



Article/Artigo

In-house ELISA method to analyze anti-*Trypanosoma cruzi* IgG reactivity for differential diagnosis and evaluation of Chagas disease morbidity

Método de ELISA *in-house* na análise da reatividade de IgG anti-*Trypanosoma cruzi* para o diagnóstico diferencial e avaliação da morbidade da doença de Chagas

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ABSTRACT

Introduction: The goal was to develop an *in-house* serological method with high specificity and sensitivity for diagnosis and monitoring of Chagas disease morbidity. **Methods:** With this purpose, the reactivities of anti-*T. cruzi* IgG and subclasses were tested in successive serum dilutions of patients from Berilo municipality, Jequitinhonha Valley, Minas Gerais, Brazil. The performance of the *in-house* ELISA was also evaluated in samples from other relevant infectious diseases, including HIV, hepatitis C (HCV), syphilis (SYP), visceral leishmaniasis (VL), and American tegumentary leishmaniasis (ATL), and noninfected controls (NI). Further analysis was performed to evaluate the applicability of this *in-house* methodology for monitoring Chagas disease morbidity into three groups of patients: indeterminate (IND), cardiac (CARD), and digestive/mixed (DIG/Mix), based on their clinical status. **Results:** The analysis of total IgG reactivity at serum dilution 1:40 was an excellent approach to Chagas disease diagnosis (100% sensitivity and specificity). The analysis of IgG subclasses showed cross-reactivity, mainly with NI, VL, and ATL, at all selected serum dilutions. Based on the data analysis, the IND group displayed higher IgG3 levels and the DIG/Mix group presented higher levels of total IgG as compared with the IND and CARD groups. **Conclusions:** These findings demonstrated that methodology presents promising applicability in the analysis of anti-*T. cruzi* IgG reactivity for the differential diagnosis and evaluation of Chagas disease morbidity.

Keywords: *In-house* ELISA. IgG subclasses. Differential Diagnosis. Chagas Disease Morbidity.

RESUMO

Introdução: O objetivo foi desenvolver um método sorológico *in-house* de alta especificidade e sensibilidade para diagnosticar e monitorar a morbidade da doença de Chagas. **Métodos:** Para tal, a reatividade sorológica de IgG e subclasses foi testada em soros de pacientes chagásicos de Berilo, Vale do Jequitinhonha/MG/Brasil. A reatividade sorológica foi também avaliada em amostras de pacientes com outras doenças infecto-contagiosas relevantes, incluindo o HIV, vírus da hepatite C (VHC), sífilis (SYP), leishmaniose visceral (LV), leishmaniose tegumentar americana (LTA) e controles não infectados (NI) para verificar o desempenho do método. Outras análises foram feitas para avaliar a aplicabilidade desta metodologia no monitoramento da morbidade da doença de Chagas. Com este propósito os pacientes com doença de Chagas foram anteriormente classificados em três grupos: indeterminados (IND), cardíacos (CARD) e digestivos/mistos (DIG/Mis) conforme seu estado clínico. **Resultados:** A análise da reatividade sorológica de IgG total na diluição 1:40 mostrou ser uma abordagem importante no diagnóstico da doença de Chagas (100% de sensibilidade e especificidade e ausência de reação cruzada com as demais infecções). A análise das subclasses de IgG mostrou reação cruzada principalmente com NI, LV e LTA em todas as diluições. O grupo IND apresentou a maior reatividade para IgG3 e o grupo DIG/Mis apresentou nível mais elevado de IgG se comparados aos grupos IND e CARD. **Conclusões:** Estes achados demonstram que o método de ELISA *in-house* apresenta uma promissora aplicabilidade no diagnóstico diferencial e na avaliação da morbidade da doença de Chagas.

Palavras-chaves: *In-house* ELISA. Subclasses de IgG. Diagnóstico diferencial. Morbidade da doença de Chagas.

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INTRODUCTION

Chagas disease is endemic to a wide region extending from Mexico to Argentina and is one of the parasitic diseases with a major social impact in Latin America, mainly due to its high morbidity and mortality, including the occurrence of sudden death¹. In the past decades, *Trypanosoma cruzi* infection has been increasingly found in non-endemic countries, such as the United States, Canada, Japan, Australia, and many European countries, mainly due to increasing population mobility².

Chagas disease has two successive phases: the acute and the chronic phase, with different clinical forms referred to as asymptomatic (indeterminate form (IND)) for many years throughout the lifetime of the majority of infected people. In approximately 30% of patients, the disease evolves to cardiac (CARD), digestive (DIG), and/or mixed clinical forms³.

During the acute phase or during reactivation due to immunosuppression, the diagnosis is carried out by the direct detection of circulating parasites. However, during the chronic phase, because of low parasitemia, the diagnosis is performed mainly by serological methods. The enzyme-linked immunosorbent assay (ELISA) is the most used serological technique due its high sensitivity and specificity, which is dependent on the antigen employed, the cutoff point used, and the ability to process large quantities of samples rapidly⁴.

However, while the serological methods used in routine diagnosis of *T. cruzi* infection are very effective in detecting infected individuals with high and medium antibody titers, they fail to detect patients with low immunoglobulin levels⁵. This and other limitations have been the main reason of the continuing research on the ELISA technique and its targeted antigens for the purpose of eliminating the occurrence of false-positive and false-negative results.

The sera reactivity of patients with chronic Chagas disease, human T-cell lymphotropic virus (HTLV), hepatitis B virus (HBV), hepatitis C virus (HCV), syphilis (SYP), visceral leishmaniasis (VL),

and American tegumentary leishmaniasis (ATL) was assessed using a whole lysate and recombinant (CRA + FRA) antigen⁶. The recombinant ELISA showed 100% sensitivity and specificity, with no cross-reactivity with other infections. However, the conventional ELISA showed serological cross-reactivity with VL. Although the use of purified antigen preparations increased the specificity of the ELISA tests, its availability in health systems is still limited due to its higher cost.

