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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

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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.

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

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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 × *g*, 10 min, room temperature) to remove cell debris. The supernatant containing most of the parasites was centrifuged at 1000 × *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: 262144) 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 × *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 × *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 20000 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

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