Parasitology

Effect of the preservative and temperature conditions on the stability of *Leishmania infantum* promastigotes antigens applied in a flow cytometry diagnostic method for canine visceral leishmaniasis

Henrique Gama Ker [a,b], Rodrigo Dian de Oliveira Aguiar-Soares [a,b], Bruno Mendes Roatt [a,b], Nádia das Dores Moreira [a,b], Wendel Coura-Vital [a,b], Cláudia Martins Carneiro [a,b], Andréa Teixeira-Carvalho [c], Olindo Assis Martins-Filho [c], Rodolfo Cordeiro Giunchetti [d], Denise da Silveira-Lemos [e], Alexandre Barbosa Reis [a,b,*]

[a] *Laboratório de Pesquisas Clínicas, Departamento de Análises Clínicas, Escola de Farmácia, Universidade Federal de Ouro Preto, Ouro Preto, MG CEP 35400-000, Brazil*
[b] *Laboratório de Imunopatologia, Núcleo de Pesquisas em Ciências Biológicas, Universidade Federal de Ouro Preto, Ouro Preto, MG CEP 35400-000, Brazil*
[c] *Laboratório de Biomarcadores de Diagnóstico e Monitoração, Centro de Pesquisas Renè Rachou - FIOCRUZ, Belo Horizonte, MG CEP 30190-002, Brazil*
[d] *Laboratório de Biologia das Interações Celulares, Instituto de Ciências Biológicas Universidade Federal de Minas Gerais, Belo Horizonte, MG CEP 31270-901, Brazil*
[e] *Laboratório de Imunologia e Genômica de Parasitos, Departamento de Parasitologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, MG CEP 31270-901, Brazil*

ARTICLE INFO

ABSTRACT

The control of canine visceral leishmaniasis (CVL) is imperative, but euthanasia of seropositive dogs has been highly criticized. Commonly used, immunodiagnostic tests, including Dual-Path Platform®, enzyme-linked immunosorbent assay, and immunofluorescent antibody test, have failed at detecting asymptomatic dogs in endemic areas. In this context, new serological methods are needed. Flow cytometry serology has demonstrated potential as a test with excellent performance for CVL. In this study, we proposed to establish the best conditions for preserving *Leishmania infantum* promastigote antigens employed in this serology test. During 12 months of follow-up, promastigotes were maintained in different preservatives (phosphate-buffered saline with 3% fetal bovine serum, phenol 0.35%, thimerosal 0.01%, and formaldehyde 0.5%) and stored at 3 distinct temperatures (25 °C, 4 °C, and −20 °C). During the study period, the morphological characteristics of the promastigotes were assessed by flow cytometry according to the forward and side scatter parameters and also under optical microscopic analysis. Reactivity performance was evaluated as the percentage of positive fluorescent parasites in the sera of naturally infected and noninfected dogs. Microbiological analysis was performed at 2 time points, the first and sixth months, to rule out contamination of stored promastigotes. Taken together, our results indicated that the best conditions to preserve fixed *L. infantum* antigens were storage in formaldehyde at 4 °C. Promastigotes presented the best morphological profile, with appropriate antigenic stability even at 4 °C, in an inexpensive preservative for a long period of conservation.

© 2013 Elsevier Inc. All rights reserved.

1. Introduction

Domestic dogs are the main reservoir hosts of *L. infantum* in endemic areas, and it is observed that the major focal areas of human visceral leishmaniasis (VL) are strongly associated with locations that present a high prevalence of seropositive dogs (Nunes et al., 2010). Canine VL (CVL) has recently emerged in various urban and periurban cities in Brazil (Coura-Vital et al., 2011a, 2011b; Drumond and Costa, 2011) and other countries such as the United States (Rosypal et al., 2003), Argentina (Salomon et al., 2009), Italy (Tarallo et al., 2010), and France (Chamaille et al., 2010). Since the early 1980s, VL has spread to the urban centers of northern Brazil and more recently to southern and western regions (Harhay et al., 2011).

For a long time in Brazil, the immunofluorescent antibody test (IFAT) was used to confirm positive cases detected by enzyme-linked immunosorbent assay (ELISA). Recently, the Brazilian Ministry of Health began using an immunochromatographic test (Dual-Path Platform® [DPP]; Biomanguinhos, Rio de Janeiro, RJ, Brazil) for initial screenings and ELISA to confirm CVL diagnoses (Ministério da Saúde, 2011). However, some authors have documented that DPP shows lower sensitivity in endemic area (Grimaldi et al., 2012). In Brazil, the results of these tests are used as criteria for decisions on the culling of seroreactive dogs in VL surveillance and control programs (Ministério da Saúde, 2011). Asymptomatic dogs play a role in the transmission of *Leishmania* parasites, but most of them cannot be detected by

conventional serological tests, such as the DPP® and ELISA (Coura-Vital et al., 2011a, 2011b; Grimaldi et al., 2012; Reis et al., 2006).

Due to the limitations of these methods, several efforts have been made aiming to create a more reliable serological test for CVL diagnosis. Flow cytometry is a well-established methodology in clinical laboratories with a large number of applications (Jaroszeski and Radcliff, 1999). The use of flow cytometry for leishmaniasis serodiagnosis was initiated by Rocha et al. (2002) who described a method for evaluation of antibodies against *Leishmania braziliensis* promastigotes. Subsequently, flow cytometry serology was adapted for determining anti–*L. infantum* antibody levels in serum samples from dogs with CVL (Carvalho Neta et al., 2006). Recently, the applicability of flow cytometry serology as a novel assay for diagnosis of CVL was reported, and this method showed a great sensitivity (95%) and specificity (100%) for CVL diagnosis (Andrade et al., 2009). However, studies are needed to build a diagnostic kit employing the flow cytometry serology as immunodiagnosis for CVL.