Several authors have already studied the relationship between the intensity of serological reactivity of different IgG subclasses and Chagas disease morbidity⁷⁻¹². Morgan et al detected increased levels of IgG2 in CARD patients using an ELISA test based on epimastigotes of the Brazil strain. Verçosa et al. also verified increased levels of IgG2 in CARD patients using an ELISA test based on the flagellar portion of the *T. cruzi*. Increased levels of IgG1 and IgG2 in all Chagas disease patients, independent of their clinical form, were found. At the same time, the highest levels of IgG2 were found in the CARD group⁹. Different results using other antigenic preparations were observed and have been verified to be predominantly IgG1 and IgG3 in all studied Chagas disease patients, while IgG2 reactivity was observed in only 50% of cases¹³. However, equal concentrations of IgG1, IgG2, IgG3, and IgG4 in all studied Chagas disease patients, independent of their clinical form, were verified⁷. Associations between other classes of immunoglobulins and clinical forms of Chagas disease have also been found. Higher levels of IgM were predominant in patients presenting the cardiac form, while higher levels of IgA were predominant in those with the digestive form¹⁰. The predominance of IgA was also verified in patients in the acute phase and in those with the digestive form in the chronic phase¹⁴, corroborating previous data from this team¹⁵.

Taking into account the limitations of the available serological tests for Chagas disease diagnosis and the existent controversy regarding the correlation between serological reactivity and clinical manifestations of Chagas disease, the objectives of this investigation were to develop an *in-house* ELISA, to propose criteria for the analysis of the reactivity of total IgG and IgG subclasses to *T. cruzi* antigens, and to evaluate the applicability of the *in-house* method in the differential diagnosis of Chagas disease and the analysis of correlations with the clinical forms of the disease.

METHODS

Study area

The municipality of Berilo is located in the Jequitinhonha Valley, northeast of Minas Gerais State, Brazil (10°57'06"S; 42°27'56"W), at an altitude of 401m. The territorial area of Berilo is 586km², and its total population is 17,549 inhabitants, 77% of whom live in the rural area. The economic activity of the population is mainly based on agriculture and livestock production.

Patients and sera samples

Sixty Chagas disease patients (CH) were selected in Berilo municipality, Minas Gerais State, Brazil, from March to November 2006. All patients presented positive serological results in the diagnosis of *T. cruzi* infection (ELISA and indirect haemagglutination assay (IHA)). The clinical evaluation was performed through anamnesis, physical examination, conventional electrocardiogram (ECG), thorax X-ray, and esophagus and colon barium-contrast X-rays¹⁶. Additionally, echocardiographic evaluation of all patients

was performed using a *Cypress* portable device with unidimensional, bidimensional, continuous, pulsate and color Doppler adapted for tissue Doppler imaging. A multifrequency transducer, at 3.0 to 3.5MHz, with simultaneous electrocardiography registration was used. A standard echocardiography protocol was performed assessing the following variables: cardiac dimensions, left ventricular systolic and diastolic functions, and the global and segmental contractility of the left ventricle. The patients were clinically classified according to the Brazilian Consensus on Chagas Disease¹⁷. Those with neither Chagas disease symptomatology nor alterations in the physical examination, ECG, echocardiography, thorax X-ray, and esophagus and colon contrast X-rays were classified under indeterminate form (IND) (n=15, including 9 males and 6 females, with ages ranging from 13 to 57 years). The CARD group (n=30, including 5 males and 25 females, with ages ranging from 25 to 60 years) included patients with any type of cardiac alteration detected through either referred symptoms or detected signs in the physical examination, ECG, and echocardiography; and without any alteration in the thorax X-ray, and esophagus and colon contrast X-rays. Another group of Chagas disease patients simultaneously presenting digestive alterations detected by esophagus and colon contrast X-rays, with or without cardiac alterations, were classified as digestive/mixed (DIG/Mix) group (n=15, including 6 males and 9 females, with ages ranging from 37 to 59 years).

All patients in this study were integrated into the clinical assistance program of the Chagas disease project of Jequitinhonha Valley, Minas Gerais, and received continuous assistance when necessary.

A control group of noninfected individuals (NI) (n=15, including 6 males and 9 females, with ages ranging from 17 to 54 years) was selected from the local population of Berilo municipality, Minas Gerais, Brazil; all of them presented negative results of Chagas disease serological diagnosis. These subjects were also clinically evaluated through anamnesis, physical examination, conventional ECG, and chest X-ray.

Sera samples from patients infected with other relevant infectious-parasitic diseases were also included in this study. These patients were categorized as non-chagasic patients (NCH) and further subgrouped as patients infected with human immunodeficiency virus (HIV) (n=8, including 6 males and 2 females, with ages ranging from 19-60 years), hepatitis C virus (HCV) (n=8, including 7 males and 1 female, with ages ranging from 18 to 54 years), syphilis (SYP) (n=8, including 4 males and 4 females, with ages ranging from 18-58 years), visceral leishmaniasis (VL) (n=7, including 5 males and 2 females, with ages ranging from 20-67 years), and American tegumentary leishmaniasis (ATL) (n=8, including 5 males and 3 females, with ages ranging from 16-54 years). All patients with other infectious-parasitic diseases were residents in Belo Horizonte (HIV, HCV, and SYP), Montes Claros (VL), and Caratinga (ATL), all situated in State of Minas Gerais, Brazil.

In-house ELISA for evaluation of anti-*Trypanosoma cruzi* total IgG and IgG subclasses

The *in-house* ELISA methodology modified¹⁸ proceeded as follows. Briefly, each well of the ELISA plate was sensitized overnight at 4°C with 100µL of *T. cruzi* Y-strain antigen (4.5µg/mL) prepared by alkaline extraction. Following washing steps and blocking procedures with phosphate-buffered saline (PBS) supplemented with 10% fetal bovine serum, 100µL of serial dilution from each sera sample (1:10, 1:20, 1:40, 1:80, 1:160, 1:320, 1:640, and 1:1,280) was added to selected wells and the plate incubated for 45min at

