

Short Communication

Effect of chaotropes in Chagas disease and leishmaniasis cross-reacting serology assays for epidemiological surveys

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Abstract

Introduction: Serological cross-reactivity between leishmaniasis and Chagas disease, especially at low titers, leads to difficulties of the seroepidemiological interpretation. **Methods:** We have studied the ability of urea as a chaotrope to select high-avidity antibodies in IgG ELISA, thus reducing low-avidity IgG cross-reactivity in serologically positive samples in both assays. **Results:** Using 0.5M urea for diluting the sample efficiently defined leishmaniasis or double infections in high-avidity IgG ELISA and eliminated false-positive results. **Conclusions:** The use of a chaotropic diluting agent is useful for improving the specificity of Chagas disease and leishmaniasis immunoassays.

Keywords: Serology. Chagas disease. Leishmaniasis. ELISA. IFAT. IgG antibody avidity.

Chagas disease and American leishmaniasis, two chronic endemic diseases in the Americas, are caused by protozoa of the order Kinetoplastida and the family Trypanosomatidae, belonging to the genera *Leishmania* and *Trypanosoma*, respectively¹. The differential diagnosis of Chagas disease and leishmaniasis are usually easy on clinical grounds in symptomatic patients. However, in asymptomatic patients, serology is a conflicting tool frequent in case of both infections; dual serological positivity could occur due to cross-reactive antibodies, owing to the genetic proximity of the two pathogens, and also due to double infections, owing to the high prevalence of both infections in the same area. Avidity assays are used to detect high-affinity antibodies; they use chaotropic agents, which eliminate low-avidity antibodies². The chaotrope could be used as a dissociating agent to elute bound immunoglobulin G (IgG) or to dilute the IgG, thus blocking low-affinity binding, by affecting the hydrogen bridges and other binding forces between the antigen and antibody³. These chaotropes can also eliminate non-specific antibodies, by their inclusion in the sample diluting solvent for high-avidity enzyme linked immunosorbent assay (ELISA)³.

Serum samples from patients with Chagas disease (n = 10) were provided by Dante Pazzanese Institute of Cardiology, São Paulo State Secretary of Health; leishmaniasis patients serum

samples (n = 10) were provided by the Seroepidemiology Laboratory of the Tropical Medicine Institute of the State of São Paulo [*Instituto de Medicina Tropical de São Paulo* (IMTSP)]. All samples were previously serologically assayed in their original laboratories and stored at -20°C until use. Field samples were also provided by Superintendency for the Control of Endemics [*Superintendência de Controle de Endemias* (SUCEN)] from the Program for the Control of Chagas Disease, originating from residents in four geographic regions of the State of São Paulo, Brazil, resulting in eleven available samples that were positive for both infections. *Leishmania amazonensis* promastigotes cultures were kept in the Laboratory of Protozoology in IMT, in Corning® culture bottles with Roswell Park Memorial Institute (RPMI 1640) medium (Cultilab®), as recommended⁴, containing 20% of sterile inactivated fetal calf serum, 1µg/L calf hemin and 0,01mg/mL gentamycin, in Biological Oxygen Demand (BOD) incubators, under controlled temperature conditions (24°C). The cultures were daily monitored by phase-contrast microscopy (40X objective). After an incubation period of five days, the promastigote suspension obtained was centrifuged under refrigeration (4°C) at 3,000rpm for 20 minutes. Purified *Leishmania amazonensis* promastigotes were added to 20 volumes of distilled water and disrupted by sonication (Sonic Dismembrator, Quigley-Rochester Inc., USA) in an ice bath using ten 30-second periods at 40Hz each; they were checked for lysis using phase contrast microscopy.

