UNIVERSIDADE FEDERAL DA BAHIA
INSTITUTO DE CIÊNCIAS DA SAÚDE
PROGRAMA DE PÓS-GRADUAÇÃO EM IMUNOLOGIA

GUSTAVO CABRAL DE MIRANDA

DISSERTAÇÃO DE MESTRADO

Detection of parasite antigens in *Leishmania infantum* infected spleen tissues by piezoelectric based immunosensors

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Detection of parasite antigens in *Leishmania infantum* infected spleen tissues by piezoelectric based immunosensors

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Dissertação apresentada ao Programa de Pós-graduação em Imunologia, Instituto de Ciências da Saúde, Universidade Federal da Bahia, como requisito para a obtenção do Título de Mestre em Imunologia.

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"Somos jovens, belos, bêbados e caretas...
Sempre em bandos e às vezes em dois...
Curtindo grandes amores, chapados, pirados...
Pelados, olhando as estrelas a espera de carinho e a procura
de um futuro que não chega." (Bob Maley).

Somos o Agora, este é meu momento!

DEDICATÓRIA

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SUMÁRIO

RESUMO 8
ABSTRACT 9
INTRODUCTION 10
MATERIAL AND METHODS 13
Animals 13
Anti-L. infantum monoclonal antibodies(αLiMAbs) 13
Parasites and parasite extracts 14
Extracts of L. infantum-infected hamster spleen 14
Determination of the reactivity and specificity of αLiMAbs 15
Immunosensor assembly and detection the of L. infantum *amastigote antigens in infected-hamster spleen* 16
RESULTS 18
DISCUSSION 19
CONCLUSION 22
REFERENCES 23
FIGURES 30

RESUMO

Doenças como a leishmaniose é uma importante causa de morbidade e mortalidade no Brasil e seus diagnósticos devem ser melhoradas. A produção de anticorpos monoclonbais anti-*L. infantum* e acoplamento ao sensor piezoelétrico, para formar um dispositivo bioeletrônicos capaz de detectar rapidamente antígenos de Leishmania sp, tanto qualitativa como quantitativamente, é uma alternativa promissora para o diagnóstico da leishmaniose, devido à sua alta especificidade e sensibilidade, portabilidade e baixo custo, em comparação aos métodos convencionais. Objetivo: Desenvolver um imunossensor capaz de detectar antígenos de *L. infantum* em tecidos de animais infectados. Material e Método: Quatro hibridomas produtores de anticorpos monoclonais anti-amastigota de *L. infantum* teve sua especificidade confirmada por ELISA. Esses anticorpos foram imobilizados sobre um cristal piesoelétrico de quartzo através de uma fina camada de 2-aminoetanotiol (cisteamina) e glutaraldeído, e submetidos a injeções de extratos de baço de hamster infectados e não-infectados com *L. infantum.* Resultados: Quatro anticorpos (5A9H8, 2B7B8, 4B6F7 e 5AB3A10B4), todos da classe IgG, foram acoplados à superfície do transdutor e foram capazes de reagir contra extrato de baço de hamster infectado com *L. infantum.* A metodologia desenvolvida foi capaz de detectar $3{,}5\times10^{4}$ amastigotas por grama de tecido infectado com os anticorpos 5A9H8, 4B6F7 e 5AB3A10B4, e $1{,}75\times10^{4}$ com o anticorpo 2B7B8. Conclusões: Os resultados demonstraram que este teste pode ser útil para quantificar amastigotas de *L. infantum* em órgãos de animais experimentais para estudos sobre a patogênese da leishmaniose visceral americana e é uma ferramenta promissora para desenvolver um diagnóstico de leismanioses humana e canina.

Palavras Chave: Leishmaniose visceral, diagnóstico, immunossensor, *Leishmania infantum*, anticorpo monoclonal

ABSTRACT

Diseases such as leishmaniases are an important cause of morbidity and mortality in Brazil and their diagnoses need to be improved. The production of monoclonal antibodies has ensured high specificity to immunodiagnosis. The development of an immunosensor, coupling a monoclonal antibody to a bioelectronic device, capable of detecting quickly *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 to conventional methods, without decreasing the sensibility. The present work aim at developing an immunosensor-based assay able to detect *Leishmania infantum* antigens in tissue of infected hosts. Four hybridomas producing monoclonal antibodies against *L. infantum* had their specificity confirmed by ELISA. These antibodies were immobilized on a gold surface, covered with a thin film of 2-aminoethanethiol (cysteamine) and glutaraldehyde, blocked with glycine and put into contact with extracts of *L. infantum*-infected and non-infected hamster spleens. All four IgG antibodies coupled to the transducer surface reacted against the extract of the spleen of *L. infantum*-infected hamster. The developed assay was able to detect $1.75x10^{4}$ amastigotes per gram of infected tissue. These results demonstrated that this assay may be useful to quantify *L. infantum* amastigotes in organs of experimental animals for studies on pathogenesis and immunity, and is a promising tool for the development of a diagnostic method, based on antigen detection, of human and dog visceral leishmaniases.