37°C. After washing with PBS 0.05% Tween 20, 100µL of mouse anti-human anti-IgG and anti-IgG subclass biotinylated antibodies (Sigma, St. Louis, USA) were added to specific wells and the plate incubated for 45min at 37°C. The standardization of each conjugate dilution was previously performed using a range of serial dilutions (anti-IgG - 1:7,500, 1:10,000, 1:15,000, and 1:25,000; anti-IgG1 - 1:500, 1:1,000, 1:2,500, and 1:5,000; anti-IgG2 - 1:2,500, 1:5,000, and 1:7,500; and anti-IgG3 - 1:1,000 and 1:2,000) to assay positive and negative sera samples at 1:80 dilution. The anti-IgG4 conjugate was also titrated and did not show any reactivity; therefore, it was excluded from further analysis (data not shown). The selected dilution for each conjugate was established considering the highest difference between the median OD for positive and negative samples (delta median - Δ) (Figure 1). By this approach, 1:10,000, 1:500, 1:2,500, and 1:1,000 dilutions were selected for anti-IgG, anti-IgG1,

anti-IgG2, and anti-IgG3 conjugates, respectively (Figure 1). Following incubation and washing procedures with PBS 0.05% Tween 20, 100µL of streptavidin/horseradish peroxidase (HRP) (Sigma, St. Louis, USA) was added to all wells and the plates incubated for 45min at 37°C. The standardization of the streptavidin/HRP dilution was previously performed using a range of serial dilutions (1:500, 1:1,000, and 1:2,500), and the dilution 1:1,000 was selected as the best condition to segregate positive and negative samples. After incubation and washing procedures with PBS 0.05% Tween 20, 100µL of substrate solution (1mg of orthophenylenediamine (OPD), 3L of H₂O₂ (30vol), and 15mL of citrate buffer) was added to each well and the plates incubated for 5min at 37°C. Following incubation, the enzymatic reaction was stopped by adding 32µL of 2.5M H₂SO₄ solution to each well. The optical density (OD) was determined using a 490nm filter (SOFTMAX® PRO 4.0-Life Sciences Edition).

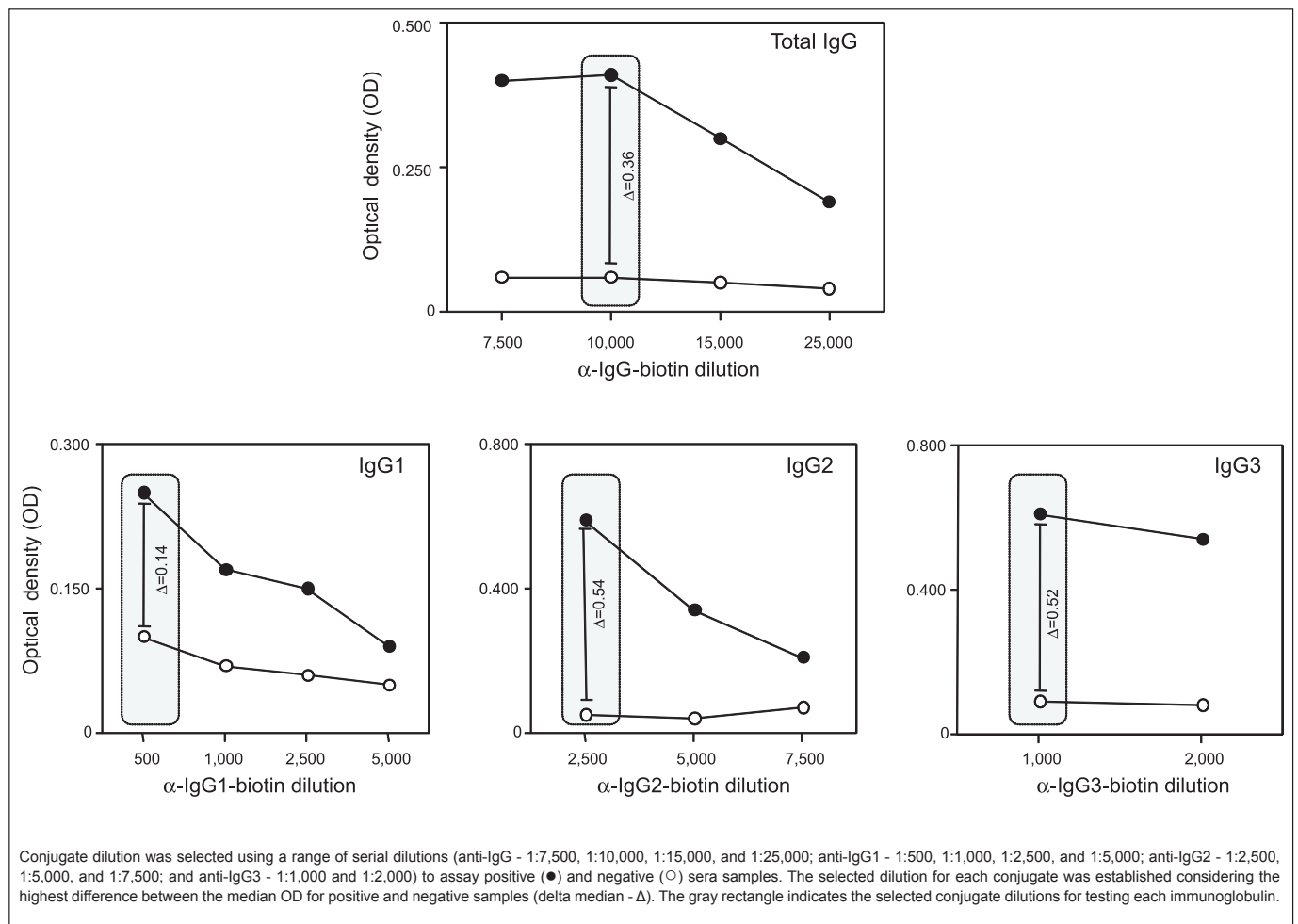
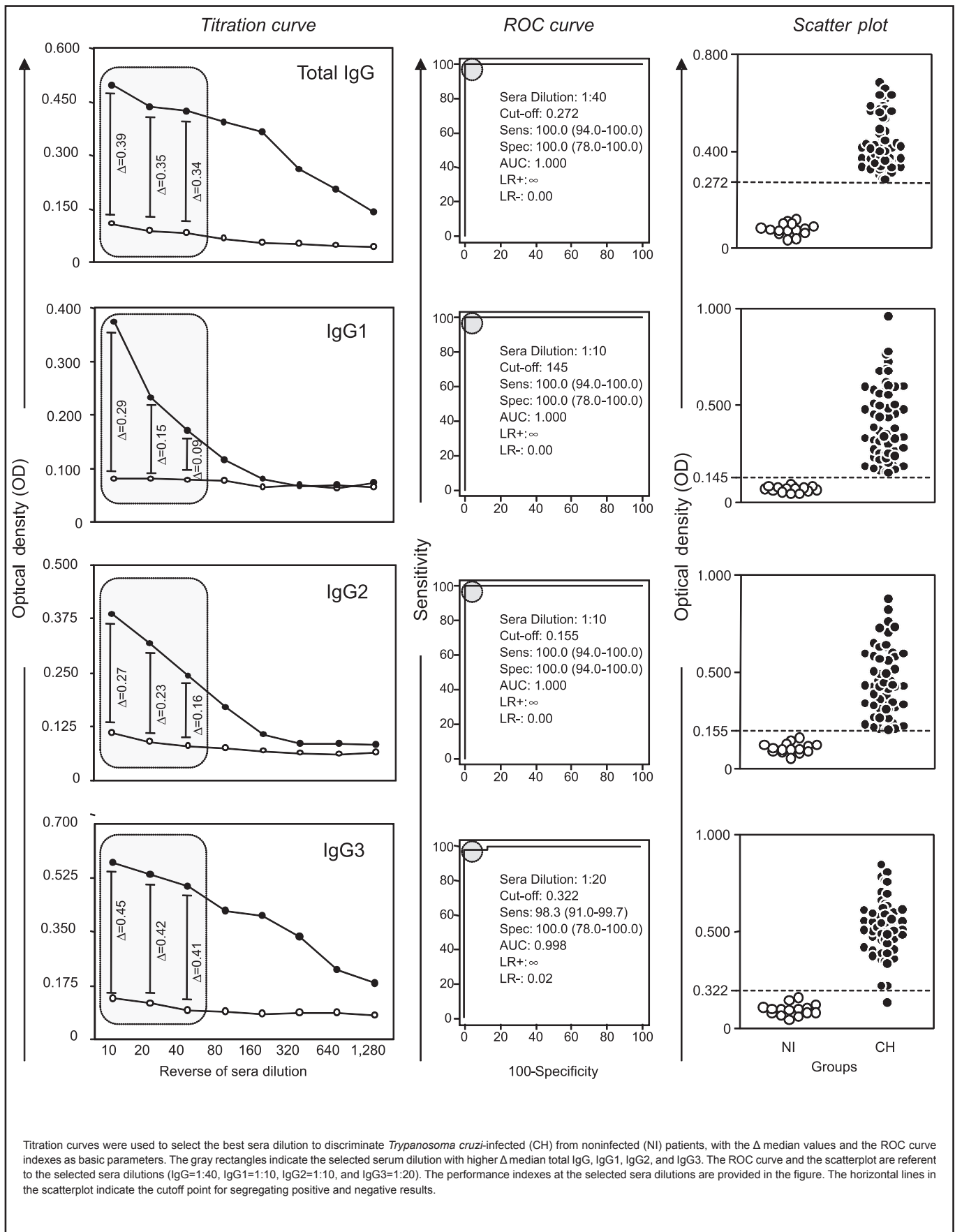


