EARLY, INTERMEDIATE, AND LATE ACUTE STAGES IN CHAGAS’ DISEASE: A STUDY COMBINING ANTI-GALACTOSE IgG, SPECIFIC SERODIAGNOSIS, AND POLYMERASE CHAIN REACTION ANALYSIS


Abstract. The acute phase of Chagas’ disease was classified as early, intermediate, and late based on the levels of anti-Gal$_1,3$Gal IgG (Gal) and specific IgM (M) and IgG (G) anti-T. cruzi reactivity. While the early phase was M+G+Gal$^-$, the intermediate phase was M+G-Gal$^-$, or M+G+Gal$, and the late phase was M-G+Gal+. This sequence of stages was consistent with our previous studies on acute-phase proteins. Analysis by the polymerase chain reaction (PCR) of parasite DNA in 65 blood samples of children living in Cochabamba, Bolivia showed a significant correlation (90.8%) between ELISA and PCR positivity. A lower correlation was observed between indirect hemagglutination, PCR (58%), and ELISA. Electrocardiographic analysis of 43 children studied by the PCR did not show any alteration typical of acute chagasic myocarditis. The PCR positivity was observed in eight samples where only Gal was increased, suggesting a very early T. cruzi infection, when specific antibodies were not yet present. By associating anti-Gal IgG with specific serology, early T. cruzi infection can be detected with greater precision. We suggest the use of anti-Gal antibody reactivity as an aid for the detection of recent T. cruzi infections, at least in endemic areas where diseases caused by other trypanosomatids do not overlap.

Chagas’ disease, which is caused by infection with Trypanosoma cruzi, is an important public health problem that affects approximately 16–18 million individuals, mainly in Latin America. Following the introduction of the infective forms by vector transmission or blood contamination, an acute phase develops with microscopically patent parasitemia. Although a strong specific immune response is able to control the acute infection, the parasite is not fully eliminated, leading the majority of T. cruzi-infected individuals to an asymptomatic, indeterminate, clinical form of the disease. Approximately 15–20% of the seropositive individuals later develop a severe chronic disease with involvement of heart or gastrointestinal tract. Serologic tests are the main laboratory procedure used to specifically diagnose Chagas’ disease during the chronic phase because the parasitemia is low, which makes the parasitologic diagnosis more difficult. The latter was improved recently by our ability to amplify parasite DNA by the polymerase chain reaction (PCR).

Specific IgM titer is the most common serologic parameter used to define the acute phase of Chagas’ disease. In recent human infections, antibodies of the IgM class appear 15 days postinfection (PI) and peak from 17 to 45 days PI. Specific IgG levels also increase soon after infection. Anti-galactose IgG captured by murine laminin is increased in chagasic persons. This response is elicited by immunogenic Gal$_1,3$Gal epitopes present on the surface of the parasite and induces lytic antibodies against trypomastigotes of T. cruzi. Anti-Gal is detected in the acute phase even at a high dilution (1:5,000), but are also found in infections caused by other kinetoplastidae.

Direct parasitemia and parasite amplification obtained by xenodiagnosis or hemoculture are the parameters used to diagnose the acute phase of infection. However, these methodologies are time-consuming and have high variability. Polymerase chain reaction assays for the detection of T. cruzi DNA have been recently described, and are considered the most sensitive methods to detect T. cruzi in chagasic patients.

In endemic countries, children are exposed to T. cruzi early in life. However, the detection of the early chagasic infection is difficult due to ancient and subjective methods currently available. Most acute cases are undetectable due to scarce and mild clinical symptoms. Detection of initial infection is of great importance since the treatment is more effective the sooner it is started. It is suspected that almost all recent infections are not diagnosed due to the fact that population serology shows that half of the children up to 12 years of age have anti-T. cruzi antibodies. In areas of high prevalence of infection, even if serology is positive, it is difficult to attribute the clinical picture to T. cruzi infection and to prescribe the specific chemotherapy.

