Detection of Parasite Antigens in *Leishmania infantum*–Infected Spleen Tissue by Monoclonal Antibody-, Piezoelectric-Based Immunosensors

Author(s): G. Cabral-Miranda , J. R. de Jesus , P. R. S. Oliveira , G. S. G. Britto , L. C. Pontes-de-Carvalho , R. F. Dutra , and N. M. Alcântara-Neves
Source: Journal of Parasitology, 100(1):73-78. 2014.
Published By: American Society of Parasitologists
DOI: http://dx.doi.org/10.1645/GE-3052.1
URL: http://www.bioone.org/doi/full/10.1645/GE-3052.1

J. Parasitol., 100(1), 2014, pp. 73–78

DETECTION OF PARASITE ANTIGENS IN *LEISHMANIA INFANTUM*–INFECTED SPLEEN TISSUE BY MONOCLONAL ANTIBODY-, PIEZOELECTRIC-BASED IMMUNOSENSORS

G. Cabral-Miranda, J. R. de Jesus, P. R. S. Oliveira*, G. S. G. Britto, L. C. Pontes-de-Carvalho*, R. F. Dutra†, and N. M. Alcântara-Neves

Universidade Federal da Bahia, Instituto de Ciências da Saúde, Departamento de Ciências da Biointeração, Salvador, Bahia, Brazil. Correspondence should be sent to: *neuzalcantara@gmail.com*

ABSTRACT: Diseases such as leishmaniases are important causes of morbidity and mortality in Brazil, and their diagnoses need to be improved. The use of monoclonal antibodies has ensured high specificity to immunodiagnosis. The development of an immunosensor, coupling a monoclonal antibody to a bioelectronic device capable of quickly detecting *Leishmania* sp. antigens both qualitatively and quantitatively, is a promising alternative for the diagnosis of leishmaniasis due to its high specificity, low cost, and portability, compared with conventional methods. The present work was aimed at developing an immunosensor-based assay for detecting *Leishmania infantum* antigens in tissues of infected hosts. Four hybridomas producing monoclonal antibodies against *L. infantum* had their specificity confirmed by enzyme-linked immunosorbent assay. These antibodies were immobilized on a gold surface, covered with a thin film of 2-aminoethanethiol (cysteamine) and glutaraldehyde, blocked with glycine, and placed into contact with extracts of *L. infantum*–infected and noninfected control hamster spleens. The assay was able to detect 1.8×10^4 amastigotes/g of infected tissue. These results demonstrated that this assay may be useful for quantifying *L. infantum* amastigotes in organs of experimental animals for studies on pathogenesis and immunity and that it is a promising tool for the development of a diagnostic method, based on antigen detection, of human and dog visceral leishmaniasis.

Visceral leishmaniasis (VL) is endemic in 62 countries, where 200 million people are at risk of acquiring the disease. About 90% of the cases are concentrated in 5 countries, namely Bangladesh, India, Nepal, Sudan, and Brazil (Desjeux, 2004). VL is mainly caused by 2 species of *Leishmania*: *Leishmania donovani* and *Leishmania infantum*. *Leishmania donovani* causes anthroponotic VL in the Old World, and *L. infantum* causes zoonotic VL in both the Old and New World (Desjeux, 2004). VL in the New World uses canines as its main reservoir and is also called American visceral leishmaniasis (AVL) (Grimaldi and Tesh, 1993; Romero and Boelaert, 2010). AVL has not been dealt with successfully by the public health services in many of the affected countries, and it still causes high morbidity and death when not treated appropriately and in a timely manner (Grimaldi and Tesh, 1993).

The serodiagnosis of AVL is usually performed by immunofluorescence, enzyme-linked immunosorbent assay (ELISA), or both, but neither of these methods has yet shown good specificity (Reithinger and Dujardin, 2007); the diagnosis is only conclusive with the identification of amastigote forms of *L. infantum* in bone marrow smears (usually in humans) and spleen aspirates (usually in dogs). However, due to economic difficulties, the diagnosis of AVL may be based exclusively on clinical criteria or serology. Indeed, in the absence of parasitological diagnosis, the therapeutic response to toxic drugs is sometimes the only way to diagnose AVL (Marsden, 1984; Bryceson et al., 1985; Chappuis et al., 2007).

Advances in the serodiagnosis of leishmaniasis have been related to the use of recombinant proteins as antigens (Braz et al., 2002; Sreenivas et al., 2002; Maalej et al., 2003). However, the detection of antibodies, despite having large relevance for epidemiological studies, is less useful for clinical diagnosis not only due to cross-reactions with other pathogens but also because high antibody levels may be related to previous or asymptomatic infections (Maia and Campino, 2008). Based on these assumptions, the detection of parasite antigens has a great advantage over detection of antibodies because, in sensu lato, it is a parasitological test, revealing the presence of parasite molecules and therefore the presence of the pathogen (Cruz et al., 2006; Gomes et al., 2008). A great advance in the diagnosis of leishmaniasis may potentially arise from the detection of parasite DNA by the polymerase chain reaction, but this technique requires a specialized technician, above-average laboratory facilities, and is relatively time-consuming (Cruz et al., 2006; Maide et al., 2008).

