EVALUATION OF ADULT CHRONIC CHAGAS HEART DISEASE DIAGNOSIS

BY MOLECULAR AND SEROLOGICAL METHODS

CHRONIC CHAGAS HEART DISEASE DIAGNOSTIC EVALUATION

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Abstract

Chagas disease caused by *Trypanosoma cruzi* is endemic in Latin America. *T. cruzi* presents heterogeneous populations and comprises two main genetic lineages, named *T. cruzi* I and *T. cruzi* II. The diagnosis in the chronic phase is based on conventional serological tests including IIF and ELISA, and in the acute phase based on parasitological methods, including hemoculture. The objective of this study is to evaluate the diagnostic procedures of Chagas disease in adult patients in the chronic phase using a PCR assay and conventional serological tests including TESA-blot as the gold standard. Samples were obtained from 240 clinical chronic Chagasic patients. The sensitivities using TESA-blot were 70% for PCR using the kinetoplast region and 75% using nuclear repetitive region, 99% by IIF and 95% by ELISA. According to the serological tests results, we recommend that researchers assess the reliability and sensitivity of the commercial kit Chagatest ELISA recombinant, version 3.0 (Chagatest Rec v3.0; Wiener Lab®, Rosario, Argentina), due to the lack of sensitivity. Based on our analysis, we concluded that PCR cannot be validated as a conventional diagnostic technique for Chagas disease. These data have been corroborated by the low concordance values with serology tests. It is recommended that PCR be used only as an alternative diagnostic support. Using the nuclear repetitive region of *T. cruzi*, PCR could also be applicable for monitoring patients receiving etiologic treatment.

Introduction

Chagas disease is a complex zoonosis caused by the parasite *Trypanosoma cruzi*. This parasite can be genetically classified into two major lineages namely *T. cruzi* I that is related to the South American northern countries while *T. cruzi* II is related to the South American southern countries of America (46). Chagas disease is a chronic systemic disease endemic to both South and Central America. *T. cruzi* is transmitted through the infected dejections of triatomine bugs, by blood transfusion, congenital infection, laboratory accidents, or by oral infection (44). Chagas disease constitutes a serious public health problem in terms of both social and economic impact. The disease currently affects 15 million people and about 28 million are at risk of acquiring the infection. In America,
nearly 41,200 new cases occur each year along with an average of 12,500 deaths per year

Chagas disease presents two distinct clinical stages. The acute phase begins about one week after initial infection and nearly 30% of the patients recall having had relevant symptoms and signs during this period. During the chronic disease stage, the parasites are no longer easily detectable in the bloodstream, but serological tests remain positive. Diagnosis of Chagas disease is based on parasitological and serological methods. Infection can usually be detected by microscopic examination or by parasitological tests such as hemoculture or PCR (7, 28). There are several targets for the detection of T. cruzi by PCR. The variable region of the minicircle kinetoplast (kDNA) and a repeat tandem sequence of nuclear DNA (stDNA) of the parasite have been the most widely used regions as target sequences for diagnosis via PCR (2, 7, 14, 32, 45). Serological diagnosis of T. cruzi infection is typically performed using two of three individual tests according to availability (44). Enzyme Linked Immunosorbent Assay (ELISA), Indirect Immunofluorescence (IIF) and Indirect Hemagglutination (IHA) are often used. These three tests also referred to as the conventional tests usually employ recombinant and/or crude antigenic T. cruzi preparations (22). The major innovation in Chagas disease diagnosis with the detection of antibodies against T. cruzi is the TESA-blot (Trypomastigote Excreted Secreted Antigens). This is an immunoblot assay that has been widely used because of its high sensitivity and specificity when compared with the conventional serological methods (38). The isolation and gene cloning of this immunodominant peptide have been intended and ELISA tests based on TESA antigens have been performed with high quality results (25) In addition, this test has shown the presence of false negatives when comparing this technique with conventional serology in a cohort from Bolivia (47). These reasons make TESA-blot one of the most feasible and available tests for the diagnosis of Chagas disease (38, 39, 40). Recently, TESA-blot has shown great usefulness in solving doubtful serology and cross-antigenicity issues with related protozoan parasites in endemic regions (41). Because of these previous reports, TESA-blot has been selected as the gold standard in several different reports due to the high sensitivity and specificity of the test.
Among the conventional techniques used for serological diagnosis of Chagas disease are ELISA because of its high sensitivity and IIF due to its specificity. However, it has been observed that these tests can detect a certain number of false positives and false negatives. This makes it necessary to search for diagnostic tests that provide more reliable results (6).