Bolivia has the highest T. cruzi infection rate (40–80%) among the Latin America countries, where it is one of the most important public health problems. The highest prevalence of the T. cruzi infection and of triatomine infestation in the houses is found in Cochabamba Department. Forty-nine to 81% of the children between one and 15 years of age were found by serology to be in the acute phase of the disease. In that study, the use of serologic markers to define the stage of acute infection was proposed. It is based on associating the detection of specific anti-T. cruzi IgM (M) and IgG (G) antibodies and the detection of anti-Gal$_1,3$Gal IgG (Gal) in highly diluted sera. The acute phase was classified as early when the samples were M$^+$, G$^-$, and Gal$^-$, as intermediate when the samples were M$^+$ plus at least another positive parameter (M+Gal$, M+G^+$, or M$^+$G+Gal$^+$), and as late when the samples were M$^-$, G$^+$, and Gal$^+$.
ELISA results. The positivity or negativity was scored based on the dilution of 1:9 and positive when reactive at a 1:81 dilution. Positive results at a dilution of 1:27 were considered negative when it was not reactive or reactive only at a dilution of 1:9 and positive when reactive at a 1:81 dilution.

Materials and Methods

Individuals and blood samples. Individuals examined were untreated children 2–15 years of age participating in epidemiologic surveys in the suburbs of the city of Cochabamba, Bolivia. Blood donation was voluntary, and written consent was obtained from authorities or the children’s parents or guardians prior to their inclusion in this study. This study was approved by the FIOCRUZ Committee of Ethics in Research according to resolution 196/96 of the National Health Council of The Brazilian Ministry of Health.

A first collection in heparinized microhematocrit capillaries was randomly made. A small volume (0.2 ml) of blood was obtained by finger puncture and the blood was used for anti- \( \text{T. cruzi} \) IgG analysis by an indirect hemagglutination assay (IHA) and an ELISA. After diagnosis, 65 children (35 girls and 30 boys, including those seropositive and seronegative) returned for the collection of a larger volume of blood for complementary ELISA and PCR. Clinical examination and electrocardiogram (ECG) were performed in 43 of the children.

Blood samples were collected in heparin for serology and in 6 M guanidine-HCl/0.2 M EDTA (GE) buffer, pH 8.0, for the PCR. In this solution, DNA remains stable for a long period. \(^{25} \) Plasma was obtained from heparinized blood, aliquoted and maintained at \(-20^\circ C\) until use. Samples were analyzed on the same day for the three antibodies at the appropriate dilution (M = 1:250, G = 1:500, and Gal = 1:5,000). The GE blood lysate (GEB) was stored at 4°C until used in the PCR.

Reagents. The ELISA reagents were obtained from Sigma (St. Louis, MO). Commercial preparations of \( \text{T. cruzi} \) DNA were obtained from Biomanguinhos (FIOCRUZ, Rio de Janeiro, Brazil). The ELISA protocol used in this study was those previously described. \(^{12,13} \)

Indirect hemagglutination assay. The IHA was performed with serum from 63 of the 65 children using a commercial diagnostic kit (Imunoserum, Biotecnologia, Sao Paulo, Brazil). Since the IHA shows good performance only in with serum from cases with chronic disease (Peralta JM, unpublished data), the results were analyzed only in the cases characterized by the ELISA as negative for anti- \( \text{T. cruzi} \) IgM and anti-Gal and positive for anti- \( \text{T. cruzi} \) IgG (indeterminate/chronic seropositive cases). The blood was considered negative when it was not reactive or reactive only at a dilution of 1:9 and positive when reactive at a 1:81 dilution. Positive results at a dilution of 1:27 were considered questionable and positivity or negativity was scored based on the ELISA results.