The main goal of this study was evaluate for the first time the morphological and microbiological characteristics of *L. infantum* promastigotes as well as the serological reactivity performance of their antigens following storage in different preservatives at varied temperatures for 12 months of follow-up for use in flow cytometry serology for CVL diagnosis.

2. Material and methods

2.1. Samples

Serum samples of 5 *L. infantum* naturally infected dogs (INF) of either genders from the endemic area of Belo Horizonte, Minas Gerais, Brazil, were selected on the basis of positivity results for serological tests (ELISA and IFAT, Biomanguinhos/Fiocruz) and also by the Polymerase Chain Reaction - Restriction Fragment Length Polymorphism method from buffy coat. A total of 5 samples from noninfected dogs (NI) from the sera-bank of the Clinical Research Laboratory, of the Pharmacy School in the Federal University of Ouro Preto, were selected. The study was approved by the ethical committees for the use of experimental animals of the Federal University of Ouro Preto (CETEA/UFOP 032/2007).

2.2. Parasite preparation

L. infantum promastigotes (MHOM/BR/74/PP75) were cultivated at 23 °C in liver infusion tryptose medium supplemented with 10% fetal bovine serum (FBS). After 9 passages *in vitro*, the parasites were harvested at stationary growth phase and centrifuged at low speed ($100 \times g$, 10 min, room temperature) to remove cell debris. The supernatant containing most of the parasites was centrifuged at $1000 \times g$ for 10 min at 4 °C. For the flow cytometry anti-fixed Leishmania infantum promastigotes IgG assay, the promastigotes pellet was washed twice in phophate buffered saline (PBS) supplemented with 3% FBS. The parasites were immediately resuspended in different preservative solutions (Phenol 0.35%, Formaldehyde 0.5%, Thimerosal 0.01%, and PBS-3% FBS) at the concentration of 5×10^7 parasites per milliliter and stored at different temperatures (25 °C, 4° C, −20°C) until use.

2.3. Morphological analysis

To identify the best preservation conditions, the morphological features of the promastigotes were evaluated through experiments using optical microscopic analysis and flow cytometry assessment. Initially, a sample of promastigotes kept in each preservative was stained with Giemsa to evaluate morphological characteristics by optical microscopy. The images were viewed with a 100× objective under oil immersion and digitized through a microscope (Leica DM5000B) coupled with a camera. In addition, a flow cytometric evaluation included an assessment of the forward (FSC) and side (SSC) scatter parameters, and FSC × SSC pseudocolor graphs were used to verify the morphological profile of the promastigotes. These analyses were performed monthly during 1 year of monitoring.

2.4. Microbiological assays

To evaluate bacterial growth in the different preservative solutions, agar plates with 5% sheep blood (MBiolog®, Contagem, MG, Brazil) were used. The plates were incubated at a temperature of 35 °C ± 1 °C and examined after 48 hours by optical microscopy. Additionally, to assess the presence or absence of fungi, culture tubes containing Sabouraud agar medium with chloramphenicol (MBiolog®) were used. The tubes were incubated for 40 days stored at 24 ± 1 °C and macroscopically examined every day. These analyses were conducted in the first and sixth month of promastigote preservation.

2.5. Detection of anti–*L. infantum* promastigotes IgG by flow cytometry serology

The anti-fixed *L. infantum* promastigotes procedure was previously described by Andrade et al. (2007) and Andrade et al. (2009). Briefly, parasite preparations (5.0×10^5 parasites/well) kept in different preservative solutions and stored at different temperatures (25 °C, 4 °C, and −20 °C) were incubated at 37 °C for 30 min in the presence of 50 μL of diluted serum samples (ranging from 1:256 to 1: 262 144) using a 96-well U-bottom plate (BD Falcon™, San Jose, CA, USA). Following incubation, the parasite suspension was washed twice with 150 μL of PBS-3% FBS ($1000 \times g$, 10 min, 4 °C) and re-incubated in the dark for 30 min at 37 °C in the presence of 50 μL of previously diluted anti-canine IgG fluorescein isothiocyanate (FITC)–labeled polyclonal antibodies (1:500; 1:1000; 1:2000 in PBS-3% FBS) purchased from Bethyl Laboratories Inc. (cat #A40-105F; Montgomery, TX, USA). After incubation (37 °C, 30 min) and 2 washing procedures with 150 μL of PBS-3% FBS ($1000 \times g$, 10 min, 18 °C), the stained parasites were fixed with FACS fix solution (10 g/L of paraformaldehyde, 10.2 g/L of sodium cacodylate, and 6.65 g/L of sodium chloride, pH 7.2; Sigma Chemical Corp., St. Louis, MO, USA) for 30 min at 4 °C and maintained for at least 30 min at 4 °C in the dark prior to flow cytometric data acquisition.

Parasites were incubated in the absence of dog serum but in the presence of the FITC-labeled secondary reagents as an internal control in all sets of experiments to monitor nonspecific binding. Flow cytometric measurements were performed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA), interfaced with an FACStation and the Cell-Quest™ software package used for data acquisition and storage. The analysis was performed in FlowJo® software (FlowJo, Ashland, OR, USA). A total of 20 000 events were acquired for each serum sample dilution tested. IgG reactivity was expressed as the percentage of positive fluorescent parasites (PPFP) (Fig. 1). These experiments were conducted monthly during 1 year of follow-up.