As a routine test for the diagnosis of Chagas disease, the WHO (World Health Organization) recommends immunological techniques according to the type of diagnosis, and a minimum of two positive serological tests are required for considering a patient to be infected with *T. cruzi*. Nevertheless, in some cases there is a need to implement other techniques for the diagnosis of *T. cruzi*. Because of the number of copies and organization of the kinetoplast and nuclear repetitive DNA of *T. cruzi* a PCR assay has been developed (29, 42). Comparative studies of PCR, hemoculture and serology showed that individuals with positive hemoculture and with positive serology had a detection rate of 36.5%. When using PCR, the detection of infection was 83.5%. These results demonstrate the higher sensitivity of PCR compared with hemoculture (14). There is great variability within the results obtained by PCR, xenodiagnosis and hemoculture that makes PCR a controversial tool of choice for the accurate diagnosis of Chagas disease.

The specificity of serological techniques has been questioned because of the cross-antigenicity between *T. cruzi* and parasites of related protozoan diseases, particularly leishmaniasis and infection with *T. rangeli* (6, 39). This questioning arises because these techniques use crude or partially purified parasite extracts, which can cause false-positive results. In order to avoid false positive results, recombinant antigens and/or synthetic peptides have been used with success (19, 27, 30, 39, 40). These problems may be overcome by using recombinant antigens containing specific *T. cruzi* epitopes that elicit an immune response in the majority of Chagasic patients (13, 15, 22, 39). Therefore, parasitological tests are still extremely necessary to detect *T. cruzi*, especially in those patients with doubtful serology and to determine treatment response.

The present study is a substudy from the BENEFIT population recruited in Colombia (23) and the objective was to evaluate the serological diagnostic tests of ELISA, IIF and TESA-blot compared to PCR amplification of the variable region of kinetoplast DNA (kDNA) and the nuclear repetitive DNA region (stDNA) of *T. cruzi* in clinical and serologically...
ascertained Chagasic patients from Colombia. We also aimed to optimize the procedure of DNA parasite amplification by selecting the most suitable DNA extraction method. Similarly, values of sensitivity, specificity, positive predictive value, negative predictive value and Kappa Index were calculated. TESA-blot was used as the gold standard due to the characteristics of this test that were mentioned above.

Materials and methods

Sample collection

A total of 240 clinical chronic Chagasic patients and 20 negative controls were included in the study. We employed the inclusion and exclusion criteria required for the BENEFIT (BENznidazol Evaluation For Interrupting Trypanosomiasis) project (23). Every patient presented clinical heart failure evidenced by echocardiogram and positive serology. A 10 mL blood sample was collected from all patients and control subjects. A 10 mL blood sample was collected from all patients and control subjects. Blood samples were mixed with an equal volume of 6 M guanidine HCl/0.2 M EDTA solution immediately after sample collection. The guanidine-EDTA blood (GEB) mixture was then maintained at room temperature and later stored at 4°C until the DNA extraction. A 2 mL blood sample was also collected for serum collection and analysis. The serum aliquots were then stored at 4°C until the serological tests were performed. The 20 individual control subjects were from a non-endemic area, and they all tested negative for Chagas disease by IIF, ELISA, PCR and TESA-blot. All of the control subjects also showed no clinical signs of heart failure.

Comparison of DNA isolation methods

The phenol-chloroform method for DNA extraction (4) was compared with a DNA commercial isolation kit (BIORAD® Aquapure Genomics for blood tissue) in 100 positive samples selected randomly, including 10 negative controls. The comparison was conducted by spectrophotometry quantification of 5 µL DNA aliquots and also by estimating the efficiency of amplification by PCR using kDNA and stDNA from T. cruzi. A statistical comparison of means (t student p<0.05 one tail) was conducted for the selection of the best
method of DNA isolation and a correlation analysis was conducted for both methods of
amplification.