An immunosensor is a device made up of an antigen or antibody species coupled to a signal transducer that detects the binding of the complementary species. Biosensors based on piezoelectric transducers can replace commonly used tests, decreasing the time of performance and cost, by using serum and tissue samples. Quartz crystal microbalance (QCM) piezosensors are considered excellent for detection of immunoreactions, in that changes in the electrode–electrolyte interface are of a sufficient magnitude to reveal the presence of the desired adsorbed components by changes on the electrical frequency (Chu et al., 2006). The principle of operation of the QCM biosensor depends on the piezoelectric effect that is governed by relationships between mass and frequency variations (Luo et al., 2007). The selectivity of the immunosensor is achieved by the proper choice of a chemically modified surface and immobilized biocomponents on the electrode surface (Marx, 2003; Dutra and Kubota, 2007). Self-assembled monolayers (SAMs) using alkanethiol films have been presented as adequate structuring for the antibody immobilization (Dutra et al., 2007). These QCM sensors can be easily packaged for routine use as portable units. The present work was aimed at developing a piezoelectric immunosensor from sensing elements (anti–*L. infantum* monoclonal antibodies) to be used as a tool to detect parasite antigens in tissues of experimentally infected animals and, as a medium-term goal, to contribute to the parasitological (sensu lato) diagnosis of human and canine VL.

Received 1 December 2011; revised 7 October 2013; accepted 16 October 2013.

* Fundação Oswaldo Cruz, Centro de Pesquisa Gonçalo Moniz, Laboratório de Patologia e Biointervenção, Salvador, Bahia, Brazil.

† Universidade Federal de Pernambuco, Laboratório de Engenharia Biomédica, Recife, Pernambuco, Brazil.

DOI: 10.1645/GE-3052.1

MATERIALS AND METHODS

Animals

Pathogen-free, 6- to 12-wk-old BALB/c mice and golden hamsters were maintained at the animal facilities of the Gonçalo Moniz Research Center, Oswaldo Cruz Foundation, Bahia (FIOCRUZ-BA), Brazil, and provided with rodent diet and water ad libitum. All procedures were approved and conducted according to the institutional Ethical Committee for the Utilization of Experimental Animals (protocol 036/2009) of FIOCRUZ, Salvador, Brazil.

Anti–*L. infantum* monoclonal antibodies (αLimAbs)

αLimAbs (5A9H8, 2B7B8, 4B6F7, and 5AB3A10B4)-producing hybridomas, previously obtained by fusing myeloma cells with *L. infantum*–infected mouse splenocytes in accordance with the methodology described by Fróes et al. (2004), were cultured and expanded in Iscove's modified Dulbecco's medium (Invitrogen, São Paulo, SP, Brazil), containing 10% fetal calf serum (FCS), 100 μg/ml glutamine, and 50 μg/ml gentamicin. The isotypes of the monoclonal antibodies (mAbs) present in supernatants of hybridoma cultures were determined by capture ELISA, using the Mono-Ab-ID kit (Invitrogen), according to manufacturer's recommendations. Large amounts of these mAbs were produced by inoculation of the hybrid cells into the peritoneal cavity of BALB/c mice, previously injected intraperitoneally with pristane (2,6,10,14-tetramethylpentadecane; Sigma-Aldrich, St. Louis, Missouri). The antibodies were semipurified from the ascitic fluid by precipitation with ammonium sulfate (pH 7.0), followed by dialysis against 0.15 M phosphate-buffered saline (PBS) at pH 7.4. The semipurified mAbs were assayed for protein content by Lowry's method (Lowry et al., 1951), aliquoted, and stored at –70 C until use.

Parasites and parasite extracts

Leishmania infantum (MHOM/BR2000/Merivaldo strain) promastigotes were obtained from in vitro cultures of homogenates from infected hamster spleens, in Schneider's medium supplemented with 10% fetal FCS (Invitrogen) and 50 μg/ml gentamicin at 25 C. Part of the promastigotes was washed by 3 centrifugations (1,620 *g*; 10 min) in cold PBS, counted in a Neubauer's chamber, and stored at –70 C for extract preparation. The remaining promastigotes were transferred to Schneider's medium containing 50 μg/ml gentamicin and 20% FCS (pH 7.2) and cultivated at 35 C for 10–13 days, in accordance with Teixeira et al. (2002), to obtain axenic amastigotes. After transformation, the axenic amastigotes were washed in cold PBS and stored at –70 C until use.

Trypanosoma cruzi amastigotes and trypomastigotes were obtained from in vitro culture of infected monkey epithelial cells (MK2) in the presence of RPMI-1640 medium (Invitrogen) containing 10% FCS and 50 μg/ml gentamicin (Piazza et al., 1994). The parasites were collected from the cell supernatants after 5 days of infection, washed, and kept frozen until use. All frozen parasites were lysed by ultrasound (Branson's Cell Disruptor, Branson Sonic Power Company, Danbury, Connecticut) in the presence of PBS and centrifuged (14,000 *g*; 10 min). The supernatants were filtered through a membrane of 0.45-μm-diameter pore size and stored at –70 C until use. The protein content was measured by Lowry's method (Lowry et al., 1951).