2.6. Analysis

We performed an exploratory analysis of data through graphical and summary measures. The results are presented descriptively. The receiver operating characteristic (ROC) curve was used to select the cut-off value to discriminate negative from positive PPFP results. The ROC curve analysis was performed using STATA version 11.0 software (Stata Corp., College Station, TX, USA).

3. Results

3.1. Morphological characteristics and microbiological assays of the L. infantum promastigotes antigens

Morphological analysis was performed on the parasites stored in different preservatives at varied temperatures. PBS served as the preservative control, and these promastigotes had poor-quality
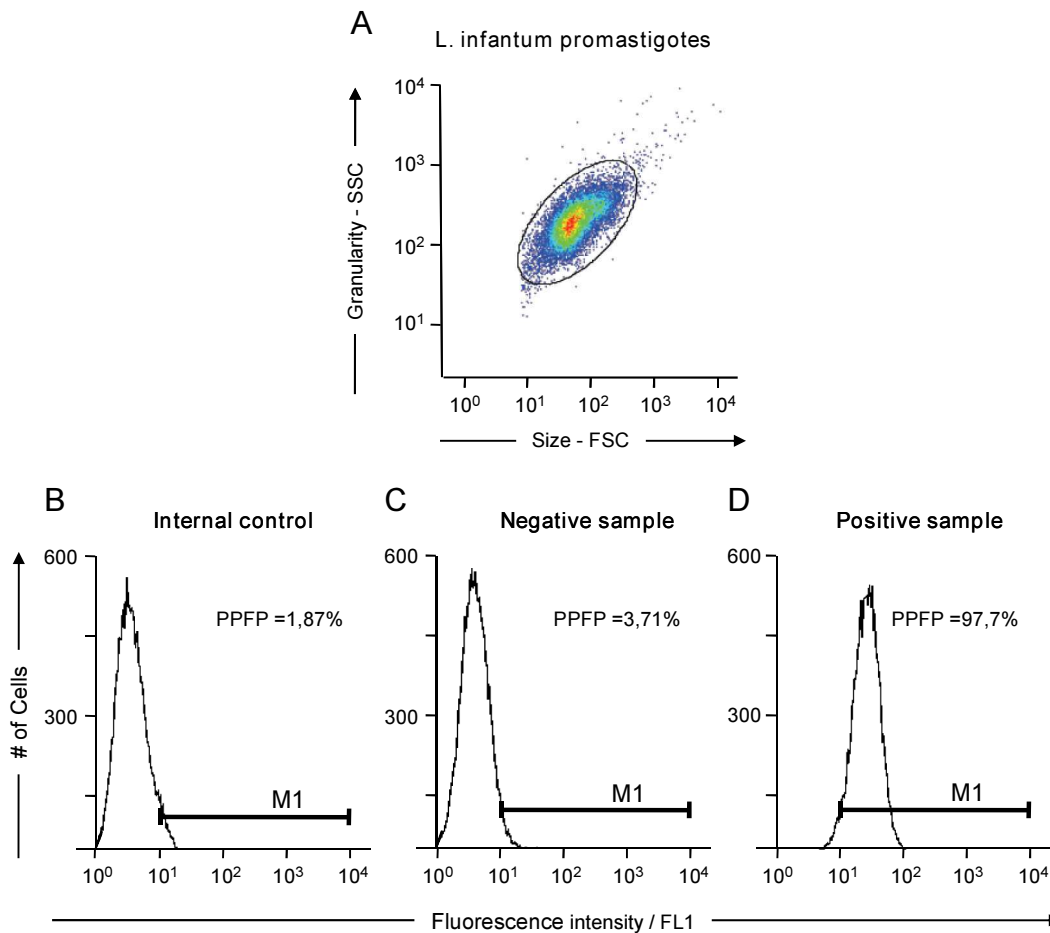
Fig. 1. Representative flow cytometry serology charts used to analyze the anti-fixed *L. infantum* promastigotes IgG reactivity in canine sera samples. Promastigotes were selected based on their FSC and SSC on flow cytometry pseudocolor graphs. Fixed parasites were found to assume homogeneous and characteristic elliptic distribution (A). The relative FL1/FITC fluorescence intensity was quantified in the absence of dog sera but in the presence of the FITC-conjugated anti-canine IgG (internal control). This condition leads to the establishing of a maximum value of reactivity and determination of the marker M1 (B). This marker was maintained to determine the reactivity in all data analyses performed in serum sample from NI (C) and INF (D) dogs. The reactivity of IgG is provided in histograms for each serum samples as PPFP, which represents the frequency of parasite shift toward higher fluorescence intensity, across the M1.

morphological characteristic as observed by optical microscopy and flow cytometry analysis (Fig. 2A). Storage in PBS was associated with changes in cellular integrity and cell structures like flagellum and kinetoplast at all storage temperatures during follow-up.

Promastigotes fixed in formaldehyde showed no changes in cellular integrity or in cell structures such as flagellum and kinetoplast. The morphological features assessed by both optical microscopy and flow cytometry demonstrated that formaldehyde yielded the best profile in pseudocolor graphs. The promastigote population distribution in the FSC × SSC pseudocolor graphs revealed that the *L. infantum* preserved in formaldehyde had a more homogeneous profile (Fig. 2B).