**DNA isolation**

The samples were immersed in boiling water for 15 min. After cooling, two 200µL aliquots
were taken from each patient blood lysate and successive phenol-chloroform extractions
were performed on this material as previously reported (4). The DNA isolation using the
commercial kit (BIORAD® Aquapure Genomics for blood tissue) was performed according
to the manufacture’s protocol. The DNA was then stored at -20°C; The DNA purity and
concentrations were determined using an Eppendorf Biophotometer 6131 at 260/280 nm
wavelengths.

**Polymerase chain reaction (PCR)**

The amplification reactions were performed in a total volume of 21µL. This reaction
consisted of, 1X Pfx Taq polymerase amplification buffer (100 mM Tris-HCl, pH 8.3;
500mM KCI; Invitrogen®), 100mM dNTP solution, 25mM MgCl₂ solution, 700mM KCl
solution, 2.5 Units/µL of Taq polymerase platinum pfx (Invitrogen®), 56 pM of T. cruzi
minicircle specific primers 121 (5´AAATAATGTACGGGKGAGATGTAGTA3´) and 122
(5´GGTTCGATTGGGGTTGGTGTAATATA3´), 3 µL of DNA template and water to
give a final total volume of 21 µL. The reaction mixture was subjected to 30 cycles of
amplification in an automatic thermocycler (MJ Research Programmable Thermal
Controller PTC-100®, USA) as reported previously (5). All of the samples were further
tested under the same conditions with two oligonucleotides from the human β-globin gene
region PC03 (5´CAACTTCATCCACGTTCACC3´) and PC04
(5´ACACAACTGTGTTCACTAGC3´) as an internal control for amplification. This was
done to check for the possibility that a resulting showing no amplification could have been
due to inhibition of the reaction (33). For the amplification of the T. cruzi nuclear repetitive
region, the amplification reactions were performed in a total volume of 21µL. This reaction
consisted of, 1X of Taq polymerase amplification buffer (100 mM Tris-HCl, pH 8.3;
Invitrogen®), 100mM dNTP solution, 25mM MgCl₂ solution, 5 Units/µL of Taq
polymerase platinum (Invitrogen®), 50 pM of T. cruzi nuclear repetitive region specific
primers cruzi1 (5’ASTCGGCTGATCGTTTTCGA3’) and cruzi2 
(5’AATTCCTCCAAGCAGCGGATA 3’) (31), 3 µL of DNA template and water to give a 
final total volume of 21 µL. The reactions were subjected to 40 cycles of amplification in 
an automatic thermocycler (BIORAD® iCycler) as reported previously (11). The possibility 
of contamination of the PCR reagents and of the solutions used for preparing DNA was 
carefully examined through the use of appropriate controls (DNA from strain VS (T. cruzi 
IIb), DNA from strain Dm11 (T. cruzi I) as positive controls, DNA from strain 444 (T. 
rangeli), DNA from blood serologically negative as negative controls and each sample was 
tested in duplicate. Twenty microliters of PCR product for each reaction was analyzed by 
electrophoresis on a 2% agarose gel and visualized by staining with SYBR Green for Gel 
staining (Invitrogen®).

Serological methods

Indirect Immunofluoresence (IIF)

IIF was carried out as reported elsewhere using formaldehyde-treated epimastigotes forms 
of T. cruzi strain X-380 (T. cruzi I) obtained from culture media as described (14). Positive 
and negative controls including those positive for anti-Leishmania spp antibodies were 
always included to validate the results obtained during the study.

Enzyme linked immunosorbant assay (ELISA)

ELISA was performed with the commercial kit Chagastest ELISA recombinant, version 3.0 
(Chagastest Rec v3.0; Wiener Lab®, Rosario, Argentina), which had a mixture of 
recombinant proteins. Each serum was analyzed in duplicate and the positive and negative 
controls were analyzed in triplicate. A sample was considered positive if the optical density 
(OD) at 450nm was equal to or greater than 0.345 and a sample was considered negative if 
the OD was less than 0.344. Positive and negative controls including sera positive for anti-
Leishmania spp antibodies, which were used for specificity control were always included to 
validate the results obtained during the study.