Extracts of *L. infantum*–infected hamster spleen

One hundred million *L. infantum* metacyclic promastigotes, obtained from cultivation in Schneider's medium, were inoculated into the peritoneal cavity of a 3-mo-old golden hamster. After 60–90 days, the animal was killed and its spleen was weighed. The spleen parasite load, estimated by a limiting dilution assay (Titus et al., 1985), performed in a culture that had been set up with 200 mg of a homogenized spleen fragment in Schneider's medium containing 20% FCS and 50 μg/ml gentamicin, was 4.9×10^9 amastigotes/g. Four hundred milligrams of the remaining infected organ fragment was homogenized in 56 ml (an 1:140 [w:v] dilution) of PBS containing 1% NP40 (Sigma-Aldrich) and a protease inhibitor mix (Amersham, São Paulo, SP, Brazil). The tissue was then sonicated, incubated at 4 C for 30 min, centrifuged (14,000 *g*; 10 min), filtered through a membrane with 0.45-μm-diameter pores, aliquoted, and stored at –70 C until use. Each milliliter of this spleen extract therefore contained the soluble antigens from 3.5×10^7 amastigotes. The spleen of a noninfected hamster was subjected to the same procedure and used as a negative control.

Determination of the reactivity and specificity of αLimAbs

The reactivity of the αLimAbs was determined by a sandwich ELISA using an extract of *L. infantum*–infected hamster spleen. In brief, wells of a microtiter plate were coated with 30 μg/ml of each semipurified monoclonal antibody, diluted in 0.15 M carbonate/bicarbonate buffer (pH 9.3). After blocking of possibly available binding sites with PBS containing 5% powdered skimmed milk (PBS-SM), 100 μl of the *L. infantum*–infected hamster spleen extract or control noninfected spleen extract was added to the wells, diluted at 1:125 in PBS-SM. The reaction was detected using *L. infantum*–infected dog serum, diluted at 1:200 in PBS-SM, followed by an anti-dog immunoglobulin antibody-peroxidase conjugate, diluted at 1:1,000; followed by a solution of 3,3′,5,5′-tetramethylbenzidine with hydrogen peroxide (Sigma-Aldrich) as substrate. Between all the steps, the plate was incubated for 1 hr at room temperature (except for the substrate that was incubated for 20 min) and washed 4 times with PBS-SM containing 0.05% Tween 20 (PBS-SM-T20). The reactions were read in a spectrophotometer at 450 nm.

An indirect ELISA was conducted to investigate the cross-reactivity of the αLimAbs against heterologous antigens (axenic amastigote and promastigote forms of *L. infantum*, and amastigote and trypomastigote forms of *T. cruzi*). In brief, parasite extracts, obtained as described above, were used at a concentration of 100 μg protein/ml to coat the wells of microtiter plates. Nonspecific reactions were blocked with PBS-SM. Undiluted αLimAbs-containing hybridoma supernatants were added to the wells, and the assays were developed using an anti-mouse immunoglobulin-peroxidase conjugate diluted at 1:2,000. The remainder of the assay was performed as described above.

Immunosensor assembly and detection the of *L. infantum* amastigote antigens in infected hamster spleen

We applied 4 μg/ml αLimAbs (5A9H8, 4B6F7, 2B7B8, and 5AB3A10B4) to quartz crystal electrode (QMC of 9 MHz; Maxtec Inc., Salt Lake City, Utah) that consists basically of a disk made of piezoelectric quartz crystal coated with a thin film of gold, with a phase lock oscillator circuit coupled to a microcomputer and a potentiostat-galvanostat (Metrohm Pensalab Instrumentação Analítica Ltda, São Paulo, SP, Brazil), that was, in turn, controlled by the GPES software (Eco Chemie B.V., AD Utrecht, the Netherlands). The αLimAbs were immobilized on the gold surface using a SAM, with 2-aminoethanethiol (cysteamine), followed by covalent binding with glutaraldehyde, according to Ramos-Jesus et al. (2011). Remaining unbound aldehyde groups were blocked with glycine. The immunosensor assembly was carried out at room temperature with the following incubation steps: cysteamine (2 hr), glutaraldehyde (45 min), and antibodies and blocking (2 hr each). Then, the *L. infantum*–containing spleen and the negative control spleen extracts, diluted 1:1,000, 1:2,000 and 1:4,000, were injected in the immunosensor surface and incubated for 15 min. Between all steps, 3 washings were performed with PBS, followed by 2 readings of the frequencies. The mean frequency of the 2 readings, expressed in hertz, is depicted in the figures. The sample was considered positive when its application on the surface of the crystal produced a reduction in the oscillation movement that, in turn, resulted in decreased resonance frequencies. The measurements of the frequency in real time permitted the monitoring of the interfacial phenomena that occur by the antigen binding to specific antibodies immobilized on the electrode surface.

RESULTS

The αLimAbs were isotyped as IgG1 (5AB3A10B4), IgG2a (5A9H8 and 4B6F7), and IgG3 (2B7B8) (data not shown). To assess their specificity to *L. infantum* amastigotes compared with hamster spleen antigens, the mAbs were assayed for reactivity against extracts from amastigote-containing and control hamster spleens in a sandwich ELISA. All reacted with the *L. infantum* antigen-containing extract more intensely than with the control extract (Fig. 1). The specificity of the mAbs, as far as *T. cruzi*, *L. infantum* epimastigote, and *L. infantum* axenic amastigote antigens is concerned, was assessed in an indirect ELISA, which is simpler than the sandwich ELISA mentioned above. None of

