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# A simple immune complex dissociation ELISA for leishmaniasis: Standardization of the assay in experimental models and preliminary results in canine and human samples

Camila Aparecida de Carvalho[a], Anette Kelsei Partata[c], Roberto Mitsuyoshi Hiramoto[b], Samanta Etel Treiger Borborema[b], Luciana Regina Meireles[a], Nanci do Nascimento[c], Heitor Franco de Andrade Jr.[a,*]

[a] Protozoology Laboratory, Instituto de Medicina Tropical de São Paulo, University of São Paulo, Av. Dr. Eneas de Carvalho Aguiar, 470, 05403-000 São Paulo, SP, Brazil
[b] Instituto Adolfo Lutz, Av. Dr. Arnaldo, 355, 01246-000 São Paulo, SP, Brazil
[c] Biotechnology Center, Instituto de Pesquisas Energéticas e Nucleares, Av. Prof. Lineu Prestes, 2.242, 05508-000 São Paulo, SP, Brazil

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## ABSTRACT

Visceral leishmaniasis, caused by *Leishmania (Leishmania) chagasi*, is a chronic parasitic disease of humans and dogs. Confirmation of the protozoal agent in bone marrow, lymph node or spleen aspirate is diagnostic, while specific-IgG serology is used mainly for epidemiology despite the general presence of high levels of serum immunoglobulin. Anecdotal reports of false-negative serology in active disease cases are known and are ascribed to the formation of immune complexes. Because dissociation of immune complexes can be accomplished by acid treatment, we devised a simple, routine enzyme immunoassay (ELISA) for the dissociation of immune complexes in serum samples using acid treatment in wells adsorbed with *Leishmania* antigen (dELISA). Confirmatory acid dot-blot was also developed for antigen detection by anti-*Leishmania* rabbit antiserum. In experimental *L. chagasi* hamster models, immune complexes interfered with ELISA mostly in the 30 and 60 days postinfection, according to both dELISA and antigen dot-blot results. In larger samples from endemic areas, dELISA was positive in 10% of seronegative dog samples (7/70) and 3.5% in negative human samples (3/88), showing that dELISA could be used in the serodiagnosis of visceral leishmaniasis. Moreover, dELISA could be used as an alternative approach to screening asymptomatic visceral leishmaniasis patients, instead of invasive confirmatory testing.

## 1. Introduction

Visceral leishmaniasis (VL), caused in the Old World by *Leishmania (Leishmania) donovani* and *Leishmania (Leishmania) infantum* or caused in the New World by *Leishmania (Leishmania) chagasi* (or *L. (L.) infantum*), is endemic in South America, and has an estimated annual incidence of 500,000 new human cases worldwide, of which 90% occur in Brazil, Bangladesh, India, Nepal and the Sudan (WHO, 2012). Most cases occur in the poorest of urban communities and are related to both migration and urbanization (Alves and Bevilacqua, 2004). Symptomatic VL is associated with hepatosplenomegaly and chronic fever, depending on disease progression, but most infected individuals remain oligo- or asymptomatic (Oliveira et al., 2010). Dogs are considered the main reservoir of the parasite, especially in South America, where a high level of canine infection is associated with a high risk of human disease (Werneck et al., 2007). VL patients mount large humoral responses characterized by hypergammaglobulinemia, which appears to be inefficient against parasites; however, the high concentration of antibodies and circulating antigen can result in the formation of circulating immune complexes (CIC) (Lambert et al., 1981). CIC can mask the actual antibody concentration present in a sample when assayed using conventional methods (Gustaw et al., 2008).

The "gold standard" for the diagnosis of VL is based on the detection of parasites in organ aspirates from the spleen, liver, lymph nodes and bone marrow. The technique is specific but invasive, and accidents occasionally occur due to bleeding, as visceral leishmaniasis causes thrombocytopenia (Chappuis et al., 2007). Serological tests for specific IgG are widely used in the epidemiological surveillance of VL, including indirect immunofluorescence, direct agglutination and ELISA, but all of these methods cross-react with other *Leishmania* parasites that are frequently found in the same endemic areas of South America (Romero et al.,

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* Corresponding author at: Protozoology Laboratory IMTSP and Department of Pathology – FMUSP, University of São Paulo, Av. Dr. Eneas de Carvalho Aguiar, 470, 05403-000 São Paulo, SP, Brazil. Tel.: +55 11 30617010.
*E-mail address:* hfandrad@usp.br (H.F. de Andrade Jr.).

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2009). Additionally, serological tests also present difficulties with regard to sensitivity, specificity and efficiency due to the use of several antigen sources, such as promastigotes, amastigotes from culture or recombinant proteins (Porrozzi et al., 2007).

IgG ELISA is considered a valuable tool in the diagnosis of VL because of its high sensitivity and its automation as part of large laboratory routines that allow the testing of large numbers of samples. IgG ELISAs were adapted for use with different antigens, which gives them the advantage in terms of sensitivity and specificity (Singh, 2006), but which also leads to the problem of CIC interference.