TESA-blot
TESA from *T. cruzi*, Y strain (*T. cruzi* II) were obtained from the supernatant of infected LLC-MK2 cells and used in immunoblotting as described in a prior study (38). Membrane strips (5 mm) were later incubated with sera diluted 1:200 in TBS–1% milk, for 2 h, with mechanical agitation. After four (5-min) washes in TBS, the bound antibodies were detected using peroxidase-conjugated anti-human IgG (Sigma®), diluted 1:2500 in TBS–1% milk, for 2 h. After new cycles of washes the immune complexes were revealed by addition of H$_2$O$_2$ and 4-chloro-1-naphthol. The reaction was stopped with deionized water.

**Statistical analyses**

To validate the reliability of the results, the following parameters were used: Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and Kappa index (27). TESA-blot was used as the gold standard due to its high sensitivity and specificity and also due to the high level of accurate results in doubtful serology samples and in cross-reaction samples that have been previously reported (25, 38, 41, 47).

**Results**

**Comparison of DNA isolation methods**

A comparison of the proposed DNA isolation methods was performed based on the efficiency of PCR amplification using the kDNA and stDNA genomic regions when testing 100 positive samples that were ELISA, IIF and TESA-blot positive as well as 10 negative control samples. Likewise a comparison of DNA concentration with spectrophotometry was also performed. A one-tailed paired Student's t test (P< 0.05) was performed. Results of PCR efficiency amplification showed that the phenol-chloroform extraction method was 17% more sensitive than the kit from Aquapure Genomics extraction for blood tissue BIORAD® for the stDNA PCR and 13% more sensitive for the kDNA PCR. Our analysis indicated that the results were statistically significant respectively (p= 0.040; p= 0.047) between both extraction methods using both PCR detection targets (Figure 1). All of the negative controls showed an absence of amplification by PCR using both methods. Statistically significant differences were observed (p=0.017) when evaluating the DNA concentration, demonstrating that the final DNA concentration obtained using the phenol
chloroform method was much higher than the commercial kit (Figure 2). Additionally, there was a positive correlation (p=0.002, P<0.05) between the results observed in the amplification by stDNA and kDNA. We have therefore concluded that the ideal method for the detection of amplified DNA from \textit{T. cruzi} is the phenol-chloroform DNA extraction method.

\textbf{T. cruzi DNA detection}

A representative PCR result from both the stDNA and kDNA reactions is shown in Figure 3A and 3B. The 20 individual controls from a non-endemic area which were negative by IIF and ELISA, were all negative by PCR. The expected 330 bp corresponding kDNA region of \textit{T. cruzi} amplified product was observed in 178 (70\%) of the 240 patients and the 166 bp amplified product, corresponding stDNA was seen in 180 (75\%) patients, however this difference was not statistically significant (p=0.125, p<0.05). Although there was no statistical difference, the stDNA target was more sensitive than kDNA for PCR amplification in the samples from chronic Chagasic patients. Positive and negative controls were used from \textit{T. cruzi} strain VS (\textit{T. cruzi} IIb) DNA and \textit{T. cruzi} strain Dm11 (\textit{T. cruzi} I) DNA as positive controls and \textit{T. rangeli} strain 444 DNA and DNA from blood negative samples were used as negative controls. The PCR reactions were performed in duplicate. To consider a sample positive both reactions had to be positive. Also, the results were validated with the amplification of the 110 bp human $\beta$-globin gene region to ensure that the absence of amplification was not attributed to PCR inhibitors. All of the human positive control samples were positive (Figure 3C). The detection limit of the PCR procedure was established being of 0.5 fg for kDNA and 0.1 fg for stDNA (data not shown) according to the protocol proposed (20).