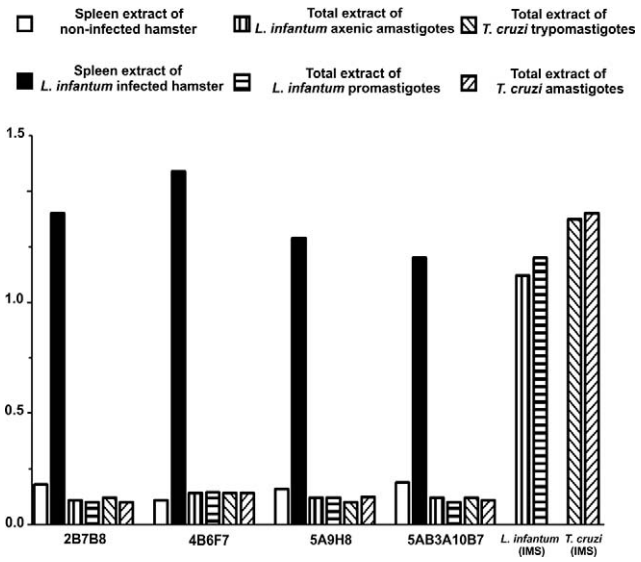

FIGURE 1. Lack of reactivity of anti–*Leishmania infantum* amastigote mAbs to heterologous antigens as determined by ELISA. To analyze the specific reaction against *L. infantum* tissue amastigotes, the wells were coated with the 4B6F7, 5A9H8, 2B7B8, or 5AB3A10B4 anti–*Leishmania* mAbs. The solid columns represent the results obtained when a hamster spleen extract containing *L. infantum* amastigotes (prepared from the spleen of an infected hamster) was added to the wells, and the open columns represent the results obtained with a control extract prepared from a noninfected hamster spleen. The presence of bound *Leishmania* antigens was shown by successive incubations with *L. infantum*–infected dog antibodies, an anti-dog immunoglobulin-peroxidase conjugate, and a chromogen–substrate mixture, as explained in Materials and Methods. To analyze the reactions against heterologous antigens, wells were coated with extracts from *L. infantum* axenic amastigotes or promastigotes and *T. cruzi* amastigotes or trypomastigotes. The reaction was developed using 2B7B8, 4B6F7, 5A9H8, or 5AB3A10B4 anti–*Leishmania* monoclonal antibodies. *Leishmania infantum*- or *T. cruzi*–infected mouse sera (IMS) were used as positive controls.

the mAbs reacted with any of these antigens (Fig. 1). Consecutive injections of 200 µl of *L. infantum*–infected hamster spleen extract, diluted 1:1,000, led to a progressive decrease in the resonance frequencies of the immunosensors coated with any of the 4 mAbs (Fig. 2). In contrast, injections of the noninfected hamster spleen extract had no effect on the frequencies; they remained stable (Fig. 2). The same data are shown in a single graph in Figure 3 to allow a better comparison among the assays performed with the different αLimAbs. The assay with the best signal was the assay that used the 2B7B8 αLimAb (Fig. 3), producing a positive result with a 1:2,000 dilution of the *Leishmania* antigen-containing containing extract (Fig. 4).

DISCUSSION

The development of diagnostic methods with high sensitivity, specificity, low cost, and easy portability, based on the detection of parasite products, may constitute an important achievement to improve the clinical management of suspected AVL cases, allowing the early initiation of specific treatment and avoiding the wrong administration of toxic drugs. In this work, the development of an immunosensor-based assay using αLimAbs and its application for the detection of *Leishmania* spp. antigens in the spleen of an infected hamster has been described. The spleen is the main organ that is infected by *L. infantum* in different mammal species (Rousseau et al., 2001).

First, it was determined that the mAbs reacted with a *L. infantum*–infected hamster spleen extract in a sandwich ELISA, producing optical densities (ODs) ranging from 1.2 to 1.6, values that were about 1 order of magnitude larger than those produced when the mAbs were incubated with a control, noninfected hamster spleen extract (range, from 0.12 to 0.19). This readily demonstrable reactivity to *Leishmania* antigens within a spleen extract indicated that the mAbs did not cross-react with a major hamster splenic antigen and could be used to detect *Leishmania* antigens in the extracts. After the characterization and semi-purification of the αLimAbs, they were successfully used to construct piezoelectric immunosensors using QCM with self-assembled, organized monolayers. These immunosensors produced clear signals when placed into contact with an extract of a spleen containing *L. infantum* amastigotes. In recent decades, QCM biosensors have found various applications in diagnosis, for example, in studies on the pathogenicity of microorganisms and in the investigation of molecular interactions, because of their attractive characteristics, such as high specificity, low cost, simplicity, and rapid generation of results (Prusak-Sochazewski et al., 1990; Plomer et al., 1992).

The mAbs used in the work reported here reacted with antigens of a *L. infantum*–infected mouse spleen extract in a sandwich ELISA, whereas they did not react with promastigote and axenic amastigote extracts of this parasite, nor with *T. cruzi* amastigote and trypomastigote extracts in an indirect ELISA. This indicates that, although similarly to cell-derived amastigotes in protein makeup (Teixeira et al., 2002), axenic amastigotes do not express the antigens that are recognized by the used mAbs. Indeed, differences in protein profile between cell-derived and axenic *Leishmania* sp. amastigotes, and between *Leishmania* sp. cell-derived amastigotes and promastigotes, have been reported previously (Teixeira et al., 2002).