We observed that the morphological profile of promastigotes maintained in thimerosal was similar to those stored in PBS (Fig. 3B). *L. infantum* promastigotes kept in phenol revealed it to be the worst preservative to ensure a morphologic conservation, especially at the −20 °C storage temperature (Fig. 3A).

To verify the sterile maintenance of *L. infantum* promastigotes in storage, microbiological assays were used to show that there was no growth of microorganisms (fungi or bacteria) in any of the preservatives or at different temperatures (data not shown).

3.2. Establishing of flow cytometry serology parameters to be applied for the diagnosis of CVL

In order to establish the flow cytometry serology to differentiate INF from NI dogs samples, it was initially necessary to select the sera

dilution and the PPFP cut-off point able to segregate the IgG reactivity data with better performance indices. Data analysis of ROC curve demonstrated that PPFP of 20% was the most appropriated cut-off to distinguish negative (PPFP ≤20%) from positive (PPFP ᴎ20%) results. Fig. 4 shows the values of IgG reactivity, expressed as PPFP in 3 different dilutions of 1:500, 1:1000, and 1:2000 of labeled antibody, obtained for pooled sera samples along the titration curve ranging from 1/256 to 1/262 144 (Fig. 4). The best differential reactivity between NI and INF samples was observed in the dilutions of conjugate 1:500 and 1:1000 were 1:2048, 1:4096, and 1:8192.

On the other hand, the 1:2000 dilution demonstrated the best segregation of INF to NI, in serum dilutions ranging from 1:1024 to 1:4096. The difference between the reactivity of positive and negatives sample (Δ) showed that the best performance in segregating these groups was the titer 1:1000 of the labeled antibody at a serum dilution of 1:4096.

3.3. Reactive performance of the flow cytometry serology employing L. infantum antigens in different preservative

The serological reaction performance of *L. infantum* antigens based on preservative and storage conditions were performed to obtain PPFP values. Aside from the morphological alterations in promastigotes, the data obtained by the analysis of reactivity showed that the best performances through 12 months were observed in PBS, formaldehyde, and thimerosal, respectively, stored at 4 °C (Fig. 5B).
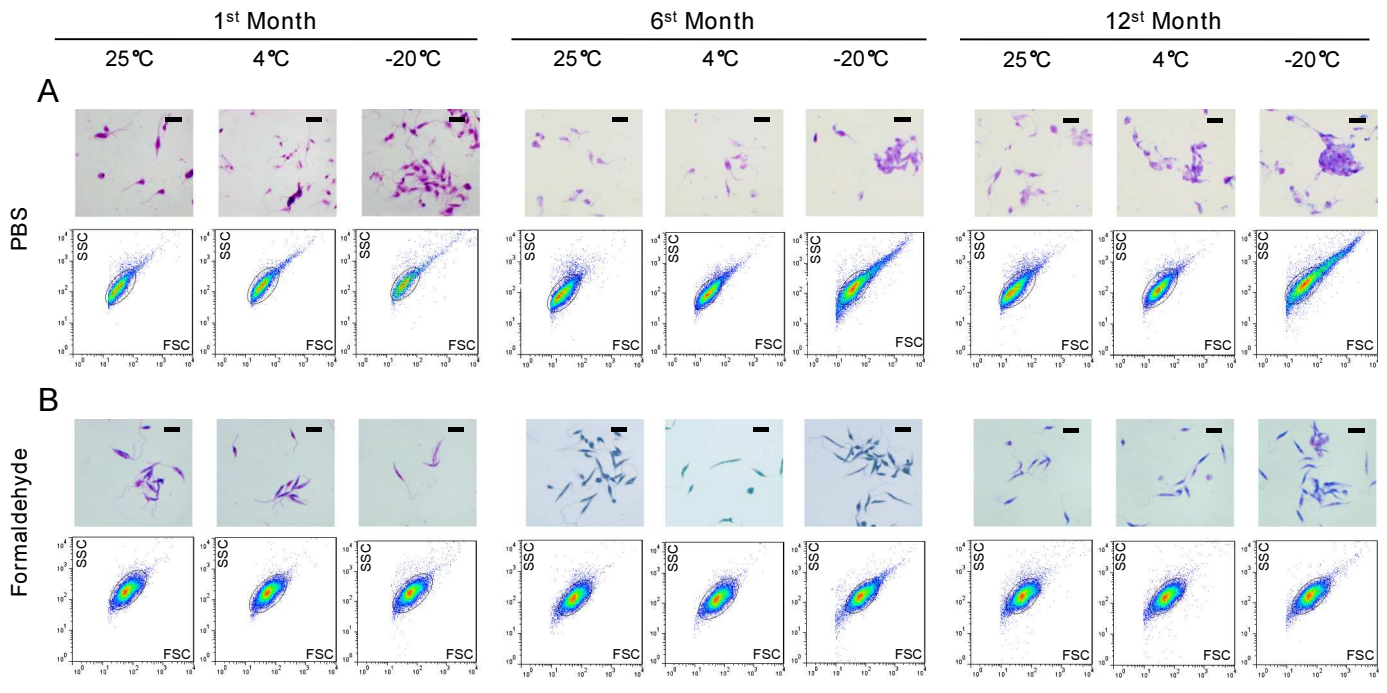
Fig. 2. Morphological evaluation of *L. infantum* promastigotes preserved in PBS (A) and formaldehyde (B) at 25 °C, 4 °C, and 20 °C at the first, sixth, and twelfth months of monitoring. The top panels represent promastigotes stained with *Giemsa* and analyzed at optical microscope. Bottom panels show the flow cytometry morphological profile obtained by FSC and SSC on pseudocolor graphs. Bar = 10 μm.