Many infectious diseases result in the formation of antigen-IgG immune complexes that worsen the chronic disease state, mainly through renal glomerular disease. These diseases may be caused by bacteria, such as *Mycobacterium tuberculosis*, *Mycobacterium leprae*, *Legionella pneumophila*, *Salmonella sp.* and *Shigella sp.* (Halstead et al., 2010); viruses, such as dengue virus (Koraka et al., 2003) and HIV (Lewis et al., 1995); fungi (Durkin et al., 2009); or parasites, such as other *Leishmania* spp. or *Schistossoma* spp. (Carvalho et al., 1983). Longstanding VL in humans or experimental animals also presents with secondary amyloidosis and vascular disease, both of which can be attributed to CIC (de Vallière et al., 2009).

Recently, some studies have attempted to elucidate the role of immune complexes in the pathology of VL. However, most of these studies focus either on the importance of CIC as a marker of disease progression (Miles et al., 2005), or on the mechanisms of the disease itself, particularly addressing the role of cytokines and the humoral immune response (Elshafie et al., 2007). Quantification assays for CIC are few and remain rare without routine use because most require expensive sample preparation (Evans and Pearson, 1988). Thus, the role of CIC in serological testing used for the diagnosis and detection of IgG antibodies to *Leishmania* is not well established.

Here, we describe the development of an easy immunoassay designed to detect CIC using a modified ELISA and pH shock to promote the dissociation of immune complexes present in VL samples obtained by experimental and natural infection.

## 2. Materials and methods

### 2.1. Parasites and animals

*L. (L.) chagasi* (MHOM/BR/1972/LD) was maintained *in vivo* through the experimental infection of Syrian hamsters (*Mesocricetus auratus*) by i.p. injection of amastigotes obtained from the spleens of previously infected hamsters. All hamsters and New Zealand rabbits used in the experiments were supplied the animal breeding facility of Faculdade de Medicina, Universidade de São Paulo, were maintained at the animal facility of Laboratório de Protozoologia at Instituto de Medicina Tropical de São Paulo, Universidade de São Paulo. Following infection or inoculation, animals were maintained in individual cages, with adequate housing and feeding, according to the protocols outlined in the "Guide for the Care and Use of Laboratory Animals" (National Research Council, 1996). This study was approved by the Ethics Committee for Animal Experimentation of the Instituto de Medicina Tropical de São Paulo, Universidade de São Paulo (No. CE-IMT/002/29042008).

### 2.2. Infection of experimental animals

Weanling hamsters were inoculated with $5 \times 10^7$ amastigotes/animal and maintained for 15, 30, 45, 60 and 90 days postinfection. Hamsters were killed by $CO_2$ narcosis, and blood was obtained by cardiac puncture and allowed to clot with serum recovery. Serum was stored at −20 °C until use. Infection was monitored by weight and parasite burden in the spleen and liver, determined according to the proportion of amastigotes to mammalian cell nuclei in Giemsa-stained imprints (Ott et al., 1967). We successfully infected 91 experimental hamsters.

Rabbits were immunized with a 250-μg dose of a soluble extract of *L. (L.) chagasi* according to a standard subcutaneous immunization protocol, using complete Freund's adjuvant in the first dose and incomplete Freund's adjuvant in subsequent doses (minimum of five biweekly doses). Antibody production was measured by ELISA at each bleeding, and when specific anti-*Leishmania* IgG reached high titers (>10,000), rabbits were submitted to anesthesia with chlorpromazine 0.75 mg/kg, midazolam 0.2 mg/kg, atropine 0.044 mg/kg, ketamine hydrochloride 35 mg/kg and xilazine 5 mg/kg by intramuscular injection. After establishment of the anesthesia, animals were exsanguinated by cardiac puncture until death. Blood was allowed to clot and the removed sera stored at −20 °C until use.

### 2.3. Dog and human negative samples

We used 203 samples taken from dogs from VL-endemic areas in the state of São Paulo; samples were kindly supplied by the main public health laboratory, the Divisão de Parasitoses Sistemicas, Instituto Adolfo Lutz. Samples had been previously tested commercial ELISA (BioManguinhos, Rio de Janeiro), which assay classified the samples in negative (70), positive (101) or inconclusive (32), when the O.D. results are in a gray zone for this assay defined by the supplier, that were excluded as confusing factor. Only 171 clearly defined dog samples were re-tested using both conventional ELISA (cELISA) and dELISA. Small fractions was also tested by commercial IFA (64) but were not used in posterior analysis.

We tested 200 human samples obtained from VL-endemic areas in the city of Araguaína, in the state of Tocantins, Brazil. As with the dog samples, human samples were tested by IFA, and dELISA (100 negative and 100 positive samples).

### 2.4. Antigen production

*L. (L.) chagasi* promastigotes were isolated from the spleens of infected hamsters (60 days postinfection), and cultured in M199 medium with 10% bovine serum and 5% male human urine at 24 °C without agitation; promastigotes were subcultured every 4 days. Promastigotes were concentrated by centrifugation at 2500 RPM for 10 min, and drained pellets were immediately frozen at −20 °C until use. For antigen preparation, drained promastigotes were suspended in ten volumes of lysis buffer (0.05 M Tris–HCl, pH 6.0; 4 M urea; 5 mM EDTA; 0.5 mM PMSF; 0.1 M KCl; and 0.5% SDS), heated at 65 °C for 15 min and centrifuged at 10,000 RPM to recover the soluble extract. Protein content was determined, and aliquots were frozen at −80 °C.