\textbf{Serological Tests:}

The 20 samples from healthy individuals were negative by IIF and ELISA, and of the 240 samples from chronic chagasic patients 99.2\% (238) were positive by IIF, while 95\% (228) were positive by ELISA. Likewise, the gold standard TESA-blot, because of its high sensitivity and specificity, showed positivity of 99.2\% (238) (Table 1). All samples were tested in duplicate. A sample was considered seroreactive in IIF when both reactions had a
titer equal to or greater than 1/32. For ELISA, a sample was considered seroreactive when both reactions showed optical densities (OD) greater than 0.345, and with TESA-blot, a sample was considered seroreactive when the 150kDa band was observed in the membrane strips. These results were previously validated with positive and negative controls for ELISA, IIF and TESA-blot. Similarly, all the six sera positive for anti-Leishmania spp. antibodies tested were negative in all methodologies, resulting in 100% specificity (Table 1).

Statistical analyses

Sensitivity, specificity, positive predictive value, negative predictive value and Kappa index were calculated. TESA-blot was used as a gold standard and the values mentioned above were obtained (27) (Table 1). Interestingly, there were some discrepancies in the ELISA results obtained. Due to these discrepancies, the 12 samples that tested as discrepant based on ELISA were sent in double-blind to the reference center in Colombia where is performed an ELISA based on crude antigenic preparation from local strains. These samples were also processed by an immunochromatographic assay Stat-Pak® (22) according to the manufacturer’s indications. The parameters were then recalculated using these results (Table 1).

Discussion

A considerable number of reports using PCR to examine the sensitivity in the detection of T. cruzi in the blood of patients with chronic Chagas disease have been published. Avila et al. described 100% sensitivity of samples from Chagasic patients when serological techniques and xenodiagnosis were compared, but not all of the patients were in chronic phase, which is evidenced by an absence of tissue failure; therefore, their probability of detecting parasites was much higher (2). In previous reports the sensitivity of PCR for samples from chronic patients ranged from 45-60% (5, 7, 14, 29, 45) when the number of parasites was relatively low. The sensitivity of PCR obtained in our study was 70% by kDNA and 75% by stDNA. This can be explained by the low number of parasites in the chronic phase of the disease, thus preventing the efficiency of amplification by PCR (1). Although some authors have demonstrated that PCR is more sensitive than hemoculture
and xenodiagnosis (7), its sensitivity is not 100% due to the fact that detecting a parasite in every 10 mL of blood is slightly complicated in terms of steric hindrance and availability of template within the same reaction. Some authors have also reported the possibility of using a Nested PCR (N-PCR) reaction in order to increase the amount of parasite template (3).

The nuclear repetitive region (stDNA) was the most suitable region for the detection of T. cruzi in chronic patients when it was compared with the variable region of kDNA minicircles. These regions have been previously selected because of the high number of copies (5000 to 10000 copies of kDNA per cell and 10% of stDNA in the T. cruzi genome) (2, 5, 24). PCR methodology for direct detection of parasite DNA in blood was standardized in this study. In addition, the limit of detection for amplification was determined to be 0.5 fg of DNA parasite per mL of blood for kDNA and 0.1 fg for stDNA (data not shown). In regard to primers used, many authors report the variable region of kDNA minicircle to amplify T. cruzi DNA (2, 5) and the nuclear repetitive DNA region (9, 21, 29). Likewise, many authors have used other regions for the detection of T. cruzi in blood. Silber et al. (35) reported the use of primers to amplify the region that encodes the flagellar protein F29 where the sensitivity was 95%. This study was carried out in chronic Chagasic patients and also in samples with a high number of parasites such as feces of infected vectors and acute patients. Lastly, Chiurillo et al. (8) reported the use of telomeric sequences with a sensitivity of 100%, where the T. cruzi detection was in artificially infected blood of mice and in triatomine feces. From these studies, we highlight the great variability of primers to detect T. cruzi in blood and similarly, it can be inferred that according to the ranges of sensitivity the best primers to detect T. cruzi in blood might correspond to the nuclear repetitive region of stDNA and the variable region of kDNA.