In contrast, the antigens preserved in phenol did not show a good performance in any storage condition. Furthermore, antigens of *L. infantum* promastigotes maintained at 25 °C demonstrated slightly lower performance from those kept at 4 °C (Fig. 5A), and any preservative showed good reactivity at −20 °C (Fig. 5C).

Considering all parameters evaluated in this study, formaldehyde provided excellent performance for at least 12 months of follow-up. In addition, although *L. infantum* promastigotes kept in PBS demonstrated a good reactivity during the study period, the morphological profile was poor. Thimerosal did not show long-term stability, and changes
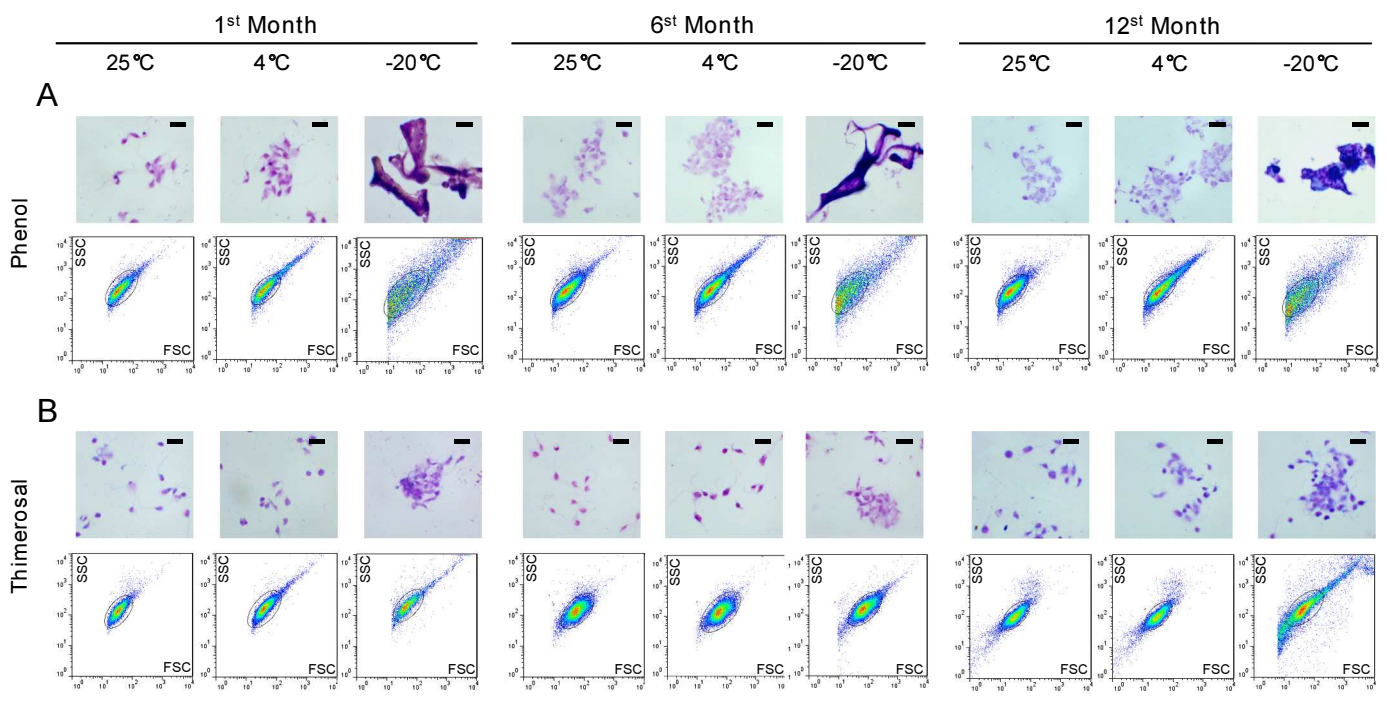

Fig. 3. Morphological evaluation of *L. infantum* promastigotes preserved in phenol (A) and thimerosal (B) at 25 °C, 4 °C, and 20 °C at the first, sixth, and twelfth months of monitoring. The top panels represent promastigotes stained with *Giemsa* and analyzed at optical microscope. Bottom panels show the flow cytometry morphological profile obtained by FSC and SSC on pseudocolor graphs. Bar = 10 μm.
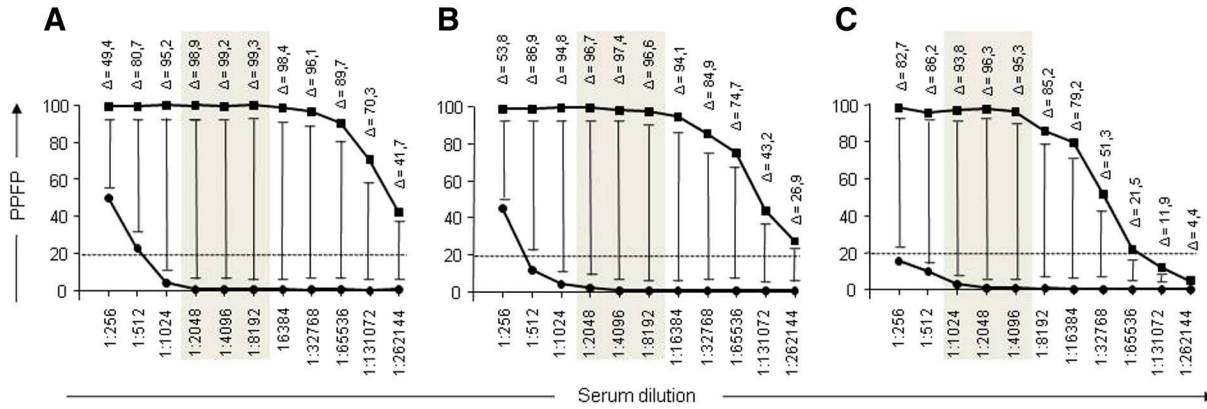
Fig. 4. Serological reactivity performance in pooled sera from INF (■) and NI (●) dogs incubated with different concentration of FITC conjugated antibody at 1:500 (A), 1:1000 (B), and 1:2000 (C). The results are expressed as PPFP at 1:256 to 1:262 144 sera dilutions. The dashed lines represent the cut-off of the test.