### 2.5. Conventional and dissociation ELISA

Serum was subjected to conventional anti-*Leishmania* IgG ELISA using 96-microwell plates adsorbed overnight with promastigote antigens of *L. (L.) chagasi* suspended in carbonate sodium buffer 0.1 M at 10 μg/ml (100 μl/well), all samples were tested in duplicate. Wells were blocked with PBS/powdered skimmed milk 5% for 1 h at 37 °C, wells were incubated with 1/100 (hamsters) or 1/200 (dog and human) diluted serum samples at 37 °C for 1 h, then washed 5 times with PBS. Bound IgG was visualized following incubation with the appropriate dilutions of anti-IgG species-specific peroxidase conjugates (anti-hamster IgG, anti-dog IgG and anti-human IgG-γ chain specific) for 1 h and subsequently washed with PBS. Bound conjugate was visualized following incubation for 30 min with the peroxidase substrates o-phenylenediamine and $H_2O_2$. The reaction was stopped with 4 N HCl (50 μl/well), and the absorbance of each well at 492 nm was determined using a microplate reader.

The procedure for dELISA was the same, except that the serum dilution was prepared at one-half of the original volume (1/50 for hamster and 1/100 for dog and human) and 50 μl was applied to each well. Immune complex dissociation was performed by adding 25 μl of 0.1 M glycine, pH 2.5, and containing phenol red as an indicator. After incubation for 5 min with occasional shaking and low pH confirmed by uniform yellow color in the well, the pH of the well was changed to pH 7.8 with 25 μl of 0.25 M Tris–HCl, pH 8.8, which turned the wells to red in the same sera dilution of control cELISA well. All reactions and non-acid treated samples were assayed in the same plate. After 1 h incubation, subsequent steps of washings, conjugate incubation and color development were performed as described for cELISA. Results were expressed as 492 nm absorbance.

The cut-off values used for cELISA and dELISA were estimated using 20 samples of non infected hamster, 20 dogs from a non-endemic area and 20 human samples from young people out of endemic area. The 99% cut-off of each assay was estimated by adding 3 standard deviations to the mean value of the result of specie-specific negative samples.

### *2.6. Artificial immune complex containing samples*

For testing artificial CIC-containing samples, 1/100 dilutions of highly positive rabbit anti-*Leishmania* antisera were incubated for 2 h with increasing amounts of whole *Leishmania* soluble antigen. These CIC samples were tested both by dELISA and by polyethylene glycol (PEG) immune complex precipitation. For PEG precipitation, 100 μl samples were added to 100 μl of 7.5% PEG in PBS and incubated overnight at 4 °C. Then, the suspension was centrifuged at 10,000 RPM for 10 min at 4 °C. The pellet was suspended in an equal volume of 3.75% PEG/PBS and centrifuged at 10,000 RPM for 10 min. The supernatant was discarded, and the pellet suspended in the initial dilution volume of PBS. PEG precipitated immune complexes restored to original volumes were subjected to cELISA and dELISA. Proportion of immune complexes was estimated by the formula (dELISA-cELISA)/dELISA in the same dilution.

### *2.7. Dot-blot for acid dissociated Leishmania antigen in serum*

*Leishmania* antigen dot-blot was performed using the same acid treatment for the dissociation of immune complexes as described previously. Samples of PEG-precipitated CIC or sample serum (5 μl) were diluted with 95 μl of 0.1 M glycine–HCl, pH 2.5, vortexed and immediately applied to an activated Immobilon-P membrane (100 μl/dot) in a 96-dot vacuum system (Bio-Dot, BioRad, Hercules, CA). After acid binding, the membrane was removed, and the free binding sites were blocked by incubating the membrane in PBS/powdered skimmed milk 5% for 1 h at 4 °C, using a rocker shaker. The membrane was washed 5 times with PBS, immersed for 1 h in a 1/200 dilution of rabbit anti-*Leishmania* antibody and then washed again 5 times with PBS. The binding of specific sera was visualized by incubating the membrane for 1 h in a 1/2000 dilution of anti-rabbit IgG peroxidase conjugate (Sigma, St. Louis). The membrane was then washed 5 times with PBS, and bound conjugate was visualized using DAB as the peroxidase substrate; visual controls of positive and negative reactions were included. The processed membrane was photographed using a digital camera. Positive and negative results by acid dot-blot were determined by visual analysis of color, where dots with the greatest color intensity were considered positive.

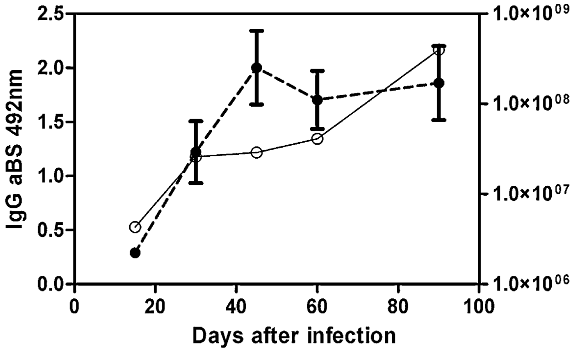

**Fig. 1.** Progression of parasite burden in hamsters experimentally infected with $5 \times 10^7$ amastigotes of *L. (L.) chagasi*, shown in association with average values of specific IgG. The open dots with continuous line show the progression of the parasite burden in spleen, while closed dots with dashed lines show the progression of anti *Leishmania* IgG levels. Error bars = SEM.

### *2.8. Statistical analysis*

Correlations between the quantitative results of dELISA and cELISA were determined using Pearson's correlation; results were expressed as $r^2$ after determination of significance. All statistical analysis was performed using the GraphPad Prisma 5.0 software (GraphPad Software, San Diego, CA, USA).