FIGURE 1 - Standardization of *in-house* ELISA method for the detection of total IgG and IgG subclasses (IgG1, IgG2, and IgG3) using *Trypanosoma cruzi* Y-strain alkaline antigen.

Data interpretation and statistical analysis

The best sera dilution to segregate the IgG reactivity of sera samples from *T. cruzi*-infected patients and NI was initially established along the titration curve, considering the highest difference between the median OD for positive and negative samples (delta median - Δ) (Figure 2). Thereafter, the sera dilution that was shown to have higher performance by the construction of the receiver operating characteristic curve (ROC) using the Medcalc statistical program (Broekstraat, Mariakerke, Belgium) was selected and identified as the

most useful condition to discriminate chagasic patients from NI individuals. The cutoff point to discriminate serological reactivity was selected for each immunoglobulin (total IgG, IgG1, IgG2, and IgG3), taking into account the ROC curve parameters, including the specificity, sensitivity index, and area under the curve (AUC) (Figure 2). The selected conditions were used to evaluate the IgG reactivity of sera samples from patients with other relevant infectious-parasitic diseases (Figure 3). The data were further analyzed with the two-graph receiver operating characteristic (TG-ROC) using the CMDT software package¹⁹.



Titration curves were used to select the best sera dilution to discriminate *Trypanosoma cruzi*-infected (CH) from noninfected (NI) patients, with the Δ median values and the ROC curve indexes as basic parameters. The gray rectangles indicate the selected serum dilution with higher Δ median total IgG, IgG1, IgG2, and IgG3. The ROC curve and the scatterplot are referent to the selected sera dilutions (IgG=1:40, IgG1=1:10, IgG2=1:10, and IgG3=1:20). The performance indexes at the selected sera dilutions are provided in the figure. The horizontal lines in the scatterplot indicate the cutoff point for segregating positive and negative results.

FIGURE 2 - Analysis of in-house ELISA reactivity of total IgG and IgG subclasses in sera samples from Chagas disease patients (CH●) and noninfected controls (NIO) using *Trypanosoma cruzi* Y-strain alkaline antigen.