were apparent by the seventh month. Preservation in phenol showed the worst results.

4. Discussion

One item of extreme importance in the development of diagnostic kits is the stability of antigens stored in different preservatives and under varied temperatures. Preservatives should provide some essential properties such as broad-spectrum activity against microorganisms, efficiency at low concentrations, and stability at different temperatures (Karabit et al., 1985). We evaluated the conservation of *L. infantum* promastigotes through

morphological and microbiological analyses as well as by the measurement of serological reactivity performance of antigens of promastigotes stored in different preservatives and at varied temperatures for 12 months to evaluate the potential use of flow cytometry serology for CVL diagnosis.

The use of phenol to preserve the immunogenic and biochemical properties of *L. braziliensis* antigens was first observed in the preservation of the Montenegro skin test in Brazil (Mayrink et al., 2006). Thimerosal has been used as an effective preservative in vaccines against human and/or canine leishmaniasis (Fernandes et al., 2008; Mayrink et al., 1979). More recently, Mayrink et al. (2010) proposed substituting thimerosal for phenol in the vaccine against
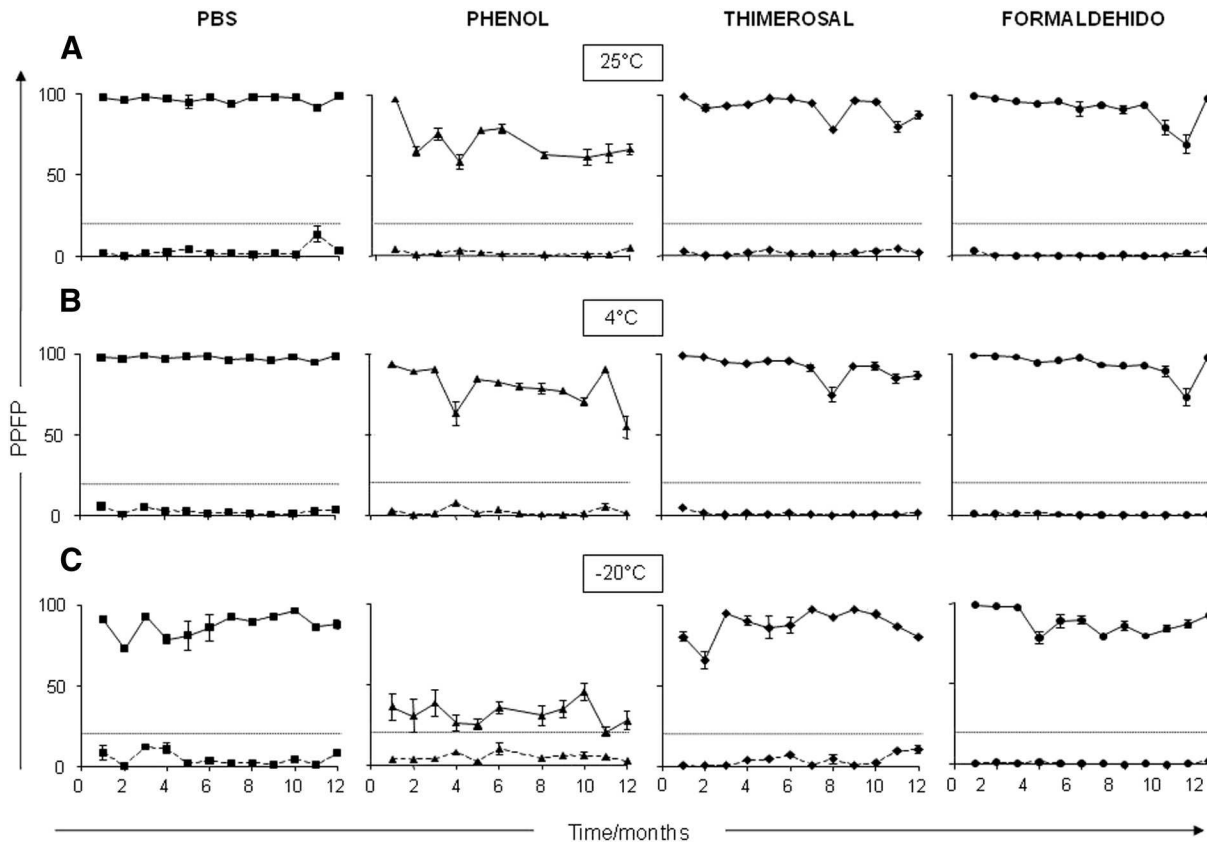

Fig. 5. Serological reactivity performance in 12 months of monitoring on individual samples from uninfected (dashed lines) and infected dogs (continuous lines) employing the sera dilution of 1:4096 and FITC conjugated antibody dilution of 1:1000. The results are expressed as PPFP for the different storage conditions: 25 °C (A), 4 °C (B), and −20 °C (C). The preservatives are represented by: PBS = ■; phenol = ▲; thimerosal = ♦; formaldehyde = ●. The dashed lines represent the cut-off of the test.