Keywords:

Visceral leishmaniases, diagnosis, immunosensor, *Leishmania infantum*, monoclonal antibodies

1. INTRODUCTION

Visceral leishmaniases (VL) are endemic in 62 countries and 200 million people are at risk of acquiring these diseases. About 90% of the cases are concentrated in five countries: Bangladesh, India, Nepal, Sudan and Brazil (Desjeux, 2004). VL are mainly caused by two species of the protozoan genus *Leishmania*: *Leishmania donovani*, which causes anthroponotic VL in the Old World, and *Leishmania infantum* (syn.to *Leishmania chagasi*), which causes zoonotic VL in both Old and New World (Desjeux, 2004). The VL in the New World has the dog as its main reservoir and is also called american visceral leishmaniasis (AVL) (Grimaldi & Tesh, 1993; Herwaldt, 1999; Romero & Boelaert, 2010). AVL has not been dealt successfully by the public health services in many of the affected countries, causing high morbidity and death when not treated timely and appropriately (Grimaldi & Tesh, 1993).

The diagnosis of AVL is usually performed by serology (IFA and/or ELISA) or associated with skin sensitivity reaction, but, as none of these method has yet shown good specificity (Reithinger & Dujardin, 2007), the diagnosis is only conclusive with the identification of amastigote forms of *L. infantum* in smears of bone marrow (usually in human beings) and spleen (usually in dogs) aspirates. 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 leishmaniaseshave been related to the use of recombinant proteins as antigens (Braz et al., 2002; Sreenivaset al., 2002; Maalejet 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 the increase of antibodies may relate to past or asymptomatic infections (Maia & 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 leishmaniases may potentially arise from the detection of parasite DNA by the polymerase chain reaction, but the carrying out of this technique requires a specialized technician, above-83 average laboratory facilities and is a relatively time-consuming method (Cruz et al., 2006; Meide et al., 2008).

An immunosensor is a device comprising an antigen or antibody species coupled to a signal transducer, which detects the binding of the complementary species. Biosensor based on piezoelectric transducers can replace commonly used tests, decreasing time of performance and price, using serum and tissue samples. The quartz crystal microbalance (QCM) piezosensors are considered excellent for on line detection of immunoreactions, in that changes in the electrode/electrolyte interface are of a sufficient magnitude to reveal the presence of the desired adsorbed analytes by changes on the electrical frequency (Xia Chu et al., 2006). The principle of operation of the QCM biosensor depends on the piezoelectric effect that is governing by relationships between mass and frequency variations (Luo et al., 2007). The selectivity of this immunosensor is achieved by the proper choice of the chemically modified surface and immobilized biocomponents on the electrode surface (Marx, 2003; Dutra & Kubota, 2007). Self assembled monolayers (SAMs) using alkanethiol films have been presented as adequate structuring for the antibodies immobilizations (Dutra et al., 2007b). These QCM sensors can be easily packaged for routine use as portable unit. The present work aim 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 (*lato sensu*) diagnosis of human and canine visceral leishmaniases.

2. MATERIALS AND METHODS

2.1 Animals

Pathogen-free, 6-12-week-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 Committe e for Animal Care and Utilization (Protocol n° 036/2009) of the FIOCRUZ-BA.

2.2 Anti-L. infantum monoclonal antibodies(αLiMAbs)

αLiMAbs-producing hybridomas, previously obtained by our team (Fróes et al., 2004), were cultured and expanded in Iscove's medium (Iscove's Modified Dulbecco's Medium, GIBCO, Grand Island, NY, USA), containing 10% fetal calf serum (FCS), 100 µg/mL glutamine, and 50 µg/mL gentamicin. The isotypes of the monoclonal antibodies (5A9H8, 2B7B8, 4B6F7, 5AB3A10B4), present in supernatants of hybridoma cultures, were determined by capture ELISA, using the Mono-Ab-ID kit (Invitrogen, Carlsbad, CA, USA), according to manufacturer's recommendations. Large amounts of these antibodies were produced by inoculation of the hybrid cells into the peritoneal cavity of BALB/c mice, previously inoculated with pristine (2,6,10,14-tetramethylpentadecane; Sigma-Aldrich Chemical Co., St. Louis, MO, USA) intraperitoneally. The antibodies were semi-purified from the ascitic fluid by precipitation with ammonium sulphate, pH 7.0, followed by dialysis against phosphate buffered saline, pH 7.4 (PBS). The semi-purified antibodies were assayed for protein content by Lowry's method (Lowry et al., 1951), aliquoted and stored at -70o C until use.