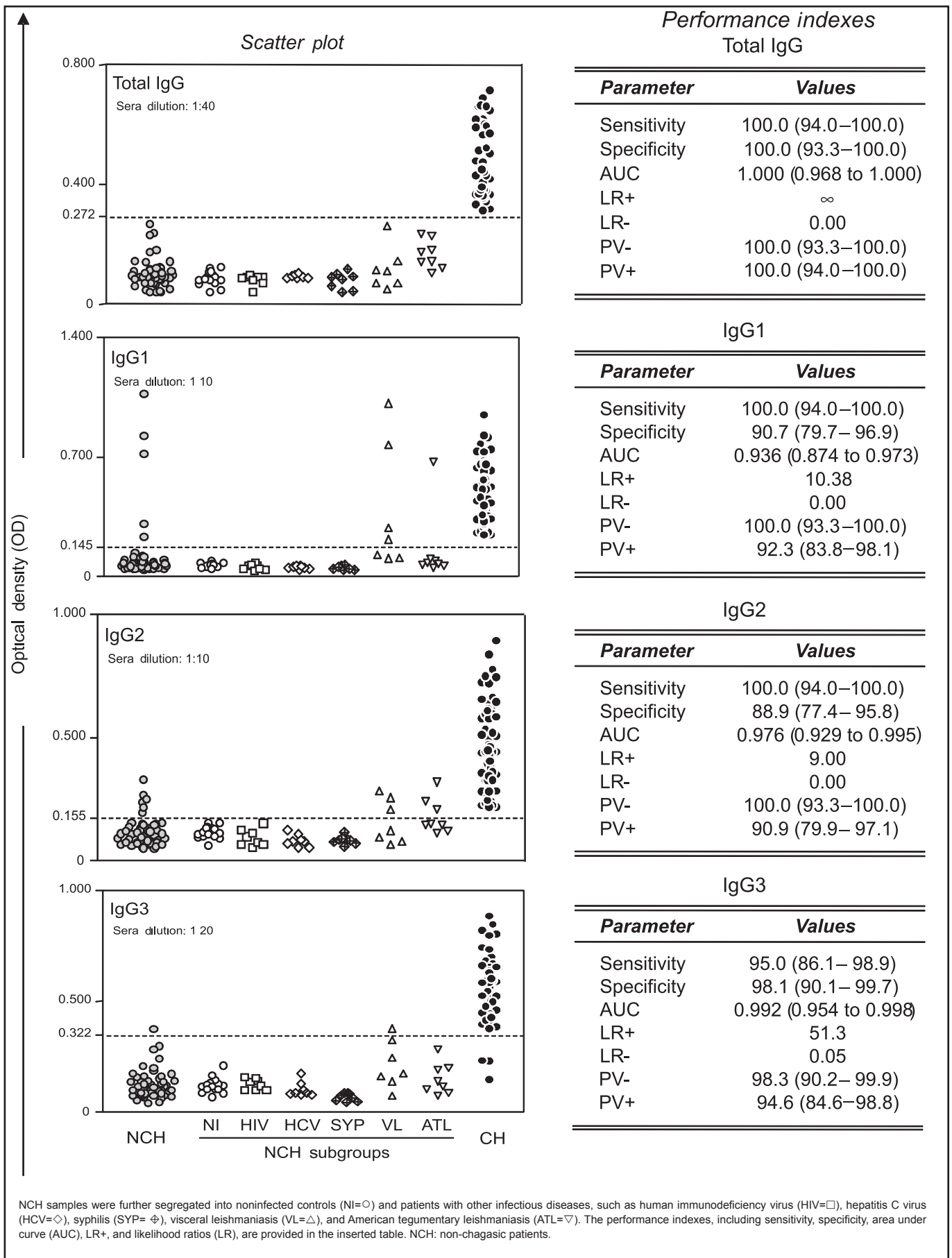


FIGURE 3 - Scatterplots of *in-house* ELISA reactivity at selected serum dilutions for total IgG (1:40) and IgG subclasses (IgG1=1:10, IgG2=1:10, IgG3=1:20) in sera samples from Chagas disease patients (CH=●) and non-chagasic samples (NCH=○) using *Trypanosoma cruzi* Y-strain alkaline antigen.

This method reads valid pairs of sensitivity and specificity considering pre-assigned cutoff values and allows the selection of the best condition for data interpretations. The cutoff of the sensitivity and specificity intersection point (θ_0) provides equal test indices. According to Greiner et al., whenever the value of θ_0 is lower than a preselected accuracy level (90% or 95%), two cutoff values are selected to represent the limits of an *intermediate range* (IR). The IR can be considered as a borderline range for the clinical interpretation of the test results. The selection of cutoff values was corroborated by graphical displays of logarithmically transformed likelihood ratios (LR) ($LR+ = Se / (1 - Sp)$ and $LR- = (1 - Se) / Sp$). The Efficiency and Youden (J) indices were also presented to support the cutoff selection (Figure 4).

Differences in the OD median values of IgG reactivity between different clinical groups of Chagas disease patients (IND, CARD, and DIG/Mix) were evaluated by the Kruskal-Wallis test, followed by Dunn's test, using the GraphPad Prism software (San Diego, CA, USA). Statistical significance in all cases was considered at $p < 0.05$ (Figure 5).

Ethical considerations

All volunteers were included in the study after they had read and signed an informed consent form approved by the Ethics Committee of the Centro de Pesquisas René Rachou (CPqRR), FIOCRUZ, Belo Horizonte, Minas Gerais, Brazil (Process 0007/02).

