. 3 Polymerase chain reaction (PCR) amplification for the detection of the DNA sequence encoding the Tc-24 protein from *Trypanosoma cruzi*. Amplification bands are visualized on a 2 % agarose gel stained with ethidium bromide. *Lanes*: (*1*) 100 bp DNA ladder, each lane corresponds to 100 base pairs, (*2*) Negative control without DNA, (*3*) Positive control with *T. cruzi* genomic DNA, (*4–8*) DNA extracted from heart tissue of mice infected with *T. cruzi*, (*9*) DNA extracted from heart tissue of a mouse infected with *T. cruzi*

determined that the Sat-PCR protocol detects a single parasite in 0.1 ml of blood, and such sensitivity should be sufficient to detect congenital infection corresponding to an acute parasitic phase, also showing a good specificity in newborns from infected and non-infected mothers. Therefore, this technique may be a useful alternative tool in the diagnosis of congenital Chagas disease.

Treatment research for Chagas disease has been considered a promising medical approach to patients in either the acute, indeterminate, or chronic phases. It is necessary to be able to monitor the efficacy of several therapeutic alternatives and to establish reliable criteria to determine the parasitological cure of patients. The high sensitivity and specificity of the PCR-based diagnosis of *T. cruzi* infection makes it a suitable tool for the follow-up of a chemotherapeutic treatment of chagasic patients. Therefore, the method could be used to assess parasite clearance from blood or cardiac tissue [37].

3.9 Loop-Mediated Isothermal Amplification (LAMP) Technique

Loop-mediated isothermal DNA amplification (LAMP) is a powerful innovative gene amplification technique emerging as a simple rapid diagnostic tool for early detection of several infectious diseases, including viral, bacterial, and parasitic diseases [38]. The mechanism of the LAMP reaction can be simplified in three steps: an initial step, a cycling amplification step, and an elongation step.

LAMP employs a DNA polymerase with strand-displacement activity (*Bst* DNA polymerase, obtained *Bacillus stearothermophilus*), along with two inner primers (FIP, BIP) and outer primers (F3,B3) which recognize six separate regions within a target DNA sequence. The LAMP assay amplifies specific sequences of DNA under isothermal conditions in the range of 65 °C, and as a result it allows the use of simple and no special reagent, no sophisticated equipment is required, it has high specificity because the amplification reaction occurs only when all six regions within a target DNA are correctly recognized by the primers, it amplifies a specific gene from a genome discriminating a single nucleotide difference, and shows high amplification efficiency (enables amplification in a shorter time) that is attributed to no time loss of thermal change because of its isothermal reaction [39]. It also produces tremendous amount of amplified products that makes a simple detection possible [40].

3.9.1 LAMP for Detection of rRNA Genes from *Trypanosoma cruzi*

1. The LAMP primer sets were designed from 18S rRNA genes of *T. cruzi* [41]. The design and operation of the two outer primers (F3 and B3) is the same as that of the regular PCR primers. Each of the inner primers (FIP and BIP) contains two distinct sequences that correspond to the sense (FIPF2 and BIP-B2) and the antisense (FIP-F1c and BIP-B1c) sequences of the target DNA, and they form stem-loop structures at both ends of the minimum LAMP reaction unit. These stem-loop structures initiate self-primed DNA synthesis and serve as the starting material for subsequent LAMP cycling reaction [42].

3.9.2 18S rRNA Genes LAMP Protocol

For LAMP reactions, the material (reaction tubes) and reagents (Loopamp Fluorescent Detection Reagent) provided by the commercial kit are used.

1. LAMP reaction mixture in a total volume of 25 μl contained: 12.5 μl of the reaction buffer (40 mM Tris–HCl pH 8.8, 20 mM KCl, 16 mM $MgSO_4$, 20 mM $(NH_4)_2SO_4$, 0.2 % Tween 20, 1.6 M Betaine, 2.8 mM of each dNTP), between 200 and 400 ng of sample DNA extracted from blood or tissue, 8 U of *Bst* DNA polymerase, 6 μl of primer mix (FIP: 5′·GGTAAAAA ACCCGGCTTTCGCAACCGGCAGTAACACTCAGA·3′, BIP: 5′·CGATGGCCGTGTAGTGGACTGTTTCTCAGGC TCCCTCTCC·3′ at 40 pmol each; F3: 5′·GGACGTCCAGCGAATGAATG·3′ and B3: 5′·CCTCCGTAGAAGTGGTAGCT·3′) at 5 pmol each; Loop-F and Loop-B at 20 pmol each, 1 μl Fluorescent Detection Reagent and water sufficient to complete the final volume.
2. The mixture is incubated at 64 °C for 40 min and then at 85 °C for 5 min.
3. An amplification reaction without DNA template is included in each PCR round as a negative control to exclude false positives