2.3 Parasites and parasite extracts

L. infantum/L. chagasi (MHOM/BR2000/Merivaldo strain) promastigotes were obtained from *in vitro* cultures of homogenates of infected-hamster spleens, in Schneider´s medium supplemented with 10% fetal calf serum (FCS; GIBCO, Grand Island, NY, EUA) and 50 µg/mL gentamicin at 25o 139 C. Part of the promastigotes was washed by three centrifugations (1,620 x g; 10 minutes) in cold PBS, counted in a Neubauer's chamber and stored at -70o141 C for antigen 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 during 10-13 days, in accordance with Teixeira and collaborators (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 cell (MK2) cells in the presence of RPMI-1640 (GIBCO, Grand Island, NY, EUA) containing 10% FCS 148 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, CT, USA) in the presence of PBS and centrifuged (14,000 x g, 10 minutes). The supernatants were filtered through a membrane of 0.45 m diameter-pore size and stored at -70°C until use. The protein content was measured by Lowry's method (Lowry et. al., 1951).

2.4 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 three month old

golden hamster. After 60 to 90 days, the animal was euthanized, the spleen was weighed and a fragment of 200 milligrams was homogenized and cultured in Schneider's medium containing 20% FCS and 50 µg/mL gentamicin, to estimate the parasite load of the spleen, through a limiting dilution assay (Titus et al., 1985). The remaining infected organ fragment (400 mg) was homogenized in PBS containing 1% NP40 (Sigma-Aldrich Chemical Co., St. Louis, MO, USA) and protease inhibitors mix (Amersham, São Paulo, SP, Brazil). The tissue was then sonicated, incubated at 4° C for 30 minutes, centrifuged (14,000 x g, 10 minutes), filtered through a membrane with 0.45 □m-diameter pore, aliquoted and stored at -70° C until use. Its protein containing was measured by the Lowry method (Lowry et. al., 1951). Spleen of a non-infected hamster was subjected to the same procedure and used as a negative control.

2.5 Determination of the reactivity and specificity of α*LiMAbs*

The reactivities of the αLiMAbs were determined by sandwich ELISA using an extract of *L. infantum*-infected hamster spleen. Briefly, wells of a microtiter plate were coated with 30 µg/mL of each semi-purified monoclonal antibody, diluted in 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 spleen extract were added to the wells, diluted at 1:125 in PBS-SM. The reaction was 181 detected by using a *L. infantum* infected dog serum, diluted at 1:200 in PBS-SM, followed by an anti-dog immunoglobulin antibody - peroxidase conjugate, diluted at 1:1000, followed by a solution of 3,3',5,5'-tetramethylbenzidine (TMB) with hydrogen peroxide (Sigma-Aldrich Chemical Co., St. Louis, MO, USA) as substrate. Between all the steps, the plate was incubated for 1 hour at room temperature (except for the substrate which was

incubated for 20 minutes) and washed four times with PBS-SM containing 0.05% Tween 20 (PBS-SM-T20). The reactions were read in a spectrophotometer at 450nm.

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*). Briefly, parasite extracts, obtained as described above, were used at a concentration of 100 µg of protein/mL to coat the wells of microtiter plates. Non-specific 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 1:2000. Substrate used, washings and reading were done as described above.

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

Four µg/mL of αLiMAbs (5A9H8, 4B6F7, 2B7B8 and 5AB3A10B4) were applied to quartz crystal electrode (QMC of 9 MHz; Maxtec Inc, Salt Lake, Utah, USA), which 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 Autolab, PGstat12, The Netherlands) which 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 self assembled monolayer (SAM), with 2-aminoethanethiol (cysteamine), followed by covalent binding with glutaraldehyde (Figure 1). Remaining unbound aldehyde groups were blocked with glycine. The immunosensor assembly was carried out at room temperature with the following incubation steps: cysteamine (2 hours), glutaraldehyde

(45 minutes), antibodies and blocking (2 hours each). Following the *L. infantum* containing spleen and the negative 214 control spleen extracts, diluted 1:1000 and 1:2000, were injected in the immunosensor surface and incubated for 15 minutes. Between all steps three washings were done with PBS followed by two readings of the frequencies. The sample was considered positive when its application on the surface of the crystal produced a reduction in the oscillation movement, which in its turn resulted in decreased resonance frequencies. The measurements of the frequency in real time permit to monitor the interfacial phenomena that occur by the antigen binding to specific antibodies immobilized on the electrode surface.