## 3. Results

### *3.1. Experimental infection*

Overall, 91 hamsters were successfully infected with *L. (L.) chagasi* amastigotes, presenting with a typical progressive infection and visceral disease as determined by the parasite load in the spleen; 17 non-infected controls were also used, resulting in 108 hamster samples. Disease progression was determined by sacrificing the hamsters at different stages of infection. Spleen parasite burden was determined as described in the methods, and blood was collected for ELISA. The parasitological and serological progression of the infection is shown in Fig. 1. Anti-*Leishmania* IgG results from cELISA are also shown, demonstrating good correlation between spleen parasite burden and serology. Serum samples were used in subsequent immune complex determinations. We used anti-*Leishmania* antisera from four rabbits; titers for specific IgG were greater than 100,000, as determined by cELISA.

### *3.2. Standardization of optimum pH shock for dELISA*

The interference of pH in IgG anti-*Leishmania* ELISA was tested with samples (negative, inconclusive and positive) from experimentally infected hamsters and is shown in Fig. 2. The interference occurred in a pH range of 2.0–3.5. The range of pH 2.5–3.5 showed excellent correlation with the dilution of samples without pH stress (pH 7.5), where adequate results was achieved without the high background resulting from the non-specific binding of acid denatured IgG, which clearly occurs at more acidic pH values (pH 2.0). Despite the absence of statistically significant differences between pHs over 2.5, we opted for the lowest pH (2.5) with no interference in ELISA for maximum immune complex dissociation.

### *3.3. Quality control of dELISA using in vitro artificial immune complex production*

For quality control, we created an artificial sample with high immune-complex content for dELISA assay. We incubated a fixed dilution (1/50) of rabbit anti-*Leishmania* antiserum with increasing

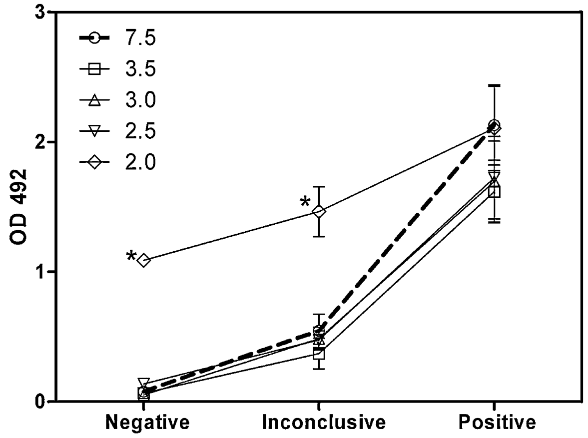

**Fig. 2.** Interference of pH stress in ELISA from anti-*Leishmania* IgG negative, inconclusive and positive samples from experimentally infected hamsters. Values of pH stress are shown in legend. Error bar = SEM. Asterisks represent *t* test significant difference from neutral pH samples.

amounts (1, 2, 4, 8, 16 and 32 μg) of soluble *L. (L.) chagasi* promastigote antigen. The mixture was incubated for 1 h and subsequently subjected to dELISA and immune-complex PEG precipitation. As shown in Fig. 3, both assays resulted in similar measurements of immune complex content, as the proportion of association detected by cELISA and the proportion of dissociation detected by dELISA were similar, especially with higher concentrations of antigen (16 and 32 μg/ml). This assay confirms the quantitative aspect of dELISA, showing the proportion of immune complexes formed and the recovery percentage for IgG after dissociation.

### 3.4. Detection of immune complexes in experimental samples in dELISA

#### 3.4.1. dELISA in experimental infection in hamsters

All experimental samples from infected hamsters were tested using cELISA and dELISA. The correlation between the quantitative data obtained from the two assays was significant ($r^2 = 0.69$, $p < 0.0001$).

A substantial increase in IgG detection following pH shock in dELISA was observed for some hamster samples. Typically, the detection of anti-*Leishmania* IgG was higher in dELISA at 30, 60 and 90 days postinfection, as compared to cELISA. Highest dELISA values for anti-*Leishmania* IgG detection were observed at 30 and 60 days postinfection, suggesting the interference of immune complexes in these periods. Immune complex proportions mean at different times were extrapolated from cELISA and dELISA ODs and shown in Fig. 4. No sample was considered positive if dELISA value was lower than mean plus 3 standard deviations of control non-infected samples. The presence of immune complex interference was observed in individual animals in all postinfection periods, which leads to individual sample analysis.

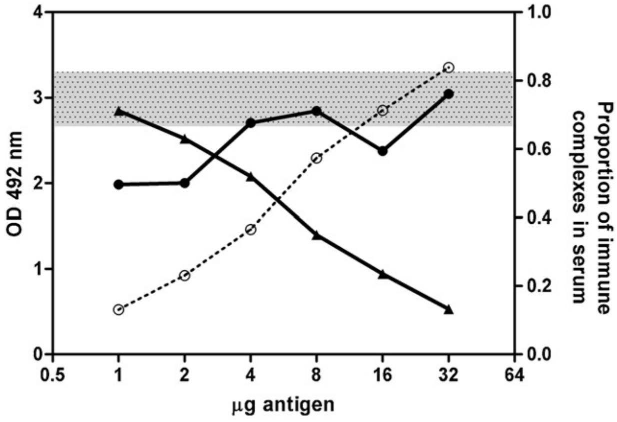

**Fig. 3.** cELISA and dELISA in artificially created *Leishmania* antigen IgG immune complexes. cELISA = solid black line with triangles; dELISA = solid black line with closed dots. The proportion immune complexes was estimated as described in Section 2 and are shown by dashed line with open dots. Dashed area = mean ± 3 SD of 10 dELISA assays of standard rabbit sera.