Analysis by the PCR. A quick extraction method was used for preparation of DNA. \(^{26} \) Aliquots of 2–3 ml of the GEB lysate were mixed with an equal volume of the lysis solution (10 mM Tris HCl, pH 8.0, 5 mM MgCl\(_2\), 32 mM sucrose, and 1% Triton X-100). The homogenate was centrifuged at 13,000 \( \times \) g for 5 min and the pellet was washed three times by centrifugation with 1 ml of lysis solution. After the last washing, the pellet was resuspended in 100 \( \mu \)l of buffer (7 mM Tris HCl, pH 8.0, 35 mM KCl, 1.7 mM MgCl\(_2\), 0.3% NP40, and 0.3% Tween 20) and incubated with 100 \( \mu \)g/ml of proteinase K for 2 hr at 55°C. The samples were boiled for 5 min, cooled, and stored at \(-20^\circ C\) until used as the DNA template for the PCR amplification.

The PCR products were performed in a final volume of 10 \( \mu \)l containing 0.2 units of Tag DNA polymerase (Cenbiot, Porto Alegre, Brazil), 200 \( \mu \)M of each dNTP, 0.75 mM MgCl\(_2\), 50 mM KCl, 10 mM Tris-HCl, pH 8.5, 10 pmol of each primer, and 2 \( \mu \)l of template DNA (Vidigal PG, unpublished data). The primers used were DIAZ 1 (\( \text{5}'\text{-CGCAAAACAGATATT-GACAGAG-3'}\)) and DIAZ 2 (\( \text{5}'\text{-TGGTCACACACTTGGACACCAA-3'}\)); these amplify a nuclear and repetitive sequence of 195 basepairs (bp) of \( \text{T. cruzi} \). \(^{27} \) The reaction mixtures were overlaid with 25 \( \mu \)l of mineral oil and subjected to amplification in a thermocycler (PTC 100; MJR Research, Inc., Watertown, MA) using one cycle at 94°C for 5 min, 30 cycles at 94°C for 1 min and 55°C for 1 min, and extension at 72°C for 5 min. After amplification, 3 \( \mu \)l of each reaction was mixed with 3 \( \mu \)l of 2\( \times \) DNA sample buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol) and subjected to electrophoresis on an 8% polyacrylamide gel. The bands were visualized by silver staining of the gel.

Several precautions were taken to avoid PCR contamination. The three stages of the reaction, 1) pre-amplification, 2) amplification, and 3) post-amplification development, were made in different rooms with their specific materials. The samples were coded and a blood sample from a nonchagasic individual was added as control of DNA extraction. Tubes without DNA and with 1 and 100 fg of \( \text{T. cruzi} \) DNA were added as controls for contamination and sensitivity of the amplification reaction, respectively. The PCR sets where the negative controls were positive or the positive controls negative were discarded. Negative PCR samples were again tested at a dilution of 1:10 containing 10 times more DNA template to overcome PCR inhibition and subamplification, respectively. Furthermore, their DNA were either amplified with human glyceraldehyde diphosphate dehydrogenase primers or added with 1 fg of \( \text{T. cruzi} \) DNA to test for the presence of PCR inhibitors. Only then were the individuals considered PCR negative.

Direct ELISA. All samples were analyzed by direct ELISA as previously described. \(^{13} \) Control sera with positive serology for \( \text{T. cruzi} \) were added to each plate, as well as three different negative sera. The mean of triplicate determinations of the negative sera plus three standard deviations was taken as the cut-off value. The mean optical density of each test sample was divided by the cut-off value of the plate to obtain the index of variation (IV). Since autoantibodies can give false-positive reactions in the serodiagnosis of \( \text{T. cruzi} \) infection, only sera with an IV > 1 were considered positive. \(^{28} \) The cases were then grouped according to the following criteria: S− = negative serology for M, G, and Gal, S+ = positive serology for M and/or G and/or Gal.

Statistical analysis. Nonparametric methods were used since most of the data did not have a normal distribution.
FIGURE 1. A 8% silver-stained polyacrylamide gel showing *Trypanosoma cruzi* polymerase chain reaction products from the blood of Bolivian children from an area endemic for Chagas’ disease. The arrows show the molecular size standards (bp = base pairs). Lane 1, 4X174 DNA digested with *Hae* III; lane 2, negative control, without DNA; lanes 3 and 4, DNA of the *T. cruzi* Y strain (1 and 100 fg, respectively); lane 5, blood from a nonchagasic individual; lanes 6–15, blood from chagasic children. Serologic data for these samples are shown in the table above the gel. M = anti-*T. cruzi* IgM; G = anti-*T. cruzi* IgG; L = anti-Gal α(1,3)Gal IgG.