The disrupted parasite cell solution was made isotonic by the addition of the same volume of 0.3M NaCl and cleared by centrifugation at 10,000g for 30 minutes at 4°C. The protein

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Received 10 November 2017

Accepted 4 April 2018



content of the supernatant was determined, after which it was stored at -70°C until use. Polystyrene 96-well microtiter plates certified for high protein binding (Costar[®]) were used. The plates were sensitized with soluble *L. amazonensis* antigen diluted in carbonate-bicarbonate buffer (0.1M, pH 9.5; 100 μL per well), at the concentrations of 1 μg and 10 μg . After incubation for 20 hours at 6°C , the plates were washed thrice, for five minutes each, with phosphate-buffered saline [(PBS); 0.02M, pH 7.2], containing 0.05% of polyoxyethylene sorbitan monolaurate (Tween 20 - Sigma[®]) (PBS-T) and blocked with a 0.3% solution of skimmed milk (Molico[®]) in PBS-T for one hour at 37°C . After blocking and three washes with PBS-T (100 μL /well), the control serum samples, positive and negative, at dilutions of 1:100, 1:200, 1:400, 1:800, 1:1,600, 1:3,200, and 1:6,400 were added to the wells in duplicate, and the plate was incubated at 37°C for one hour. After three further washes, a conjugated anti-human IgG peroxidase, (Sigma[®]) at a dilution of 1:20,000 (100 μL per well), was added to each well and the plate was incubated again for one hour at 37°C .

After washing, the revelation of the reaction was performed by addition of 0.05M sodium citrate solution (pH 5.8), containing 0.4mg/mL of ortho-phenylenediamine (OPD), and 0.03% hydrogen peroxide (H_2O_2). The reaction was stopped after 30 minutes by adding 4N HCl. The absorbance [optical density (OD)] readings were performed at 492 nm using a colorimeter equipped with an automatic evaluation system for microplates (Labsystems Multiskan MS[®]).

For chaotrope ELISA, the standardization of the optimal concentration of dissociating agents (chaotropes) was made by using the 20 serum samples previously assayed for both infections. The chaotrope used in the standardization of the ELISA was a urea solution further diluted to the following concentrations: 0.125M, 0.25M, 0.5M, 1M, and 2M. The samples were used at the following dilutions: 1:100, 1:400, 1:1,600, and 1:6,400. This diluent was used for sample dilution, and the ELISA was performed as described above. The titers for an optical density value of 1 in ELISA could be estimated in each sample by log-log transformation. In order to establish if a low antigen concentration would be sufficient to eliminate cross-reaction, we tested serially diluted standard positive and negative samples using 1 $\mu\text{g}/\text{mL}$ and 10 $\mu\text{g}/\text{mL}$ concentrations of the soluble antigenic extract in solid phase in ELISA. Both assays showed high levels of antibody detection and were equally accurate in discriminating positive and negative controls until the most diluted sample, presenting a typical log response of titers. After analyzing the curve by nonlinear regression, it was seen that a lower antigen concentration in the solid phase yielded better negative results, without affecting the positive sera results, with parallel estimated regression, while higher concentration tends to cross curves between the negative and positive serum samples. Thus, we have chosen the lower concentration (1 μg antigen/mL) for use in the ELISA. We tested several chaotrope concentrations in sample dilutions of both infections to verify the effects on IgG ELISA cross-reactive antibodies. The reactions were performed with the chaotrope (urea) concentration varying from 0.125M to up to 2M. We used two standard dilutions of sera, 1/100 and 1/400, and estimated

the serum titers for 1.0 ELISA OD by plotting dilutions in log-log plots. We used the 20 serum samples previously screened externally by using commercial Indirect Immunofluorescence Antibody Test (IFAT): ten seropositive for Chagas disease and ten seropositive for leishmaniasis. Interestingly, at the higher dilution, the *Leishmania* patients' sera reacted more intensely than the Chagas patients' sera if the co-infected conditions were removed; this allows a better distinction of some sera. Despite these effects, based only on the ELISA absorbances, cross reactive antibodies were not completely eliminated.