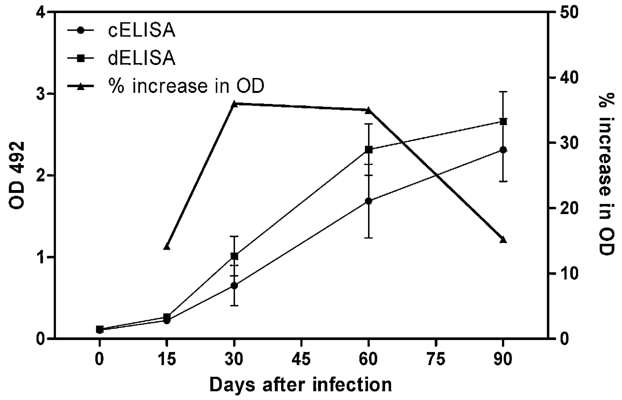

**Fig. 4.** Longitudinal analysis of anti-*Leishmania* IgG ELISA in hamsters experimentally infected with *L. (L.) chagasi*. Dots represent cELISA, squares represent dELISA and triangles represent percent increase in dELISA. Error bars = SEM.

#### 3.4.2. Individual analysis of samples with an increased IgG in dELISA

Each individual sample assay was considered with immune complex interference when negative cELISA becomes dELISA positive. The cutoff values used for cELISA and dELISA were calculated assaying 17 samples from non-infected hamsters, adding 3 standard deviations to the mean value of each assays. The frequencies and proportions for qualitative results for cELISA and dELISA are shown in Table 1 and indicate higher positivity in the detection of anti-*Leishmania* IgG by dELISA. Usually, dELISA reactivity was also higher than cELISA in most positive animals, but we opt for analyze only more definitive comparisons.

A significant fraction of cELISA negative samples (6/16, or 37.5%) were dELISA positive samples. We also detected a large increase in dELISA reactions in some samples compared to initial cELISA reactions, and Fig. 5A shows the individual increases in dELISA reactivity for these samples. The typical increase from negative to positive in dELISA can be seen in most of the samples, but the higher increases were only seen in a few samples belonging to hamsters 30 and 60 days postinfection. This distribution of reactive samples was related to the course of infection, increasing as the infection worsened, though not in all infected animals. During later stages of infection, at the 90th day postinfection, cELISA was positive for all animals, but the large increases in dELISA were observed in only two animals, suggesting that at later stages when the humoral anti-*Leishmania* response is strong, immune complex interference is not powerful enough to abolish positivity reactivity.

#### 3.4.3. dELISA in samples from dogs with suspected VL

Samples from dogs with suspected VL from an epidemic area of São Paulo were previously tested by commercial ELISA as described in methods and were classified as positive (101) or negative (70). Samples classified as inconclusive according the supplier (32) were removed from the analysis. Samples were re tested by cELISA and dELISA. The correlation between the quantitative results for cELISA and dELISA was significant ($r^2 = 0.96$, $p < 0.0001$). The qualitative results for sample reactivity by cELISA and dELISA are shown in

**Table 1**
Qualitative analysis of anti- *L. (L.) chagasi* IgG using both conventional and dissociation ELISA on experimental samples from infected hamsters.

| Conventional ELISA ($n = 91$) | | Dissociation ELISA ($n = 91$) | Agreement | Negative cELISA and positive dELISA | |
|---|---|---|---|---|---|
| Positive | 75 (82.5%) | Positive | 75 | 100% | 0 |
| | | Negative | 0 | | |
| Negative | 16 (17.5%) | Positive | 6 | 10/16 (62.5%) | **6/16 (37.5%)** |
| | | Negative | 10 | | |

**Table 2**
Qualitative results for both conventional and dissociation anti-*Leishmania* IgG ELISA in suspected dog samples from epidemic areas.

| Conventional ELISA ($n = 171$) | | Dissociation ELISA ($n = 171$) | Agreement | Negative cELISA and positive dELISA | |
|---|---|---|---|---|---|
| Positive | 101 (59%) | Positive | 101 | 100% | 0 |
| | | Negative | 0 | | |
| Negative | 70 (41%) | Positive | 7 | 63/70 | **7/70** |
| | | Negative | 63 | 90% | **10%** |

Table 2. To 10% (7/70) fraction of positive samples not detected by cELISA was detected by dELISA, suggesting that the dissociation of immune complexes allows for the detection of IgG anti-*Leishmania* complexed to antigen, undetectable in cELISA.

Among samples that appeared negative by cELISA, a small portion (10%) was reactive in dELISA, and some samples showed a significant increase in IgG. The data for individual samples are presented in Fig. 5B.