The IV obtained for M, G, or Gal were ranked and processed according to the Mann-Whitney U test.

RESULTS

Parasitemia detected by PCR. The PCR was used to detect the parasitemia in the blood samples of children suspected of having *T. cruzi* infections. Figure 1 shows a typical result obtained in which a band of 195 bp was observed as a product of specific amplification in positive controls (*T. cruzi* DNA in lanes 3 and 4) and in seropositive children (lanes 6–15). No reaction was observed in the negative controls without DNA (lane 2) and in a sample from a seronegative individual (lane 5). Samples from two of eight individuals who had high levels of anti-Gal IgG but no specific anti-*T. cruzi* IgM or IgG were PCR+ (Table 1) and are shown in lanes 14 and 15. This indicated that the detection of anti-Gal α₃Gal IgG correlated with the detection of *T. cruzi* DNA by the PCR. These individuals were then considered seropositive for the next step of the analysis.

Distribution of cases among acute-phase stages according to serologic profiles and correlation between serodiagnosis and PCR results. The 65 samples were analyzed serologically for anti-Gal and anti-*T. cruzi* IgM and IgG and parasitologically by the PCR (Table 1). A group positive by the PCR and anti-Gal+ but negative for anti-*T. cruzi* IgM and IgG (group 2) was also included (S+). Most of the cases (n = 32) were distributed in the acute stages, 14 in the early stage, 11 in the intermediate stage, and seven in the late stage. Only one seronegative individual was PCR+, indicating a false-negative serologic result. In the first four acute-phase groups (groups 2–5 = 24 individuals), a 100% correlation was found between serology and PCR analysis, but only 16 individuals were M+. Thus, anti-Gal serology detected eight false-negative results that were not detected in a specific serologic test. The 10 anti-Gal− cases (groups 3 and 5) that were PCR+ and M+ or G+ did not minimize the usefulness of anti-Gal as an aid to decrease false-negative serologic results. Disagreement between results was observed in the late stage of acute infection (one of six individuals = 16.6%) and in the indeterminate/chronic phase (three of 17 individuals = 17.6%). Only one of 65 plasma samples analyzed that were PCR− showed increased levels of the three immunoglobulins assayed (group 6), thus obviating any conclusion on a correlation between serology and the PCR in this group. This one case was a borderline case for IgM and anti-Gal and could be interpreted as an indeterminate/chronic case. The ELISA and PCR showed an agreement of 90.8%.

Quantitative analysis of the ELISA results showed that
The ELISA and IHA results showed a significant correlation in the distribution of IgM levels, with Cases 1 and 2 having higher levels of IgM (0.521) than noninfected samples. This indicates the beginning of bradicardia in group 2 (PCR+), which is relatively low (Table 1, group 1). Thus, IgM in group 2 was 26% higher than in group 1, indicating the beginning of bradicardia.

The IHA was performed with 63 samples and compared with the ELISA and PCR. Among 14 S− individuals, nine were IHA+, indicating false-positive results. Among the 26 S+ acute cases, 12 were IHA−, indicating false-negative results by the IHA. The ELISA and IHA results showed a correlation in only 47.5% of the acute cases. In indeterminate/chronic cases, 11 of the 17 S+ cases (G+, M−, Gal−) were IHA+ (correlation = 51.6%). The correlation between the PCR and the IHA with the seronegative and indeterminate disease/chronic groups was 58%.