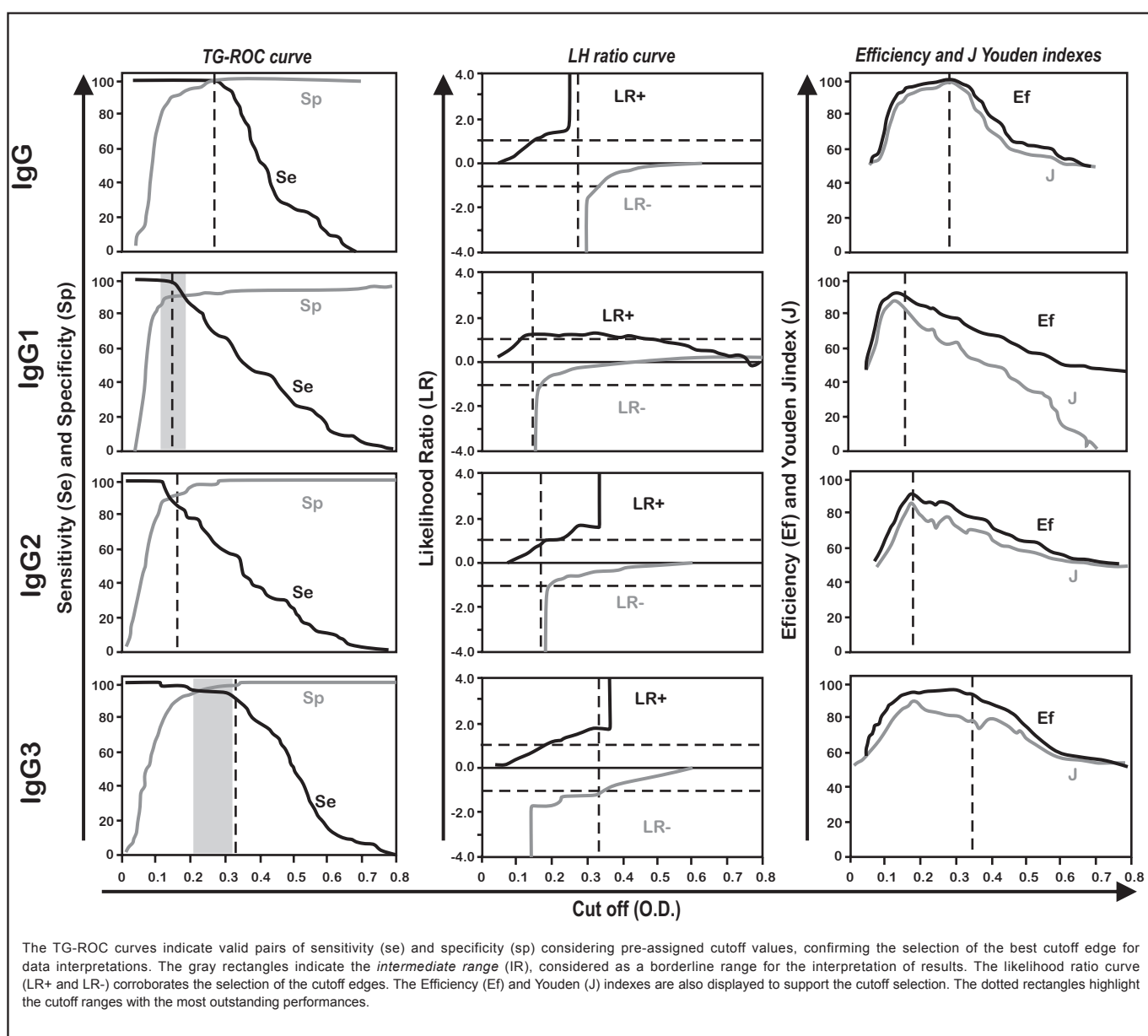


FIGURE 4 - Analysis of *in-house* ELISA reactivity at selected serum dilutions for total IgG (1:40) and IgG subclasses (IgG1=1:10, IgG2=1:10, IgG3=1:20) in sera samples from Chagas disease patients and non-chagasic samples using *Trypanosoma cruzi* Y-strain alkaline antigen.

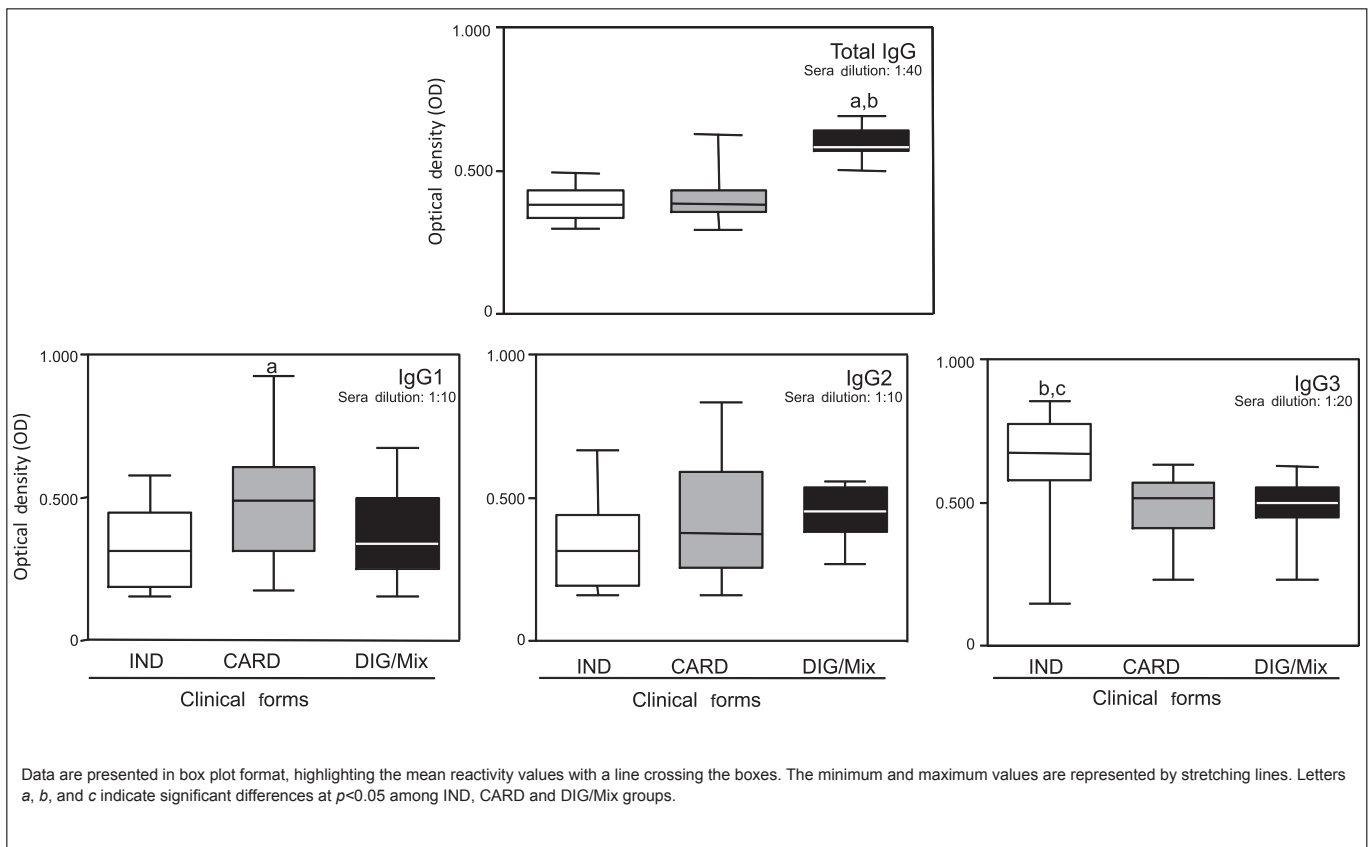


FIGURE 5 - Analysis of *in-house* ELISA reactivity at selected serum dilutions for total IgG (1:40) and IgG subclasses (IgG1=1:10, IgG2=1:10, IgG3=1:20) in sera samples from Chagas disease patients from Berilo municipality, Jequitinhonha Valley, State of Minas Gerais, Brazil, categorized under indeterminate (IND), cardiac (CARD), or digestive/mixed (DIG/Mix) group.