### *3.4.4. dELISA in human samples from an endemic area*

Human samples (200) were obtained from an area endemic for human VL and human cutaneous leishmaniasis (Araguaína, Tocantins, Brazil). According to previous IFA testing, 100 samples were positive and 100 were negative. Samples were re-tested using cELISA and dELISA. The correlation between results for human samples using cELISA and dELISA, independent of groups, was high ($r^2 = 0.66$). The qualitative analysis of IFA-negative samples that were re-tested by cELISA and dELISA are shown in Table 3; the data indicate that 3.5% (3/88) of the dELISA positive samples were negative according to both IFA and cELISA. There are 3.5% (3/88) human samples cELISA negative dELISA positive, suggestive of interference of immune complex in *Leishmania* serology.

The analysis of individual samples with increase IgG in dELISA is shown in Fig. 5C. The samples presented clear increase of specific IgG detection by dELISA after acid dissociation, suggesting immune complex interference.

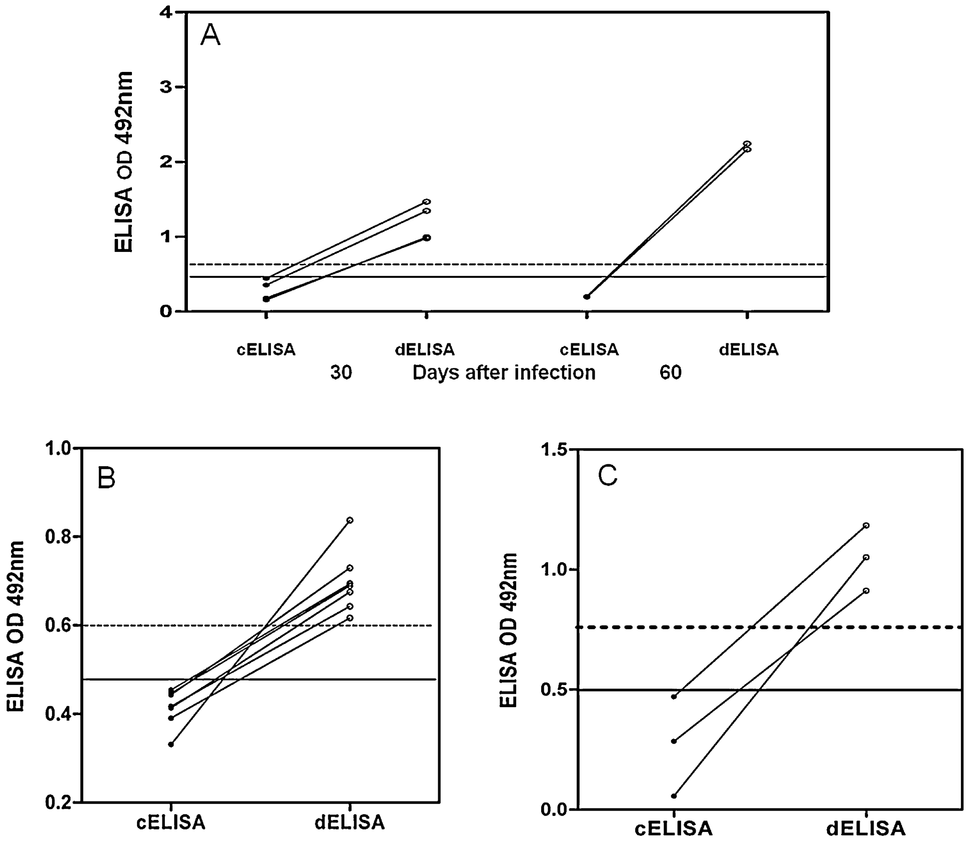

**Fig. 5.** (A) cELISA and dELISA in individual samples from infected hamster samples at 30 and 60 days postinfection. (B) cELISA and dELISA in dogs samples. (C) cELISA and dELISA in human samples. Horizontal lines show cut-offs values, cELISA (solid line) and dELISA (dashed line). Individual reactions are linked by solid lines.

**Table 3**
Qualitative distribution of conventional and dissociation ELISA for anti-*Leishmania* IgG in IFA tested human samples from endemic areas.

| IFA ($n = 200$) | Conventional ELISA ($n = 200$) | | Dissociation ELISA ($n = 200$) | | Agreement | Negative cELISA and positive dELISA |
|---|---|---|---|---|---|---|
| Positive ($n = 100$) | Positive | 100 (100%) | Positive | 100 | 100% | 0 |
| | Negative | 0 | Negative | 0 | 100% | 0 |
| Negative ($n = 100$) | Positive | 12 (12%) | Positive | 12 | 100% | 0 |
| | | | Negative | 0 | | |
| | Negative | 88 (88%) | Positive | 3 | 85/88 | **3/88** |
| | | | Negative | 85 | 96.5% | **3.5%** |

### 3.5. *Confirmation of circulating antigen by dot-blot in samples with increased dELISA activity*

We devised an acid dot-blot to confirm the presence of antigen in samples using an acid buffer for binding serum proteins to a membrane. All of the samples that were negative in cELISA but positive in dELISA were tested using dot-blot. Positive reactions were visually defined as darker impressions on the membranes compared to the negative results obtained with the serum from uninfected animals. No attempt was made to quantify this reaction. All samples were defined qualitatively as positive (antigen-containing serum) or negative (antigen-free serum). The qualitative results for this test are shown in Table 4. Most of the samples (93.7%) that were negative by cELISA and positive by dELISA were positive in the acid dot-blot. The only negative result for acid dot-blot from a dELISA positive sample demonstrated low reactivity near the cutoff level for the reaction, representing a low concentration of antigen that was undetectable in our system.