**Electrocardiographic analysis.** The most frequently cited cardiovascular manifestations in acute Chagas’ disease are tachycardia, changes in T waves, first-degree AV block, low voltage QRS complex, primary alterations in ventricular repolarization, and ventricular extrasystole. Among the 43 individuals evaluated by ECG and serology, 13 (30%) were S− and 30 (70%) were S+. Eleven of these 43 individuals showed ECG alterations (Table 2) that were not typical for acute chagasic myocarditis. Of these, six cases were PCR− and S−, suggesting that most of these alterations were not due to the *T. cruzi* infection. Furthermore, of the five cases with ECG alterations and positive serology, four were

### Table 1
Distinction of individuals in the serologic groups corresponding to different stages of acute phase of *Trypanosoma cruzi* infection and results from the polymerase chain reaction (PCR) analysis for parasite DNA

<table>
<thead>
<tr>
<th>Test*</th>
<th>S− (n = 16)</th>
<th>Acute phase (n = 32)</th>
<th>Indeterminate/chronic phase (n = 49)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group</td>
<td>Early (n = 14)</td>
<td>Intermediate (n = 11)</td>
<td>Late (n = 7)</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Anti-Gal</td>
<td>Median IV</td>
<td>0.485</td>
<td>1.799</td>
</tr>
<tr>
<td></td>
<td>p25/p75</td>
<td>0.346/0.578</td>
<td>1.187/2.581</td>
</tr>
<tr>
<td>IgM</td>
<td>Median IV</td>
<td>0.589</td>
<td>0.740</td>
</tr>
<tr>
<td></td>
<td>p25/p75</td>
<td>0.410/0.686</td>
<td>0.491/0.794</td>
</tr>
<tr>
<td>IgG</td>
<td>Median IV</td>
<td>0.488</td>
<td>0.561</td>
</tr>
<tr>
<td></td>
<td>p25/p75</td>
<td>0.311/0.603</td>
<td>0.357/0.699</td>
</tr>
<tr>
<td>PCR + (n = 45)</td>
<td>1/16</td>
<td>8/8</td>
<td>6/6</td>
</tr>
<tr>
<td>PCR − (n = 20)</td>
<td>15/16</td>
<td>0/8</td>
<td>0/6</td>
</tr>
</tbody>
</table>

* Anti-Gal = anti-Gal, Gal IgG; IV = quantitative levels (indices of variation); p25/p75 = percentile 25% and percentile 75%; IgM = anti- T. cruzi/IgM; IgG = anti- T. cruzi/IgG.

**S−** and **S+** = seronegative and seropositive individuals, respectively.

### Table 2
Age, sex, serologic, and polymerase chain reaction (PCR) characterization of children with electrocardiographic (EKG) alterations*

<table>
<thead>
<tr>
<th>ID no.</th>
<th>Sex</th>
<th>Age (years)</th>
<th>Serology†</th>
<th>PCR</th>
<th>Chagasic</th>
<th>ECG alterations</th>
<th>Common in chagasic myocardiitis</th>
</tr>
</thead>
<tbody>
<tr>
<td>64</td>
<td>M</td>
<td>9</td>
<td>Neg</td>
<td>Neg</td>
<td>No</td>
<td>Left shift of electric axis</td>
<td>Acute</td>
</tr>
<tr>
<td>71</td>
<td>F</td>
<td>14</td>
<td>Neg</td>
<td>Neg</td>
<td>No</td>
<td>Left shift of electric axis</td>
<td>No</td>
</tr>
<tr>
<td>90</td>
<td>M</td>
<td>10</td>
<td>Neg</td>
<td>Neg</td>
<td>No</td>
<td>Left anterior hemiblock; left shift of electric axis</td>
<td>No</td>
</tr>
<tr>
<td>93</td>
<td>F</td>
<td>8</td>
<td>Neg</td>
<td>Neg</td>
<td>No</td>
<td>Left anterior hemiblock; left shift of electric axis</td>
<td>No</td>
</tr>
<tr>
<td>95</td>
<td>F</td>
<td>10</td>
<td>Neg</td>
<td>Neg</td>
<td>No</td>
<td>Left antero-superior hemiblock</td>
<td>No</td>
</tr>
<tr>
<td>105</td>
<td>M</td>
<td>14</td>
<td>Neg</td>
<td>Neg</td>
<td>No</td>
<td>Relative bradicardia</td>
<td>No</td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>5</td>
<td>Pos</td>
<td>Yes</td>
<td>Yes</td>
<td>Left posterior hemiblock</td>
<td>No</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>11</td>
<td>M=G+Gal−</td>
<td>Pos</td>
<td>Yes</td>
<td>Incomplete right bundle branch block</td>
<td>No</td>
</tr>
<tr>
<td>13</td>
<td>M</td>
<td>8</td>
<td>M=G+Gal−</td>
<td>Neg</td>
<td>Yes</td>
<td>Right ventricular overload</td>
<td>No</td>
</tr>
<tr>
<td>51</td>
<td>M</td>
<td>11</td>
<td>M=G+Gal−</td>
<td>Pos</td>
<td>Yes</td>
<td>Right ventricular overload</td>
<td>No</td>
</tr>
<tr>
<td>72</td>
<td>F</td>
<td>8</td>
<td>M=G+Gal−</td>
<td>Pos</td>
<td>Yes</td>
<td>Right bundle branch conduction disturbance</td>
<td>No</td>
</tr>
</tbody>
</table>