RESULTS

Discrimination between *Trypanosoma cruzi*-infected and noninfected individuals by the differential reactivity of total IgG and IgG subclasses

Upon its standardization, the *in-house* ELISA (Figure 1) method was used to evaluate the reactivity of total IgG and IgG subclasses, with the aim of discriminating *T. cruzi*-infected (CH) from noninfected individuals (NI). During the standardization procedure, the best dilution of second-step reagents to be applied for diagnosis purposes was chosen by considering the delta median (Δ median) between positive (CH) and negative samples (NI), as well as the ROC curve indexes for each immunoglobulin. Using a similar approach to select the best sera dilution to be applied for diagnosis purposes, our findings demonstrated that only the first three serum dilutions presented an outstanding ability to discriminate the reactivity of total IgG, and IgG1, IgG2, and IgG3 subclasses along the titration curve, to segregate patients with *T. cruzi* infection (CH) from noninfected controls (NI). The data analysis demonstrated distinct Δ median values for IgG (0.39, 0.35, and 0.34), IgG1 (0.29, 0.15, and 0.09), IgG2 (0.27, 0.23, and 0.16), and IgG3 (0.45, 0.42, and 0.41) at serum dilutions of 1:10, 1:20, and 1:40, respectively. Although the Δ median value was higher at 1:10 dilution for all IgG reactivity, the selected sera dilution also took into account the best performance in the ROC curve analysis including sensitivity, specificity, and AUC. Using these criteria, the sera dilution 1:40 was elected for IgG, 1:10 for IgG1 and IgG2, and 1:20 for IgG3 (Figure 2).

The use of the cutoff edge indicated by the ROC curve analysis revealed outstanding performance indexes for IgG, IgG1, and IgG2, with 100% sensitivity and specificity, and AUC=1.0. Moreover, the LR ratio analysis by the ROC curve further confirmed these indicators (Figure 2). The analysis of IgG3 demonstrated indexes with lower power to discriminate the clinical groups CH and NCH, with 98.3% sensitivity and AUC=0.998 (Figure 2).

Discrimination between *Trypanosoma cruzi* infection and other infectious diseases by the differential reactivity of total IgG and IgG subclasses

Considering the excellent performance of the *in-house* ELISA in discriminating chagasic patients from noninfected controls, an additional analysis was subsequently performed to evaluate the reactivity of sera samples from chagasic patients in parallel with samples from noninfected controls and also from patients with other relevant infectious-parasitic diseases, including HIV, HCV, SYP, VL, and ATL.

The analysis of total IgG at serum dilution 1:40 was able to discriminate patients with *T. cruzi* infection from patients with the other studied infectious diseases (HIV, HCV, SYP, VL, and ATL) and NI individuals, with maximum performance indexes, including 100% sensitivity and specificity, AUC of 1.0, and LR+ and LR- values indicating infinite probability of finding a positive result for CH and a negative result for NCH (Figure 3).

Although the analysis of IgG1 at serum dilution 1:10 showed 100% sensitivity, indicating that all CH presented a positive result, the low specificity (90.7%) indicated the presence of cross-reactivity in 5 out of 54 tested samples, including 7.4% false-positive results due to

four VL samples and 1.9% due to one ATL sample. The AUC=0.936, LR+=10.38, and LR-=0 indicated lower performance as compared with total IgG (Figure 3).

The evaluation of IgG2 reactivity at serum dilution 1:10 also showed 100% sensitivity but also revealed lower specificity (88.9%), with cross-reactivity in 11.1% of the tested samples, due to the existence of false-positive results in three VL and three ATL samples. The AUC=0.976, LR+=9, and LR-=0 indicated lower performance as compared with total IgG (Figure 3).

The analysis of IgG3 reactivity at serum dilution 1:20 demonstrated low sensitivity (95%), with three out of 60 patients with *T. cruzi* infection showing OD values below the cutoff point. The specificity of 98.1% was due to cross-reactivity observed in one VL sample. The AUC=0.992 showed high accuracy. Although the LR+=51.3 indicated that a positive result has higher probability of belonging to the *T. cruzi*-infected population as compared with NCH, the LR-=0.05 showed borderline performance in identifying negative results (Figure 3).

Considering the good performance of the *in-house* ELISA when applied to the diagnosis of Chagas disease and aiming to validate our findings with supplementary performance analysis, the data were further evaluated using novel parameters, including the TG-ROC, the LR ratio in log base graphs, and the simultaneous analysis of the Efficiency and Youden indexes (Figure 4). In the first approach, valid pairs of sensitivity and specificity were read for pre-assigned cutoff values directly from the TG-ROC plots¹⁹. The TG-ROC analysis showed that the *in-house* ELISA used to detect total IgG reactivity did not present any intermediate range (IR) that can be considered a borderline range for the clinical interpretation of test results, therefore indicating that the method displayed 100% valid data with no incorrect results. These findings were also confirmed by the maximum LR+ and LR-, and the Efficiency and Youden index analysis (Figure 4). Together, these analyses showed that the anti-*T. cruzi* IgG detected by the *in-house* ELISA at sera dilution 1:40 using the cutoff point=0.272 was adequate to confirm the diagnosis of Chagas disease and exclude non-chagasic samples (Figure 4).

Although the analysis of IgG2 performance also evidenced the absence of an intermediate range in the TG-ROC graph, as reported in the previous analysis (Figure 3), it was not possible for the IgG2 reactivity to conclusively discriminate *T. cruzi*-infected individuals from non-chagasic patients, considering the cross-reactivity with sera from VL and ATL patients.

The most unsatisfactory results were found in the analysis of IgG1 and IgG3 reactivity. The analyses of IgG1 and IgG3 anti-*T. cruzi* reactivity revealed the presence of an intermediate range, corresponding to the existence of gray zones that do not guarantee a clear discrimination between *T. cruzi*-infected and noninfected individuals. Moreover, the LR analysis of IgG1 reactivity did not show outstanding performance. Therefore, together with the previous analysis (Figure 3), these findings did not recommend the use of the IgG1 and IgG3 detected by the *in-house* ELISA proposed in this study as a valuable method for diagnosis of Chagas disease (Figure 4).

Applicability of the *in-house* ELISA in analyzing total IgG and IgG subclasses reactivity and monitoring Chagas disease morbidity

The analysis of IgG reactivity at serum dilution 1:40 revealed that the DIG/Mix group presented significantly higher OD reactivity as compared with the IND and CARD groups (Figure 5). On the

other hand, the IgG1 reactivity at serum dilution 1:10 and the IgG3 reactivity at serum dilution 1:20 demonstrated that the median OD for the IND group was significantly lower as compared with that for the CARD group (Figure 5). No significant differences were observed in IgG2 reactivity at sera dilution 1:10 among the distinct clinical groups evaluated (Figure 5).