As it is, the *L. infantum* antigen-reactive immunosensor described in this report could be of great clinical importance if it proved to be sensitive and specific enough to detect *Leishmania* sp. antigens in AVL patients' bone marrow aspirates. It would be also interesting to determine whether the αLimAbs recognize other *Leishmania* species, particularly *L. donovani* amastigotes, because this would extend their use to other important endemic areas of VL, such as those in the Indian subcontinent and in Sudan. The use of the developed immunosensor is a promising procedure to replace the visualization of amastigotes in bone marrow or spleen aspirates by microscopy, the method that is usually conducted to confirm the diagnosis of VL and requires a highly skilled technician (Chappuis et al., 2007).

The assay using the 2B7B8 αLimAb-coated sensor produced a positive result with a 1:2,000 dilution of a *Leishmania* sp.–infected hamster extract. This dilution corresponded to a 1:280,000 dilution of the soluble antigens that were present in the spleen tissue (4.9×10^9 amastigotes/g of tissue that was diluted 1:140 [w:v] in the neat extract). The assay should therefore be able to produce a positive result in an extract in which the tissue of a spleen containing 7×10^6 parasites/g was diluted 1:400 (w:v). The developed assay is already potentially useful for quantifying amastigotes in studies on pathogen-free experimental animals, such as those dealing with pathogenicity, and on assessing vaccination procedures. Nonetheless, an improvement in the

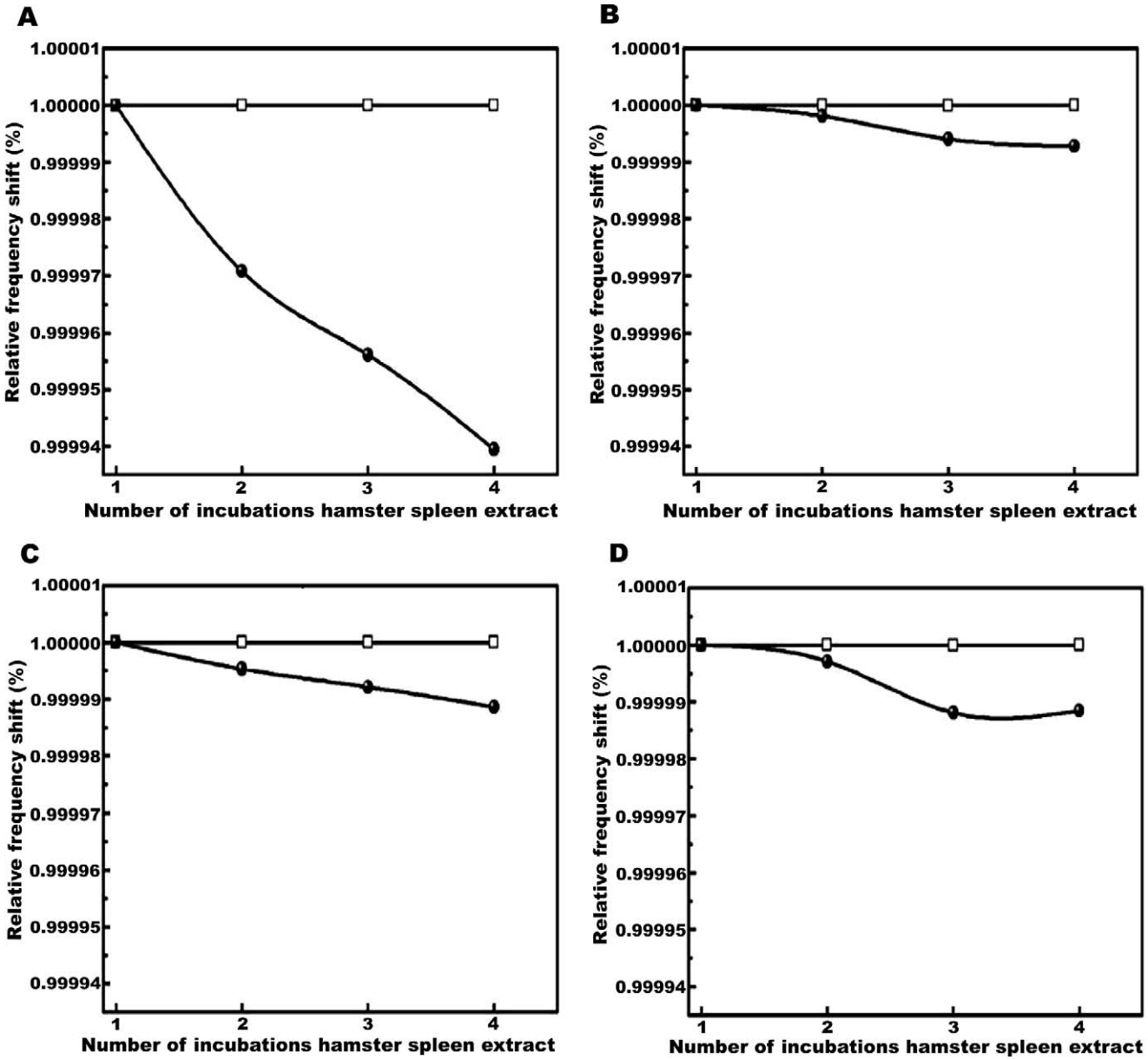

FIGURE 2. Reaction of αLimAbs against extracts of spleens from *Leishmania infantum*–infected (●) or noninfected (□) hamsters on a piezoelectric effect-based sensor. Four injections of 1/1,000 dilutions of spleen extracts were applied onto the immunosensor surface that had been precoated with the 2B7B8 (**A**), 4B6F7 (**B**), 5A9H8492 (**C**), and 5AB3A10B4 (**D**) αLimAbs. Each point of the curves represents the mean frequency of 2 reaction readings in the same immunosensor.