For further analysis, we performed the determination of reactive titers for each chaotrope concentration; these data are shown in **Figure 1A** and **Figure 1B**. Titers are more proportional for antibody concentration in ELISA due to the exquisite sensitivity of enzymatic reactions. It is clearly seen in **Figure 1A** that two Chagas patients' serum samples and one leishmaniasis serum sample presented no significant decay of titer despite the increasing chaotrope concentration, showing high avidity. We also estimated the ratio of the decay of those reactions according to the mean of the absorbance of reactive samples, which was expressed as OD in specific dilution or titers (**Figure 1B**). This shows that at the 1/100 dilution, there is an inflexion point in this ratio in case of 0.5M urea in the diluent. The same inflexion was observed for 0.25M urea when the 1/400 dilution was analyzed, while titer the shows an intense decay. Due to feasibility, we define that this assay would be performed best using 0.5M urea in a 1/100 dilution of the serum sample, as titer determination is complex and costly, and the 1/100 dilution usually has low OD results. For studying the use of this methodology in serum samples, we tested cross-reacting positive samples from the state of São Paulo, Brazil; these results are shown in **Figure 2A** and **Figure 2B**. The OD values associated with one sample remained invariant, but a reduction was observed in case of the further ten samples (**Figure 3**).

The dilutions of samples in PBS and 0.5M urea yielded the best results in terms of discrimination of cross-reactions (**Figure 3B**). Three positive samples presented no decrease of high-avidity ELISA reactivity, suggesting that those samples represented double infections (**Figure 3C**).

Six other samples presenting low-grade reactivity in conventional ELISA showed negative high-avidity ELISA reactivity; these represented only the cross-reacting Chagas disease sera.

Besides these defining results, this assay also presented two samples negative for both assays; this represents a screening error or true false-positive reaction for both assays. This result suggests that high-avidity ELISA could be used with field samples for improving the resolution of ELISA assays in epidemiological studies (**Figure 3C**).

We successfully standardized a high-avidity ELISA using standard sera from American visceral leishmaniasis and American trypanosomiasis samples, defining that high-avidity antibodies could be determined both by conventional OD ELISA or more precise IgG titers in multiple ELISA reactions, and also using an optimal urea concentration in the serum diluent solution, which allows the distinction between coinfection, cross-reacting antibodies, and false-positive results.