* Neg = negative; Pos = positive.
† M = IgM; G = IgG; Gal = anti-Gal, Gal IgG.
served that only 35% of the Bolivian children are PCR+, and of the 93 PCR+ cases, only 18 were IgM+. However, these investigators used a different combination of DNA primers, as well as different antigens to capture anti-*T. cruzi* IgM antibodies. Therefore, the data cannot be directly compared.

We found a single false-seronegative case with a positive PCR result, and it is unlikely that this could be a false-positive result since several measures were taken to avoid contamination. This five-year-old girl was probably a case of a recent infection in this endemic region since she had increased levels of haptoglobin and fibronectin, two inflammatory proteins (Antas PRZ, unpublished data). Although these proteins do not specify *T. cruzi* infection, their increase strongly suggests inflammation triggered by the parasite, which was detected by the PCR. Repeated serodiagnosis and PCR analysis in the discrepant cases would undoubtedly confirm any of these hypotheses. Unfortunately, due to difficulties in following these individuals, we were unable to perform these assays.

The ECG analysis of 43 children that were studied by PCR did not show alterations typical of acute chagasic myocarditis, and we found more seronegative individuals with ECG alterations (6 of 13 [46%]) than seropositive ones (5 of 32 [15%]). In a study of another hyperendemic area of Cochabamba, ECG abnormalities were found in seven (11%) of 64 chagasic individuals (age range = 5–35 years old), but in only one (2.7%) of 37 nonchagasic persons, suggesting a relative early morbidity of *T. cruzi* infection in young people.19 These apparently discrepant results are probably due to the different age range of the individuals studied, and/or to the distinct approach taken by us in distinguishing serologically acute cases from indeterminate/chronic ones. However, of the four S+, PCR+ cases showing some type of ECG alteration, one presented a conduction disturbance, which was typical for chronic chagasic myocarditis. This eight-year-old child already had the serologic and ECG profiles of a case with chronic disease. Despite the fact that all the cases analyzed were clinically asymptomatic, morbidity in Chagas’ disease can start very early in childhood, as previously suggested.

Our findings that some children in an endemic area with positive PCR results but negative specific serology merits consideration of starting chemotherapy immediately after the results of anti-Gal serology. Despite the power of PCR diagnosis, it will not be easy to be introduced as a routine procedure in such areas, where the microhematocrit method is widely used and economically feasible. Combining specific serology with anti-Gal IgG serology resulted in the detection of early *T. cruzi* infections with great precision.

The level of anti-Galα3,4Gal IgG in the sera of chagasic people indicates that it increases whenever the parasitic load increases: during the acute phase in patients with chronic diseases with chagasic cardiomyopathy, and as we showed here, in PCR-positive samples.11,14,32 We believe that the strong correlation between anti-Galα3,4Gal IgG quantitation and the PCR analysis suggests the use of the former as an aid for the detection of recent *T. cruzi* infections, at least in the endemic areas where diseases caused by trypanosomatids (such as Chagas’ disease, infection by *T. rangeli*, and leishmaniasis) do not overlap, as is the case in Cochabamba.
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