The primers used to amplify the variable region of T. cruzi kDNA have been designed from sequencing T. cruzi strains from Brazil (T. cruzi II). Similarly, all reports for the detection and amplification of DNA from T. cruzi are based on the kDNA of T. cruzi II strains. According to some authors (18, 34, 36), genetic variability of strains of T. cruzi I can lead to the inference that a difference in sequence between kDNA may exist between strains belonging to the T. cruzi I lineage compared to those belonging to lineage T. cruzi II. Phylogenetic analysis based on the variable region of T. cruzi kDNA demonstrated genetic
variability when comparing isolates belonging to the lineages *T. cruzi* I and *T. cruzi* II (40).

Also, the influence of stDNA copy number has been established between the *T. cruzi* lineages (12, 16, 24), where the number of stDNA copies in *T. cruzi* I is lower than the number seen in *T. cruzi* II. It is important to mention that parasites circulating in Colombia belong to *T. cruzi* I but there is evidence of patients infected with *T. cruzi* II parasites (26, 46). This fact could affect the sensitivity of the PCR due to changes in the sequence and copy number seen in the parasites according to the primers used. This may be a factor that can explain the lower sensitivity when using PCR. Virreira et al. (43) observed that the intensity of DNA bands might vary according to the genetic lineage of *T. cruzi*, when the amplification was carried out with primers used for the detection of *T. cruzi* kDNA. These facts corroborate the importance of considering genetic variation and virulence factors in the detection of the parasite.

Although previous reports suggest that the use of recombinant antigens is the best choice for the serodiagnosis of Chagas disease (13, 40) the sensitivity of commercial kit ELISA Chagastest recombinant, version 3.0, resulted in 95% of sensitivity when tested in Colombian Chagasic patients. The origin of recombinant protein is always argued since the majority of theses peptides are obtained from *T. cruzi* II. Since *T. cruzi* that circulate in Colombia and in the northern countries of South America are predominantly *T. cruzi* I (26).

Likewise, it would be ideal to clone, express and purify recombinant antigens from *T. cruzi* I strains to assess their sensitivity in the diagnosis of chronic Chagasic Colombian patients. In a multicenter study, performed with 53 Chagasic chronic patients from an endemic area of Brazil, where it is known that infections are reported to be from *T. cruzi* II, the ELISA Chagastest recombinant showed 100% sensitivity (6). Chagastest recombinant was also evaluated, in other study, when five commercially available ELISAs were evaluated to determine their diagnostic accuracy for Chagas disease in Brazilian and Panamanian Chagasic patients. The sensitivity of 100% was obtained with Brazilian patients but 81.25% with Panamanian Chagasic patients where *T. cruzi* I is predominant. Interestingly, in this study the chronic Panamanian Chagasic patients showed lower titer in all the five ELISAs than Brazilian samples (6). Likewise, it has already been reported that there is a difference in immunoglobulins profiles between the different genotypes of *T. cruzi*, another factor that can explain the low sensitivity of the commercial kit used (11). These developments have a
great epidemiological and social impact due to the blood bank screening where these kits are often used to determine if a sample is positive or negative for anti-*T. cruzi* antibodies in blood for transfusions.

The values of sensitivity, specificity, PPV, NPV and Kappa indexes were considered (Table 1). TESA-blot was chosen as the gold standard test based on its sensitivity and specificity. IIF results showed a Kappa index of 1, but with the discrepant results obtained with ELISA the sensitivity dropped to 95%. In relation to the 12 ELISA discrepant samples, it was observed that sensitivities and specificities were 100% in the ELISA results provided by the reference center, highlighting the limitations of the commercially available ELISA kits and the high potential of Stat-Pak® as a rapid diagnostic test in the chronic phase of Chagas disease (22). However, it is also necessary to consider the usefulness of PCR in doubtful or negative serology, because two samples that were negative by IIF, ELISA and TESA-blot were positive using the PCR by stDNA and kDNA. In the PCR comparison, the 70% sensitivity by kDNA and 75% sensitivity by stDNA highlight the disadvantages of using this test in the diagnosis of chronic Chagas disease. In addition, the Kappa indexes showed a very low concordance of PCR with serological methods, although the stDNA PCR showed higher concordance than kDNA PCR, suggesting that PCR may not be a reliable diagnostic test for Chagas disease but alternatively as a diagnostic tool. The commercial kit ELISA, Chagatest recombinant, version 3.0 (Chagatest Rec v3.0; Wiener Lab ®, Rosario, Argentina) provided false negative results in the diagnosis of chronic patients. It is important to consider that the reference center in Colombia uses crude antigenic extracts to perform ELISA from *T. cruzi* I strains. The use of commercial kits must be evaluated due to the high proportion of false negative results that may lead to serious public health implications given the fact that these kits are used for screening of anti-*T. cruzi* antibody in blood banks (6).