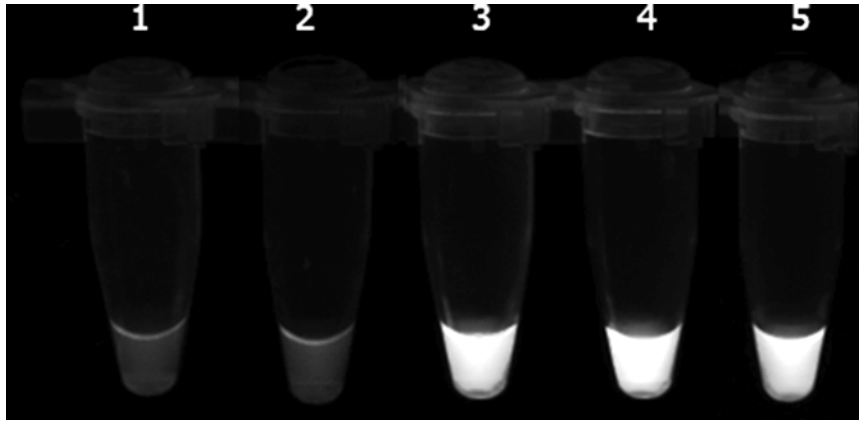

Fig. 4 Loop-mediated isothermal DNA amplification (LAMP) for the detection of the 18S rRNA genes from *Trypanosoma cruzi*. Amplification product is visualized directly on the reaction tubes using a molecular imager. *Tubes*: (*1*) Negative control without DNA, (*2*) 760 ng of DNA extracted from heart tissue of a non-infected rat, (*3*) 760 ng of DNA extracted from heart tissue of an infected rat, (*4*) 1 pg of genomic DNA from *T. cruzi*, (*5*) 140 ng of DNA extracted from blood of a patient with Chagas disease in acute phase

resulting from contamination of reagents. A *T. cruzi* positive sample from a patient and genomic DNA from *T. cruzi* are also included as positive controls.

4. Amplification product is observed in a Molecular Imager ChemiDoc XRS (BioRad) (Fig. 4).

In a study developed by Concepción et al. (not published) the LAMP technique was used to detect *T. cruzi* DNA in samples of rat heart tissue and human blood, using as a negative control DNA extracted from heart of a non-infected rat, and as a positive control DNA from cultured *T. cruzi e*pimastigotes. As shown in Fig. 4, no signal was observed when no DNA was added to the mix and in the negative control, however, a strong signal was detected in the positive samples, confirming the high sensitivity of the LAMP method.

3.9.3 Advantages of the LAMP Technique

LAMP is an established nucleic acid amplification method that offers rapid, accurate, and cost-effective diagnosis of infectious diseases [43]. This molecular tool can be used for rapid field diagnosis in rural areas where there is no access to sophisticated equipment for detection. This technique eradicates the need for expensive thermocyclers used in conventional PCR; it may be a particularly useful method for infectious disease diagnosis in the non-developed countries. An additional advantage of LAMP over PCR-based methods is that DNA amplification can be monitored spectrophotometrically and/or with the naked eye without the use of special dyes [44].

LAMP assay allows the detection of parasitic infections. Thekisoe et al. in 2010 [45] developed two loop-mediated isothermal amplification (LAMP) assays for specific detection of *Trypanosoma cruzi* and *Trypanosoma rangeli* based on the 18S ribosomal RNA (rRNA) and the small nucleolar RNA (snoRNA) genes, respectively. The *T. cruzi* 18S LAMP assay specifically amplifies *T. cruzi* DNA without amplifying negative control with *T. rangeli*, vector insects, and human host DNA. The detection limit of the assay was 100 fg of serially diluted *T. cruzi* DNA. In this study, six LAMP primers were used for amplification of each target trypanosome DNA. In this sense eight distinct regions were recognized on the target gene, thereby ensuring specificity, high sensitivity, and rapid reaction whereby amplification is achieved within 60 min. This result is a very important approach as the non-pathogenic *Trypanosoma rangeli* shares the same geographical location and same insect vectors with *T. cruzi* parasites, hence being necessary an accurate differential diagnosis. This study brings LAMP to the forefront as an alternative for molecular diagnosis and confirmation of differential *T. cruzi* and *T. rangeli* infections in vectors, clinical samples, transfusion blood samples, and organs for transplantation.

4 Notes

1. Vortexing is required for complete release of the genomic DNA. Although vortexing will result in limited shearing of the genomic DNA, it will have no effect upon the performance of the genomic DNA in applications which require high molecular DNA.
2. If any liquid remains in the Miniprep column after centrifugation, extend the centrifuge time or increase the *g*-force.
3. Pre-warming Buffer TE or milli-Q water at 65 °C will generally improve elution efficiently.

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