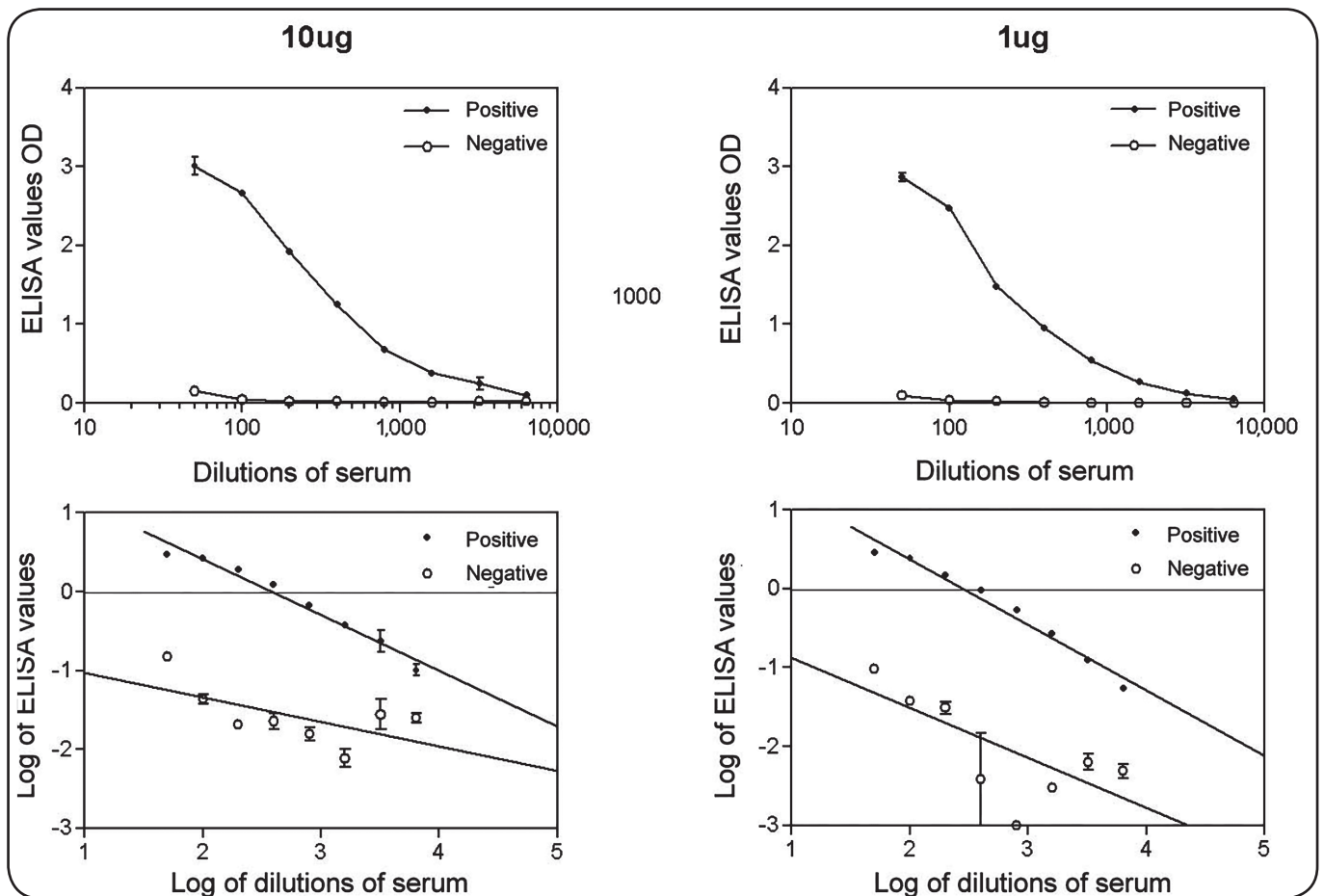


FIGURE 1: Evaluation of the effect of antigen concentration in the solid phase for the detection of anti-*Leishmania* IgG antibodies. The upper graphs indicate the optical density obtained in duplicate wells, with the SEM. The lower graphs indicate the log-log conversion and linear regression assays of the same data. Note the almost parallel slope of curves in case of the lower antigen concentration. **ELISA:** enzyme linked immunosorbent assay; **OD:** optical density; **IgG:** immunoglobulin G; **SEM:** standard error of the mean.

For epidemiological studies, the diagnosis must be conducted in serum samples. The overlapping geographical distribution of Chagas disease and leishmaniasis leads to incorrect estimations due to the diverse occurrence of either *Trypanosoma cruzi* or *Leishmania* cases in a given region; such incorrect estimations could be minimized by using an immunoassay specific enough to produce a significant reduction of such cross-reactions^{5,6}.

We standardized a high-avidity ELISA that yielded good results despite standard sera presenting true cross-reactions. This determination allows to choose a chaotrope concentration that is feasible for use with field samples. The strategies available for overcoming these difficulties for the diagnosis of leishmaniasis in epidemiological studies were reported as the use of a lower cut-off than that recommended for Chagas disease⁷, the use of different antigens, including recombinant antigens⁸, in serological reactions^{9,10}, analysis of sera by western blot with different *Leishmania* antigens utilized for the serological diagnosis of American cutaneous leishmaniasis¹¹, or subsequent retest by means of the polymerase chain reaction (PCR)¹² and flow cytometry¹³. All these procedures have sampling or feasibility problems due to high cost or

need for resampling. A chaotrope-containing sample diluent provides a reasonable distinction between Chagas disease and leishmaniasis double infections or cross-reacting sera without requiring new sampling and at a low cost; this strategy could be applied to epidemiological surveys, for improving their distinctive potential without the need of new techniques or equipment. Four chaotropes are most frequently used as sample diluents: thiocyanate salts, diethylamine, guanidine, and urea, out of which urea is associated with lower costs and the absence of high toxicity, unlike the other substances. From a clinical and individual standpoint, the diagnostic reliability and usefulness of such a serological technique is reduced, requiring association with other diagnostic tests and the clinical picture presented by the patient. Mismanagement caused by misdiagnosis can cause a delay in the prescription of proper treatment or even lead to an incorrect treatment¹⁴. Besides this, reported IgG avidity evolution in leishmaniasis is complicated by the absence of a clear marker of the beginning of the infection, resulting in high-avidity antibodies in most patients¹⁵. These characteristic high-avidity antibodies could be low-avidity antibodies associated with a heterologous antigen, explaining the urea removal found in our data.