Based on the evaluation and comparison of the five tests, we concluded that the serological tests have higher sensitivity and specificity for the diagnosis of Chagas disease compared to PCR. Thus, it is advisable to evaluate serological techniques for the Chagas disease diagnosis based on crude extracts of *T. cruzi* strains that are circulating at the site where the transmission occurs. Others studies, as reported by Caballero et al. 2007 (6) are
recommended to assess available commercial diagnostic kits for Chagas disease due to their tendency to increase false negative results. PCR may be an alternative diagnostic technique tool and particularly valuable for the confirmation of doubtful results by serology and possibly screening in blood banks. Some evidence also suggests that PCR may be a useful method to determine response to etiologic treatment as the reversion of serology may take several decades (11, 28). Our study made a maximum optimization of the conventional PCR method, obtaining a sensitivity of 75% by stDNA and 70% by kDNA and a specificity of 100%. The selection of the phenol-chloroform method was shown to be the most reliable DNA extraction method for the detection of parasite DNA by PCR. Finally, it is recommended to use the available serological tests, and we recommend corroborating these results with PCR, using the nuclear repetitive region of T. cruzi in order to validate the parasitological and serological diagnosis of patients with chronic Chagas disease.

ACKNOWLEDGMENTS

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Figure 1: Results comparing the DNA phenol-chloroform isolation method and Aquapure genomics for blood tissue BIORAD® based on 100 positive samples. The results were statistically significant by stDNA and kDNA amplification respectively (p= 0.040; p=0.047)
Figure 2: Results comparing the DNA phenol-chloroform isolation method and Aquapure genomics for blood tissue using BIORAD® assessed with DNA concentration measured by spectrophotometry. The results were statistically significant (p=0.017)
Figure 3A. Results of 166 bp PCR fragment amplifications with *T. cruzi* nuclear repetitive region specific primers cruzi1 and cruzi2 from DNA extracted. Lane 1: patient sample 1. Lane 2: patient sample 2. Lane 3: patient sample 123. Lane 4: patient sample 220. Lane 5: patient sample 72. Lane 6: positive control Dm11. Lane 7: positive control VS *T. cruzi* II strain. Lane 8: weight marker.

Figure 3B. Results of 330 bp PCR fragment amplifications with *T. cruzi* minicircle-specific primers 121 and 122 from DNA extracted. Lane 1: weight marker. Lane 2: patient sample 1. Lane 3: patient sample 2. Lane 4: patient sample 123. Lane 5: positive control VS *T. cruzi* II strain. Lane 6: positive control Dm11. Lane 7: negative control 1. Lane 8: negative control 2.

Figure 3C. Results of 110 bp PCR fragment amplification of human β-globin region for internal control of amplification. Lane 1: patient sample 1. Lane 2: patient sample 2. Lane 3: patient sample 123. Lane 4: patient sample 200. Lane 5: patient sample 127. Lane 6: patient sample 122. Lane 7: patient sample 256. Lane 8: weight marker.
Table 1. Values of sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and kappa index (KJ) calculated according to the results of PCR and serological tests (IIF and ELISA) using TESA-blot as Gold Standard.

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</tbody>
</table>

<sup>a</sup> Values calculated without the results of corrected discrepant samples

<sup>b</sup> Values calculated with the results of corrected discrepant samples

<sup>c</sup> Less than 0.40 = poor agreement

0.40 – 0.60 = Fair agreement

0.61 – 0.80 = Good agreement

More than 0.80 = Excellent Agreement