sensitivity of the immunosensor is highly desirable. One way to achieve this goal is to increase the QMC binding area for the biological component. This will allow an increase in the amount of antigen–mAb complex due to an increase in the amount of the solid phase mAb in the immunosensor, increasing the assay's sensitivity. An additional possibility is to test whether coating the QMC with more than 1 mAb would also increase the assay sensitivity. Still another possibility is to replace glutaraldehyde with a molecule directed to the carboxyl residue of the Fc region; glutaraldehyde does not bind specifically to the NH_2 residues in the Fc region of the antibody and therefore may interfere with the antibody antigen-binding region. This would increase the amount of operationally active mAb in the immunosensor. Future investigation aimed at decreasing the assay costs would also be desirable. Finally, the assay may also be useful in the following cases: (1) for the detection of amastigote antigens in bone marrow aspirates of suspected human cases of AVL, and in spleen aspirates of dogs suspected of being infected with *L. infantum*, replacing the parasitological examination, as discussed above; (2) detection of amastigote antigens in the blood of *L. infantum* and human immunodeficiency virus (HIV) co-infected patients; and (3) detection of *L. donovani* amastigote antigens in the blood of anthroponotic VL. Although *L. infantum* amastigotes are not easily found in the blood of patients with AVL, their presence in

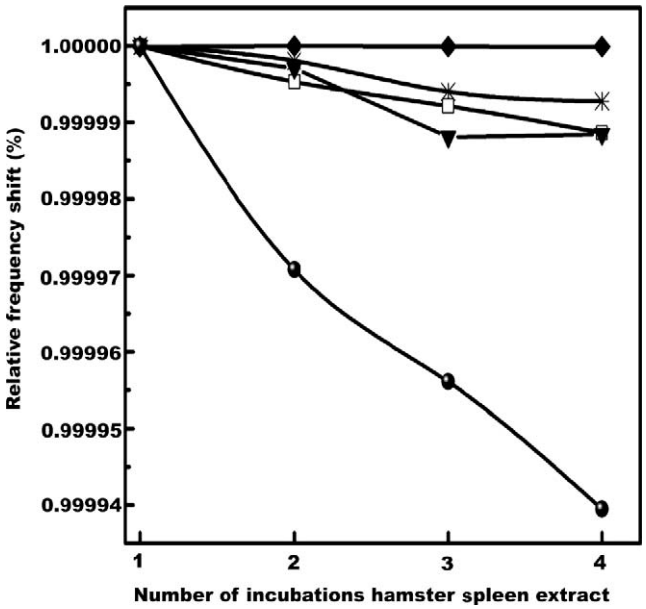

FIGURE 3. Signal strengths obtained by using different αLimAbs in a piezoelectric effect-based sensor. The curves represent data obtained with immunosensors prepared with the 2B7B8 (●), 4B6F7 (▼), 5A9H8 (*), and 5AB3A10B4 (□) αLimAbs, injected 4 times with 1:1,000 dilutions of extracts from *L. infantum*–infected (ISE) or noninfected (NiSE) hamster spleens. The curves obtained with the NiSE in the assays with the 4 different αLimAbs were identical and are shown as a single curve (αLimAbs + NiSE). Each point of the curves represents the mean frequency of 2 reaction readings in the same immunosensor.

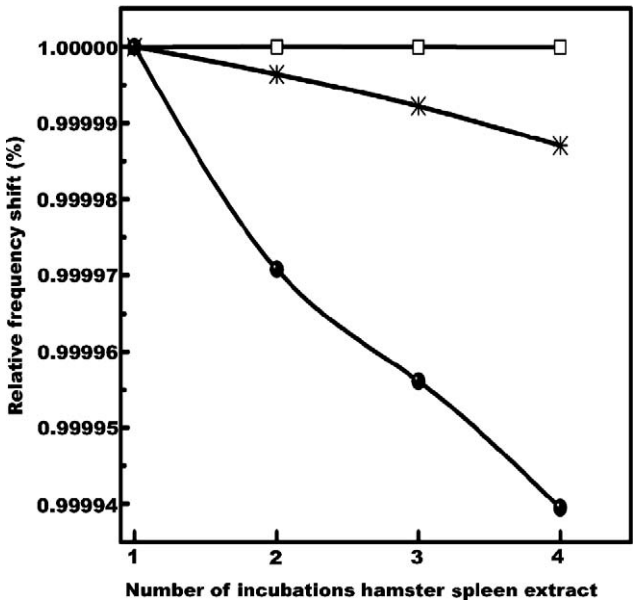

FIGURE 4. Dilution curve of the *Leishmania infantum*–infected hamster spleen extract assayed in an immunosensor prepared with the 2B7B8 αLimAb. *Leishmania infantum*–infected spleen extract was diluted 1:1,000 (●), 1:2,000 (*), and 1:4,000 (□) and applied 4 times onto the surface of an immunosensor prepared with the 2B7B8 αLimAb. Each point of the curves represents the mean frequency of 2 reaction readings in the same immunosensor.