American tegumentary leishmaniasis because it is considered to be less toxic. *Leishmania* antigen preservation by formaldehyde is common in CVL diagnostic tests. Formaldehyde is used as a preservative of *Leishmania major* promastigote antigens in diagnostic tests like the IFAT (Bio-Manguinhos/Fiocruz, Rio de Janeiro). Furthermore, fixation with this preservative improved parasite processing for antigen preparation for the DAT test and provided an extended shelf-life to at least 9 months at 4 °C (el Harith et al., 1989).

Serology by flow cytometry has shown the potential for diagnosing CVL and prompted our group to conduct the present investigation. In previous studies that investigated flow cytometry serology, the test was frequently performed with live parasites, and the fixation of *L. infantum* antigens occurred just before the test was completed (Carvalho Neta et al., 2006; Andrade et al., 2007; Andrade et al., 2009). This process requires constant production of promastigotes, making routine implementation difficult. In this context, we suggest that formaldehyde can ensure the preservation of promastigotes for a long period and consequently reduces the cost of the reaction and facilitates their large-scale employment since it allows storage of large amounts of antigens.

Regarding the dilution of the anti-IgG FITC-labeled conjugate employed in the method, our results showed that the 1:1000 titer permitted a more certain differentiation between positive and negative serum samples in comparison with the 1:500 dilution. According to previous data from Carvalho Neta et al. (2006), a higher dilution of anti-IgG FITC-labeled conjugate (1:2000) can generate lower sensitivities in flow cytometry serology. Furthermore, other authors also confirmed that the labeled antibody at 1:1000 is the best titer to be applied in flow cytometry serology in dogs (Andrade et al., 2007, 2009; Carvalho Neta et al., 2006).

According to Andrade et al (2009), flow cytometry in CVL diagnosis showed higher performance indices with the serum dilution of 1:8192 when compared to 1:2048. However, the lowest dilution (1:2048) presented higher sensitivity in asymptomatic dogs, and the authors recommended the use of both serum dilutions to detect all CVL clinical forms. Similar results were found in the present study; the observed segregation among negative and positive samples was wide in serum dilutions of 1:2048, 1:4096, or 1:8192. Some authors have been demonstrated that asymptomatic dogs usually have low titers of the anti-*Leishmania* antibodies and their animals may not be detected by conventional test such as ELISA and IFAT (Coura-Vital et al., 2011a, 2011b; Reis et al., 2006, 2009). In order to avoid the false positive and false negative results, we recommended the use sera dilution 1:4096. Moreover, it is important to consider that this dilution showed higher segregation in all conjugate conditions tested (1:500, 1:1000, 1:2000).

From this work, we show for the first time that the morphological assessment of *L. infantum* parasites is important to verify the homogeneous morphometric distribution on FSC versus SSC. Only formaldehyde was associated with no change in the morphological features, which was verified by flow cytometry and optical microscopy. Under all preservative conditions, the reactivity of the antigens kept at 4 °C was slightly higher than for those maintained at 25 °C. This result also indicated that antigens may exhibit good stability when subjected to a temperature change ranging between these two. Moreover, the maintenance of *L. infantum* antigens at −20 °C was not indicated due to this temperature being associated with the worst morphological characteristics and the poorest reactivity. Microbiological analysis revealed no contamination in the *L. infantum* promastigotes among all preservatives and storage temperatures. It is important to highlight that a possible microorganism contamination of the antigens could lead to an unreliable flow cytometry evaluation of the serological reactivity as well as the morphological characteristics.

Taken together, our findings demonstrate that preservation in formaldehyde and storage at 4 °C provides the best conditions for preservation of *L. infantum* promastigotes. We verified that these conditions ensure the best stability of morphological characteristics and excellent antigen reactivity through 12 months of storage, as well as sterility of the antigen preparation. In this way, these preservative and storage conditions can be very useful for developing new tests using crude antigens for immunodiagnosis of CVL. With the results obtained in this study, it was possible to define the best conditions to use in developing a new kit for serological detection of CVL by flow cytometry.