DISCUSSION

In this study, an *in-house* ELISA method was standardized to better determine the reactivity of total IgG, and IgG1, IgG2, and IgG3 subclasses in *T. cruzi*-infected and noninfected patients. Additionally, the *in-house* ELISA technique was applied to verify the reactivity of these immunoglobulin classes and subclasses in groups of individuals with *T. cruzi* infection and other relevant infectious-parasitic diseases (HIV, HCV, SYP, VL, and ATL). Initially, after the standardization procedures to define the best dilutions of the second step reagents (Figure 1), the goal of the study was to verify the best serum dilutions able to discriminate Chagas disease patients from the other selected groups. Our findings demonstrated that only total IgG reactivity showed no cross-reactivity with sera from patients without *T. cruzi* infection. However, the IgG1 and IgG2 analysis showed cross-reactivity with sera samples from VL and ATL patients. IgG2 showed higher cross-reactivity with sera samples from ATL as compared with IgG1 and IgG3. This finding is in agreement with previous reports showing that *T. cruzi*-infected patients presented high levels of cross-reactivity of IgG2 with *Leishmania braziliensis* antigens²⁰.

The cross-reactivity between anti-*Leishmania* and anti-*T. cruzi* IgG2 may explain these data. Although IgG3 showed lower cross-reactivity with other diseases, with only one false-positive result, the performance of this subclass was not validated due to the low sensitivity index. These findings agreed with the data in the literature demonstrating the existence of shared antigens between *T. cruzi* and *Leishmania sp.*, such as carbohydrate epitopes²⁰⁻²³. On the other hand, the use of crude antigens in this *in-house* ELISA may explain this cross-reactivity since the same phenomenon was not observed in the sera of patients with VL, ATL, SYP, HIV, and HCV when CRA+FRA recombinant antigens were used⁶. Thus, the discovery and availability of purified antigens or antigenic fractions from *T. cruzi* with higher specificity and similar sensitivity to avoid cross-reactions among these and other infections may represent a next step in further investigations to validate *in-house* ELISA methods. Nevertheless, the present study demonstrated that the detection of total IgG using an *in-house* ELISA technique with *T. cruzi* crude alkaline antigenic extracts, as proposed by our group at the *Laboratório de Doença de Chagas* of the *Universidade Federal de Ouro Preto*, represent a valuable tool for the diagnosis of *T. cruzi* infection, with outstanding performance indexes. The major changes performed in the present investigation as compared with the original methodology proposed¹⁸ included the use of biotinylated second-step reagents, the use of serial dilutions for all tested serum samples, and a standardized incubation temperature of 37°C. The use of biotinylated reagents may lead to increased performance of this *in-house* ELISA due to the reagents' ability to expand the detection capacity of the system from that in the original methodology. Moreover, these reagents require a shorter development time (5min) as compared with that in the original method (15min), which may reduce the occurrence of cross-reactivity and false-positive results.

The second goal of this study was to verify whether the reactivity of total IgG and the different IgG subclasses detected by the *in-house* ELISA was associated with the different clinical forms of the disease. Firstly, this analysis showed that total IgG and IgG3 showed the highest OD values in all patients, followed by IgG2 and IgG1. The total IgG was higher in patients from the DIG/Mix group than in the other clinical groups. The total IgG reactivity was significantly different among the different clinical forms of the disease and presented a positive correlation with the existence of clinical manifestations of the disease. These results agree with a study²⁴ that used the IHA based on *T. cruzi* antigens of the Y strain, which verified that the total IgG levels were slightly higher in patients from the State of Goiás, Brazil, with severe cardiomyopathy and digestive manifestations. However, the same authors did not observe the same correlation when they used an ELISA and a complement fixation reaction (CFR), indicating that the biological meaning of these findings is not understood. Patients with chagasic cardiomyopathy from Virgem da Lapa, the municipality next to Berilo, presented higher total IgG reactivity independent of the use of different serological tests (conventional ELISA, recombinant ELISA, IHA, and indirect immunofluorescence (IIF)) and antigenic preparations²⁵. Differently, no association between levels of IgG and clinical manifestations of Chagas disease was observed when 225 patients with different clinical forms of Chagas disease, 121 patients with other diseases (SYP, toxoplasmosis, kalazar, mucocutaneous leishmaniasis, connective tissue diseases, malaria, rubeola, and *Pemphigus foliaceus* infection), and 160 healthy individuals were evaluated using antigens at different stages of the parasite life cycle (Y strain), and ELISA, IHA, CFR, and IIF techniques¹⁴. Similarly, no correlation was observed by Cordeiro et al⁸, when they evaluated other groups of chagasic patients from Argentina and State of Minas Gerais, Brazil.

The analysis of the IgG1 subclass demonstrated lower reactivity in the IND group as compared with the CARD group. This finding is discrepant to those previously reported by several other authors^{7-10,13,26-27}. Only at serum dilution 1:10 was a significant difference in the OD of IgG1 observed between the IND and CARD groups. No correlation (neither positive nor negative) with morbidity of Chagas disease was observed with the IgG1 subclass, possibly due to the low OD values observed. The results were different from those found by other authors who, even when using different serological tests, verified IgG1 predominance over the other IgG subclasses. The measure of IgG1 was also suggested as a prognosis marker for Chagas disease since the level was found to be significantly higher in patients with the indeterminate form of the disease when compared with the other clinical groups⁸. In fact, we do not have a convincing explanation for the obtained results regarding IgG1.

The evaluation of IgG2 did not reveal a direct association with cardiac involvement, in contrast to other authors, who reported higher reactivity of this IgG2 subclass in patients with different levels of cardiopathy^{9-10,12}. On the other hand, high levels of IgG3 were detected, especially in the IND group as compared with the CARD and DIG/Mix groups. However, several studies considering the IgG3 subclass did not find the same association with higher levels of this immunoglobulin in all patients, independent of the clinical form of the disease^{7,10,13,26}.

The discrepancy of these results may be especially attributed to methodological variations and the diverse antigens used in the different techniques. Additionally, other variables regarding the selected casuistry may be involved, such as the geographical origin of the patients and consequent predominant *T. cruzi* population, and the length of the infection^{7-10,12-13,26}.

Finally, the authors believe that this type of study is of great importance because *in-house* ELISA methods, when well established, may serve as important tools for the diagnosis and prognostic study of Chagas disease, especially in developing countries, where the low availability of financial support for research may represent a remarkable limitation for population serological surveys.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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