the blood of *L. infantum* and HIV co-infected patients has been reported previously (Orsini et al., 2002; Catorze, 2005). In the case of anthroponotic visceral leishmaniasis, *L. donovani*–reactive mAbs would have to be used in the immunosensor. Due to the strong homology between *L. donovani* and *L. infantum* (Mauricio et al., 2001; Jamjoom et al., 2004), it is possible that the immunosensor whose development is described in the present paper could be used for the diagnosis of anthroponotic visceral leishmaniasis. Although the clinical features of anthroponotic VL are similar to those of AVL, *L. donovani* amastigotes are more frequently found in the blood than *L. infantum*, facilitating their transmission among humans (Chappuis et al., 2007). In addition, their secreted-excreted antigens circulate in the bloodstream and can be detected by antigen-capture methodology (Desjeux, 2004; Singh et al., 2006; Chappuis et al., 2007; Gorski et al., 2010). Thus, the use of immunosensors as described in the present work should be investigated as a diagnostic tool, both in the case of co-infection by *L. infantum* and HIV and of infection by *L. donovani*.

The immunosensor-based assay using αLimAbs that has been described in the present work is a promising tool for the detection of *Leishmania* antigens in infected experimental animals, and it may be useful for the development of a diagnostic method based on antigen detection, of AVL in humans and dogs and of anthroponotic VL.

ACKNOWLEDGMENTS

We thank the Conselho Nacional de Pesquisa e Desenvolvimento-CNPq (project no. 576496/2008), RENORBIO (project no. 3773/05/2007) and CNPq/INCT-CITECS (process no. 57386/2008-9) for financial support for this work. The following institutions provided scholarships to: GCM and JRJ (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior-CAPES); PRSO and GSGB (Fundação de Apoio á Pesquisa e Extensão do Estado da Bahia-FAPESB; LCPC and NMAN (CNPq).

LITERATURE CITED

BRAZ, R. F., E. T. NASCIMENTO, D. R. MARTINS, M. E. WILSON, R. D. PEARSON, S. G. REED, AND S. M. B. JERÔNIMO. 2002. The sensitivity and specificity of *Leishmania chagasi* recombinant K39 antigen in the diagnosis of American visceral leishmaniasis and in differentiating active from subclinical infection. American Journal of Tropical Medicine and Hygiene **67:** 344–348.

BRYCESON, A. D., J. D. CHULAY, AND M. MUGAMBI. 1985. Visceral leishmaniasis unresponsive to antimonial drugs. II. Response to high dosage sodium stibogluconate on prolonged treatment with pentamidine. Transactions of the Society for Tropical Medicine and Hygiene **79:** 705–714.

CATORZE, M. G. B. 2005. Leishmaniasis and AIDS. Medicina Cutánea Ibero-Latino-Americana **33:** 237–250.

CHAPPUIS, F., S. SUNDAR, A. HAILU, H. GHALIB, S. RIJAL, R. W. PEELING, J. ALVAR, AND M. BOELAERT. 2007. Visceral leishmaniasis: What are the needs for diagnosis, treatment and control? Nature Reviews Microbiology **5:** 873–882.

CHU, X., Z. ZI-LONG, S. GUO-LI, AND Y. RU-QIN. 2006. Quartz crystal microbalance immunoassay with dendritic amplification using colloidal gold immunocomplex. Sensors and Actuators B: Chemical **114:** 696–704.

CRUZ, I., C. CHICHARRO, J. NIETO, B. BAILO, C. CAÑAVATE, M. C. FIGUERAS, AND J. ALVAR. 2006. Comparison of new diagnostic tools for management of pediatric Mediterranean visceral leishmaniasis. Journal of Clinical Microbiology **44:** 2343–2347.

DESJEUX, P. 2004. Leishmaniasis: Current situation and new perspectives. Comparative Immunology, Microbiology and Infectious Diseases **27:** 305–318.

DUTRA, R. F., AND L. T. KUBOTA. 2007. An SPR immunosensor for human cardiac troponin T using specific binding avidin to biotin at carboxymethyldextran-modified gold chip. Clinica Chimica Acta **376:** 114–120.

———, R. K. Mendes, V. L. da Silva, and L. T. Kubota. 2007. Surface plasmon resonance immunosensor for human cardiac *troponin* T based on self-assembled monolayer. Journal of Pharmaceutical and Biomedical Analysis **43:** 1744–1750.

Fróes, A. M., C. V. dos Santos, M. L. Penha-Filho, M. C. Teixeira, T. M. Correa Silva, G. G. Oliveira, W. L. dos Santos, L. C. Pontes-de-Carvalho, and N. M. Alcântara-Neves. 2004. Sub-clinical infection as an effective protocol for obtaining anti *Leishmania chagasi* amastigote antibodies of different animal species. Veterinary Immunology and Immunopathology **99:** 135–141.

Gomes, Y. M., M. Paiva Cavalcanti, R. A. Lira, F. G. C. Abath, and L. C. Alves. 2008. Diagnosis of canine visceral leishmaniasis: Biotechnological advances. Veterinary Journal **175:** 45–52.

Gorski, S., S. M. Collin, K. Ritmeijer, K. Keus, F. Gatluak, M. Mueller, and R. N. Davidson. 2010. Visceral leishmaniasis relapse in southern Sudan (1999–2007): A retrospective study of risk factors and trends. PLoS Neglected Tropical Diseases **4:** 705.

Grimaldi Jr., G., and R. B. Tesh. 1993. Leishmaniasis of the New World: Current concepts and implications for future research. Clinical Microbiology **6:** 230–250.