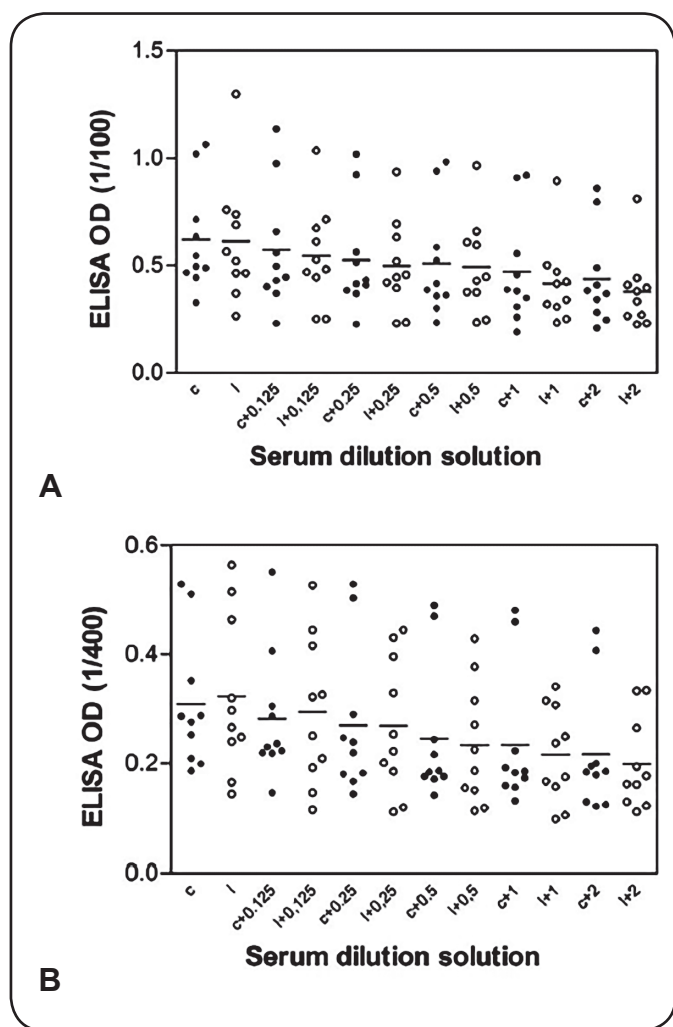


FIGURE 2: Effect of Urea (chaotrope) in anti-*Leishmania* IgG ELISA initial serum dilution solution. Codes: c = Chagas patients samples (closed dots); l = Leishmaniasis patients samples (open dots); the bars represent the mean values. **A:** Serum samples diluted 1/100. Serum samples diluted 1/400. **ELISA:** enzyme linked immunosorbent assay; **OD:** optical density; **IgG:** immunoglobulin G.

In this study, we propose the use of a sample dilution solution containing 0.5M urea as a chaotrope, which promotes the efficient performance of ELISA by reducing cross-reactions, especially with regards to retesting near cut-off samples. The use of chaotropes as sample diluents has proved to be useful and adequate for investigating the incidence and prevalence of leishmaniasis in areas with prevalence of American trypanosomiasis.

Conflict of interest

The authors declare that there is no conflict of interest.

Financial support

This work was used partially for the master’s dissertation in Parasitology [Departamento de Parasitologia, Instituto de Biologia, Universidade de Campinas (UNICAMP)] of AP Santos. HF Andrade Jr was a fellow of Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and