## 4. Discussion

In this study, we devised and standardized an ELISA method using a dissociation step for immune complexes that allowed the detection of anti-*Leishmania* IgG antibody-antigen complexes that were otherwise non-detectable in cELISA. Our procedure involves the acid dissociation of IgG–antigen complexes followed by the formation of new associations with excess antigen in solid phase, thus minimizing soluble antigen interference in a simple, quick ELISA. We used acid as a chaotropic reagent for the dissociation of immune complexes. The preparation of serum samples to allow dissociation of immune complexes has been established previously by other authors in studies on tuberculosis (Imaz et al., 2008), Alzheimer's disease (Gustaw et al., 2008), histoplasmosis (Swartzentruber et al., 2009), schistosomiasis (Galvão-Castro et al., 1981), allergic diseases (Paganelli et al., 1981), amoebiasis (Vinayak et al., 1986) and pneumococcal pneumonia (Holloway et al., 1993), but all require a two-step, time consuming procedure. Our method uses the single-reaction sample dissociation treatment included in cELISA to create a one-step ELISA reaction. Samples were subjected to acid dissociation at several pH values to check stability relative to the conventional technique, allowing us to determine an optimum pH for dissociation. We also confirmed that extreme pH conditions induced IgG denaturation and the false detection of IgG, thus leading to a false positive interpretation. This phenomenon was also observed in a study on Alzheimer's disease, where acid dissociation under extreme conditions triggered the false detection of IgG in ELISA (Li et al., 2007). The dissociation and re-association agents used in our study were the same as those reported in the detection of dissociated immune complexes involving p24 HIV antigen (Lewis et al., 1995). Our tests showed high sensitivity, but low pH could induce a drop in the specificity of the test and result in the detection of false positives. This finding may be related to the duration of exposure to the dissociation agent, as it has been reported elsewhere that stronger or longer exposures could induce artifacts of IgG in the ELISA (Li et al., 2007).

**Table 4**
Qualitative acid DOT-ELISA results in hamster, dog and human samples. Only cELISA-negative, dELISA-positive samples were tested.

| Samples | Negative cELISA and Positive dELISA | Positive DOT-ELISA |
|---|---|---|
| Hamster | 6 | 6/6 (100%) |
| Dog | 7 | 6/7 (85.7%) |
| Human | 3 | 3/3 (100%) |
| Total | 16 | 15/16 (93.7%) |

We successfully created an experimental, soluble antigen–IgG complex which was negative in cELISA but was perfectly identifiable in dELISA, thus confirming the ability of our assay to detect complexes of antigen–IgG in leishmaniasis. This reagent demonstrated the feasibility of dELISA using the production *in vitro* immune complexes. The reagent was also subjected to PEG precipitation, an alternative and established technique for IgG immune complex determination; our reagent demonstrated the same dose–response reaction to different antigen concentrations reported in a similar study (Paganelli et al., 1981), showing interference in serology by progressive antigen concentrations. The precipitation of immune complexes produced *in vivo* and *in vitro* in visceral leishmaniasis by PEG has been previously described (Casali and Lambert, 1979), confirming the presence of antigen–IgG complexes by the detection of complement and anti-*Leishmania* IgG by electrophoresis and radial immunodiffusion. These recent data were similar to our data in the concentrations of *in vivo* and *in vitro* produced immune complexes.

We also performed confirmatory assays using acid dissociation and adsorption of whole serum samples to high-capacity solid substrates. Sample dissociation at acidic pH allows for the binding of the free soluble antigen present in the sample and subsequent reaction for its immune detection. A similar dissociation step, one that used a longer incubation period, was used in a study on the acid dissociation of p24 immune complexes in HIV infection (Miles et al., 1993). This acid dot-blot was standardized to a dose–response curve that allowed detection in lower ranges (<20 ng/ml antigen in sera). The use of dot-blot for the detection of antigen in leishmaniasis has been described with consistent results between antigen dot-blot and ELISA using specific monoclonal antibodies (Senaldi et al., 1996). Commercial assays for the rk39 antigen were also extensively tested, but they demonstrated a sensitivity of only 92% (Maia et al., 2012). Antigen detection by acid dissociation in dot-blot has only been described for the detection of dengue virus antigen–IgG complexes; results were similar to ours, except that the dengue study used more concentrated samples without dissociation ELISA (Koraka et al., 2003). Such high correlation with dELISA results allows the use of dELISA data to confirm suspicions of immune complex interference in serology assays. Despite the possibility of immune complex interference in serological testing used for the diagnosis and detection of IgG anti-*Leishmania*, however, no attempts were found to avoid this problem in routine laboratory screening, most likely as a result of expensive sample preparation (Evans and Pearson, 1988; Soares et al., 2001).

In experimentally infected hamsters, cELISA assays showed progressively higher specific-IgG titers in response to parasite burden, which is consistent with results described by other authors (Afrin and Ali, 1997). As expected during the course of disease, specific antibodies were produced and detected correlated with the progress of experimental infection, providing an ideal model for reproducing natural infection (Nieto et al., 2011). The results for dELISA showed immune complex interference in the serology of the samples 30 days postinfection, encompassing the acute phase of the illness. This result was similar to the report of a higher proportion of immune complexes found in a study evaluating renal function associated with the presence of immune complexes (ElShafie et al., 2006). Using PEG precipitation, the detection of immune complexes in experimentally infected mice was reported to be a promising method for identifying active disease and monitoring treatment by ELISA (Azazy et al., 1997). Whereas previous studies used the cumbersome technique of direct antigen detection ELISA, our study uses a simple, indirect technique without sample pretreatment and with the same efficiency, as indicated by the presence of circulating immune complexes in experimental infection.