References

Andrade RA, Reis AB, Gontijo CM, Braga LB, Rocha RD, Araujo MS, et al. Clinical value of anti-*Leishmania* (*Leishmania*) *chagasi* IgG titers detected by flow cytometry to distinguish infected from vaccinated dogs. Vet Immunol Immunopathol 2007;116: 85–97.
Andrade RA, Silva Araujo MS, Reis AB, Gontijo CM, Vianna LR, Mayrink W, et al. Advances in flow cytometric serology for canine visceral leishmaniasis: diagnostic applications when distinct clinical forms, vaccination and other canine pathogens become a challenge. Vet Immunol Immunopathol 2009;128:79–86.
Carvalho Neta AV, Rocha RDR, Gontijo CMF, Reis AB, Martins-Filho AO. Citometria de fluxo no diagnóstico da leishmaniose visceral canina [Flow cytometry used in canine visceral leishmaniasis diagnosis]. Arq Bras Med Vet Zootec 2006;58:480–8.
Chamaille L, Tran A, Meunier A, Bourdoiseau G, Ready P, Dedet JP. Environmental risk mapping of canine leishmaniasis in France. Parasit Vectors 2010;3:31.
Coura-Vital W, Marques MJ, Giunchetti RC, Teixeira-Carvalho A, Moreira ND, Vitoriano-Souza J, et al. Humoral and cellular immune responses in dogs with inapparent natural *Leishmania infantum* infection. Vet J 2011a;190:e43–7.
Coura-Vital W, Marques MJ, Veloso VM, Roatt BM, Aguiar-Soares RD, Reis LE, et al. Prevalence and factors associated with *Leishmania infantum* infection of dogs from an urban area of Brazil as identified by molecular methods. PLoS Negl Trop Dis 2011b;5:e1291.
Drumond KO, Costa FA. Forty years of visceral leishmaniasis in the State of Piaui: a review. Rev Inst Med Trop 2011;53:3–11.
el Harith A, Slappendel RJ, Reiter I, van Knapen F, de Korte P, Huigen E, et al. Application of a direct agglutination test for detection of specific anti-*Leishmania* antibodies in the canine reservoir. J Clin Microbiol 1989;27:2252–7.
Fernandes AP, Costa MM, Coelho EA, Michalick MS, de Freitas E, Melo MN, et al. Protective immunity against challenge with *Leishmania* (*leishmania*) *chagasi* in beagle dogs vaccinated with recombinant a2 protein. Vaccine 2008;26:5888–95.
Grimaldi G, Teva A, Ferreira AL, dos Santos CB, Pinto IS, de-Azevedo CT, et al. Evaluation of a novel chromatographic immunoassay based on dual-path platform technology (DPP® CVL rapid test) for the serodiagnosis of canine visceral leishmaniasis. Trans R Soc Trop Med Hyg 2012;106:54–9.
Harhay MO, Olliaro PL, Costa DL, Costa CH. Urban parasitology: visceral leishmaniasis in Brazil. Trends Parasitol 2011;27:403–9.
Jaroszeski MJ, Radcliff G. Fundamentals of flow cytometry. Mol Biotechnol 1999;11: 37–53.
Karabit MS, Juneskans OT, Lundgren P. Studies on the evaluation of preservative efficacy. I. The determination of antimicrobial characteristics of phenol. Acta Pharm Suec 1985;22:281–90.
Mayrink W, Williams P, Coelho MV, Dias M, Martins AV, Magalhaes PA, et al. Epidemiology of dermal leishmaniasis in the Rio Doce Valley, State of Minas Gerais, Brazil. Ann Trop Med Parasitol 1979;73:123–37.
Mayrink W, Coelho GL, Guimaraes TM, de Andrade HM, Castro Peres E, da Costa CA, et al. Immuno-biochemical evaluations of phenol and thimerosal as antigen preservatives in montenegro skin test. Acta Trop 2006;98:87–93.
Mayrink W, Tavares CA, Deus RB, Pinheiro MB, Guimarães TM, Andrade HM, et al. Comparative evaluation of phenol and thimerosal as preservatives for a candidate vaccine against American cutaneous leishmaniasis. Mem Inst Oswaldo Cruz 2010;105:86–91.
Ministério da Saúde (2011) Esclarecimento sobre substituição do protocolo diagnóstico da leihsmaniose visceral canina; nota técnica conjunta nº 01/2011 - cgdt-cglab/devit/svs/ms;.

Nunes CM, Pires MM, da Silva KM, Assis FD, Goncalves Filho J, Perri SH. Relationship between dog culling and incidence of human visceral leishmaniasis in an endemic area. Vet Parasitol 2010;170:131–3.

Reis AB, Teixeira-Carvalho A, Vale AM, Marques MJ, Giunchetti RC, Mayrink W, et al. Isotype patterns of immunoglobulins: hallmarks for clinical status and tissue parasite density in brazilian dogs naturally infected by *Leishmania* (*leishmania*) *chagasi*. Vet Immunol Immunopathol 2006;112:102–16.

Reis AB, Martins-Filho OA, Teixeira-Carvalho A, Giunchetti RC, Carneiro CM, Mayrink W, et al. Systemic and compartmentalized immune response in canine visceral leishmaniasis. Vet Immunol Immunopathol 2009;128:87–95.

Rocha RD, Gontijo CM, Eloi-Santos SM, Teixeira Carvalho A, Correa-Oliveira R, Marques MJ, et al. Anti-live *Leishmania* (*viannia*) *braziliensis* promastigote antibodies, detected by flow cytometry, to identify active infection in american cutaneous leishmaniasis. Rev Soc Bras Med Trop 2002;35:551–62.

Rosypal AC, Troy GC, Zajac AM, Duncan RB, Waki K, Chang KP, et al. Emergence of zoonotic canine leishmaniasis in the United States: isolation and immunohisto-chemical detection of *Leishmania infantum* from foxhounds from Virginia. J Eukaryot Microbiol 2003;50:691–3.

Salomon OD, Quintana MG, Bruno MR, Quiriconi RV, Cabral V. Visceral leishmaniasis in border areas: clustered distribution of phlebotomine sand flies in Clorinda, Argentina. Mem Inst Oswaldo Cruz 2009;104:801–4.

Tarallo VD, Dantas-Torres F, Lia RP, Otranto D. Phlebotomine sand fly population dynamics in a leishmaniasis endemic peri-urban area in southern Italy. Acta Trop 2010;116:227–34.