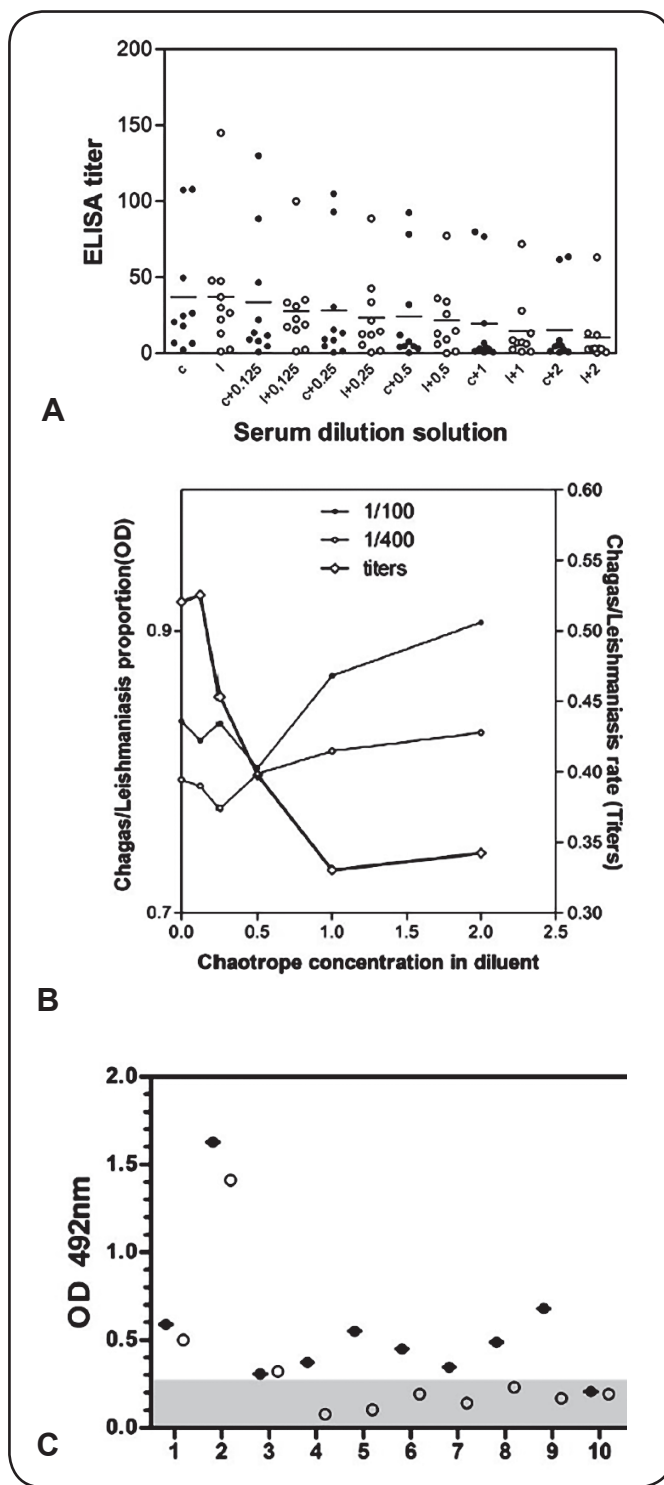


FIGURE 3: Determination of anti-*Leishmania* IgG titers in the serum samples from Chagas or Leishmaniasis patients, after serially diluting the sera using different chaotropes in the diluent solution. c = Chagas patients samples (closed dots); l = Leishmaniasis patients samples (open dots); the bars represent the mean values. **A:** Effect of chaotrope on the titer of standard sera. Titers are expressed as the serum dilution that gives a 1.0 OD ELISA reaction. **B:** Reactivity proportion in OD and titers from sera of both diseases, in different chaotrope concentrations. **C:** Field cross-reacting samples for leishmaniasis and Chagas disease tested by high-avidity leishmaniasis ELISA. The open dots indicate high-avidity ELISA, while the closed dots indicate conventional anti-*Leishmania* ELISA. The shaded area represents the negative area for high-avidity ELISA using mean + 2 SD of the negative samples. **ELISA:** enzyme linked immunosorbent assay; **OD:** optical density; **IgG:** immunoglobulin G; **SD:** Standard Deviation.

Fundação Faculdade de Medicina (FFM). This work was partially supported by Laboratórios de Investigação Médica 49 (LIM 49), Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo (HCFMUSP) and Laboratório de Imunoepidemiologia, Superintendência de Controle de Endemias (SUCEN).

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