Analyzed as a whole, most of the results for the hamster samples were consistent between cELISA and dELISA. Those samples in agreement presented no diagnostic or interpretive challenge, but cELISA-negative, dELISA-positive samples showed significant interference of immune complexes in serological results. We confirmed antigen presence by acid dot-blot in most discrepant samples, but one sample was negative for antigen. This one sample could represent a false negative acid dot-blot, due to the range of possible antigen detection, which was similar to ranges previously described (Azazy, 2004), or a false positive dELISA, attributed to the specific denaturation of IgG in individual samples.

The assessment of cELISA and dELISA was performed similarly in dog samples, using as the main sample the discordant cELISA negative, dELISA positive samples. Canine samples showed a high frequency of concordant cELISA/dELISA samples, with a large discordant fraction occurring only in the cELISA negative pool, a 10% fraction, which was also positive in acid dot-blot. The results of dELISA were compared and with adequate agreement with to previous results obtained from commercial or in house ELISA performed by our reference public health laboratory at Instituto Adolfo Lutz, São Paulo, SP. In a previous study, the detection of immune complexes in dogs with natural infection using sandwich ELISA for the detection of IgM and IgA immune complexes was related to glomerular deposition and consequent renal injury (Margarito et al., 1998). Dogs, as hosts, are extremely susceptible to VL, showing extensive and widespread disease (Solano-Gallego et al., 2009). The presence of a significant fraction of dog samples with immune complexes is therefore not surprising.

The same approach was used for human samples collected from endemic areas, and dELISA was analyzed for human samples by comparing the results with previously screened IFA and cELISA. Most IFA samples agreed with ELISA assays but with discordant samples, but all cELISA positive samples were dELISA positive. The discrepancy between IFA and ELISA has been observed in a comparative study of diagnostic methods for VL applied to human sera (Iqbal et al., 2002; Pedras et al., 2008), where the occurrence of IFA false negative results, in agreement with our assays, indicates lower sensitivity of the IFA in detecting anti-*Leishmania* IgG antibodies when compared to ELISA. The optimization of the IFA antigen in detecting anti-*Leishmania* IgG could improve this test, as the cross-reacting *L. (L.) major* IFA antigen currently used could be substituted for by a specific *L. (L.) chagasi* antigen as reported elsewhere (Mancianti et al., 1995). We found a proportion of cELISA-negative, dELISA-positive samples, suggesting that some samples contained circulating immune complexes that interfered in serological analysis. This fact is not surprising due to the low susceptibility to VL after infection of the human host (Stanley and Engwerda, 2007), which results in controlled infection in most infected persons. We did not conduct follow-up studies on these samples to confirm human disease. Techniques for detecting circulating antigen can be used to inform treatment plans for patients because the presence of circulating antigen in samples after dissociation may correspond to active disease (Evans and Pearson, 1988) or to the risk of recurrence due to factors relating to immunosuppression, such as in HIV-positive patients (Cota et al., 2011).

The diagnosis of VL is complex due to overlapping clinical syndromes and the frequent flare-ups and remissions of human disease (Srivastava et al., 2011). Once infection is suspected, parasitological evaluation is the preferred approach to diagnosis, using microscopic observation and the culturing of amastigotes from aspirates of spleen, liver, lymph node and bone marrow to detect parasites (Singh et al., 2006). Needle aspiration of the spleen or liver is most frequently used but is associated with hemorrhagic complications, thus requiring trained professionals and the infrastructure necessary for its execution (da Silva et al., 2005; Kar, 1995). Alternatively, serological testing is increasingly being used for the diagnosis of VL. ELISA can be used to detect anti-*Leishmania* antibodies with high sensitivity and specificity; it can also be used in the detection of active disease by detecting high levels of antibodies, and in the monitoring of treatment (Kumar et al., 2001). Despite the feasibility of such tests, however, the high sensitivity and specificity of the detection of *Leishmania* antibodies is mainly restricted to epidemiological studies (Palatnik-de-Sousa and Day, 2011). Infection with *L. (L.) chagasi* is characterized by the production of high levels of antibodies that do not contribute to the immune defense of the individual. Instead, when high parasitemia is established, it triggers the production of IgG antibodies that act on the amastigote surface coating, resulting in the formation of immune complexes (Halstead et al., 2010). However, we could not find any studies examining these immune complexes. Our data confirms the interference of immune complexes in conventional serology, and although interference is small, it changes the performance criteria for serology testing. The presence of immune complexes could indicate recent infection, as demonstrated in our experimental models that may correspond to oligosymptomatic patients, but follow-up studies were not performed for our human samples so the use of immune complexes as an indicator of recent infection remains speculative. The use of dELISA in asymptomatic patients may be useful in the screening of patients for subsequent invasive parasitological testing.

Our data show that soluble immune complexes can form during VL and interfere with serological assays; however, a simple dissociative assay could help reduce false negative samples in suspected leishmaniasis cases in humans or animals. Despite these promising results, further testing is needed to improve our assay prior to clinical use.

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