Jamjoom, M. B., R. W. Ashford, P. A. Bates, M. L. Chance, S. J. Kemp, P. C. Watts, and H. A. Noyes. 2004. *Leishmania donovani* is the only cause of visceral leishmaniasis in East Africa; previous descriptions of *L. infantum* and "*L. archibaldi*" from this region are a consequence of convergent evolution in the isoenzyme data. Parasitology **129:** 399–409.

Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. Journal of Biological Chemistry **193:** 265–275.

Luo, J., Q. Zhang, Y. Huang, G. Liu, and R. Zhao. 2007. Quartz crystal microbalance biosensor for recombinant human interferon-gamma-detection based on antisense peptide approach. Analytica Chimica Acta **590:** 91–97.

Maalej, I. A., M. Chenik, H. Louzir, A. Ben Salah, C. Bahloul, F. Amri, and K. Dellagi. 2003. Comparative evaluation of ELISAs based on ten recombinant or purified *Leishmania* antigens for the serodiagnosis of Mediterranean visceral leishmaniasis. American Journal of Tropical Medicine and Hygiene **68:** 312–320.

Maia, C., and L. Campino. 2008. Methods for diagnosis of canine leishmaniasis and immune response to infection. Veterinary Parasitology **158:** 274–287.

Maide, W., J. Guerra, G. Schoone, M. Farenhorst, L. Coelho, W. Faber, I. Peekel, and H. Schallig. 2008. Comparison between quantitative nucleic acid sequence-based amplification, real-time reverse transcriptase PCR, and real-time PCR for quantification of *Leishmania* parasites. Journal of Clinical Microbiology **46:** 73–78.

Marsden, P. D. 1984. Selective primary health care: Strategies for control of disease in the developing world. XIV. Leishmaniasis. Reviews of Infectious Diseases **6:** 736–744.

Marx, K. A. 2003. Quartz crystal microbalance: A useful tool for studying thin polymer films and complex biomolecular systems at the solution-surface interface. Biomacromolecules **4:** 1099–1120.

Mauricio, I. L., M. W. Gaunt, J. R. Stothard, and M. A. Miles. 2001. Genetic typing and phylogeny of the *Leishmania donovani* complex by restriction analysis of PCR amplified gp63 intergenic regions. Parasitology **122:** 393–403.

Orsini, M., M. Silva, Z. M. P. Luz, J. Disch, O. Fernandes, D. Moreira, A. C. M. Guedes, and A. Rabello. 2002. Identification of *Leishmania chagasi* from skin in *Leishmania*/HIV co-infection: A case report. Revista da Sociedade Brasileira de Medicina Tropical **35:** 259–262.

Piazza, R. M., H. F. de Andrade Júnior, E. S. Umezawa, A. M. Katzin, and A. M. Stolf. 1994. In situ immunoassay for the assessment of *Trypanosoma cruzi* interiorization and growth in cultured cells. Acta Tropica **57:** 301–306.

Plomer, M., G. G. Guilbault, and B. Hock. 1992. Development of a PZ immunosensor for detection of enterobacteria. Enzyme and Microbial Technology **14:** 230–235.

Prusak-Sochazewski, E., J. H. T. Luony, and G. G. Guilbault. 1990. Development of a piezoelectric immunosensor for detection of *Salmonella typhimurium*. Enzyme and Microbial Technology **12:** 173–177.

Ramos-Jesus, J., K. A. Carvalho, R. A. S. Fonseca, G. G. S. Oliveira, S. M. Barrouin Melo, N. M. Alcantara-Neves, and R. F. Dutra. 2011. A piezoelectric immunosensor for *Leishmania chagasi* antibodies in canine serum. Analytical and Bioanalytical Chemistry **401:** 917–925.

Reithinger, R., and J. C. Dujardin. 2007. Molecular diagnosis of leishmaniasis: Current status and future applications. Journal of Clinical Microbiology **45:** 21–25.

Romero, G. A. S., and M. Boelaert. 2010. Control of visceral leishmaniasis in Latin America—A systematic review. PLoS Neglected Tropical Diseases **4:** e584.

Rousseau, D., S. Demartino, F. Anjuére, B. Ferrua, K. Fragaki, Y. Le Fichoux, and J. Kubar. 2001. Sustained parasite burden in the spleen of *Leishmania infantum* infected BALB/c mice is accompanied by expression of MCP-1 transcripts and lack of protection against challenge. European Cytokine Network **12:** 340–347.

Singh, R. K., H. P. Pandey, and S. Sundar. 2006. Visceral leishmaniasis (kala-azar): Challenges ahead. Indian Journal of Medicine **123:** 331–344.

Sreenivas, G., N. A. Ansari, R. Singh, B. V. Subba Raju, R. Bhatheja, N. S. Negi, and R. Salotra. 2002. Diagnosis of visceral leishmaniasis: Comparative potential of amastigote antigen, recombinant antigen and PCR. British Journal of Medical Science **59:** 218–222.

Teixeira, M. C. A., R. J. Santos, R. B. Sampaio, L. Pontes-de-Carvalho, and W. L. C. dos-Santos. 2002. A simple and reproducible method to obtain large numbers of axenic amastigotes of different *Leishmania* species. Parasitology **88:** 963–968.

Titus, R. G., M. Marchand, T. Boon, and J. A. Louis. 1985. A limiting dilution assay for quantifying *Leishmania major* in tissues of infected mice. Parasite Immunology **7:** 545–555.