3. RESULTS

The αLimAbs were isotyped as IgG1 (5AB3A10B4), IgG2a (5A9H8 and 4B6F7), and IgG3 (2B7B8) (data not shown), and all reacted with the extract from amastigote containing hamster spleen (Figure 2). However, they did not react with: *L. infantum* axenic amastigote and promastigote extracts or *T. cruzi* amastigote and trypomastigote extracts (Figure 2). The *L. infantum*-infected hamster spleen contained 4.9x109 amastigotes per gram, and the spleen extract contained 3.5 x 107 amastigotes per mL. Consecutive injections of 200µL of this extract diluted 1:1000, led to a progressive decrease in the resonance frequencies of the immunosensors coated with any of the four monoclonal antibodies (Figure 3). In contrast, injections of the non-infected hamster spleen extract had no effect on the frequencies, which remained stables (Figure 3).

A comparison among the assays performed with the different αLiMAbs, plotting the results of the injection of the *L. infantum*-containing spleen extract and of the control extract, and having as the starting point the same frequency, were done using the ORIGIN software version 8.0, (Microsoft, USA). The linear fit to obtain the power of connection, or decay, showed that the assay with the best signal was the one that used the 2B7B8 αLimAb (Figure 4). The assay using the 2B7B8 monoclonal antibody produced a positive result with a 1:2000 dilution of the *Leishmania*-containing extract (Figure 5), which corresponded to an undiluted extract of a spleen containing 1.75x104 amastigotes per gram of infected tissue.

4. DISCUSSION

The development of diagnostic 247 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 anti-*L. infantum* amastigote monoclonal antibodies, and its application for the detection of *Leishmania* 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).

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 cut signals when put into contact with extracts of a spleen containing *L. infantum* amastigotes. In recent decades, the QCM biosensors have found various applications in diagnosis, 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 fast obtaining of results (Prusak-Sorechazewski et al., 1990; Plomer et al., 1992; Xia Chu et al., 2006). The monoclonal antibodies used here are specific for conventional *L. Infantum* amastigotes, since they did not react with promastigote and

axenic amastigote extracts of this parasite, nor with *T. cruzi* amastigote and trypomastigote extracts. Although, as it is, the *L. infantum* antigen-reactive immunosensor described in this report can be of great clinical importance, it would be interesting to find out whether the αLiMAbs recognize other *Leishmania* species,

particularly *L. donovani* amastigotes, since this would extend their use to other important endemic areas of visceral leishmaniasis, 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, which is usually carried out to confirm the diagnosis of VL and need a highly skilled technician (Chappuis et al., 2007).

The developed assay is already potentially useful for quantifying amastigotes in studies on experimental animals, such as those on pathogenicity and on assessing vaccination procedures. However, an improvement in the sensitivity of the immunosensor is highly desirable. One way to achieve this is to increase the QMC binding area for the biological component. This 280 will allow an increase in the amount of antigen monoclonal antibody complex due to the increase in the amount of the solid phase monoclonal antibody in the immunosensor, increasing the assay sensitivity. An additional possibility is to test whether coating the QMC with more than one monoclonal antibody would also increase the assay sensitivity. Still another possibility is to change the glutaraldehyde, which does not bind specifically to the NH2 residues in the Fc region of the antibody, and therefore may interfere with the antibody antigen binding region, for a molecule directed to the carboxyl residue of the Fc region. This would increase the amount of operationally active antibody in the immunosensor. Future investigation aimed at decreasing the assay costs would also be desirable. The assay may also be useful in the following cases: (a) 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; (b) detection of amastigote antigens in

the blood of *L. infantum* and HIV co-infected patients; (c) 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 the blood of *L. infantum* and HIV co-infected patients have been reported (Orsini et al., 2002; Catorze, 2005). In the case of anthroponotic VL, *L. donovani*-reactive monoclonal antibodies would have to be used in the immunosensor. Due to the great homology between *L. donovani* and *L. infantum* (Mauricio et al., 2001; Jamjoom et al., 2004), it is possible that the immunosensor whose development was described in the present paper could be used for the diagnosis of anthroponotic VL. 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 from man to man (Chappuis et al., 2007). In addition, their secreted306 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 the one 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 anti-*L. infantum* amastigote monoclonal antibodies that has been described in the present work is a promising tool for the detection of *Leishmania* antigens in infected experimental 313 animals and it may be useful for the development of a diagnosis method based on antigen detection, of AVL in humans and dogs and of anthroponotic VL.

5. CONCLUSION

In this work we developed an immunosensor based on anti-*L. chagasi* amastigote monoclonal antibodies which will be a promising toll for the detection of this parasite antigens in infected experimental animals and it may be useful for developing an antigen-based diagnosis method of American visceral leishmaniasis in humans and dogs.

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7. FIGURES

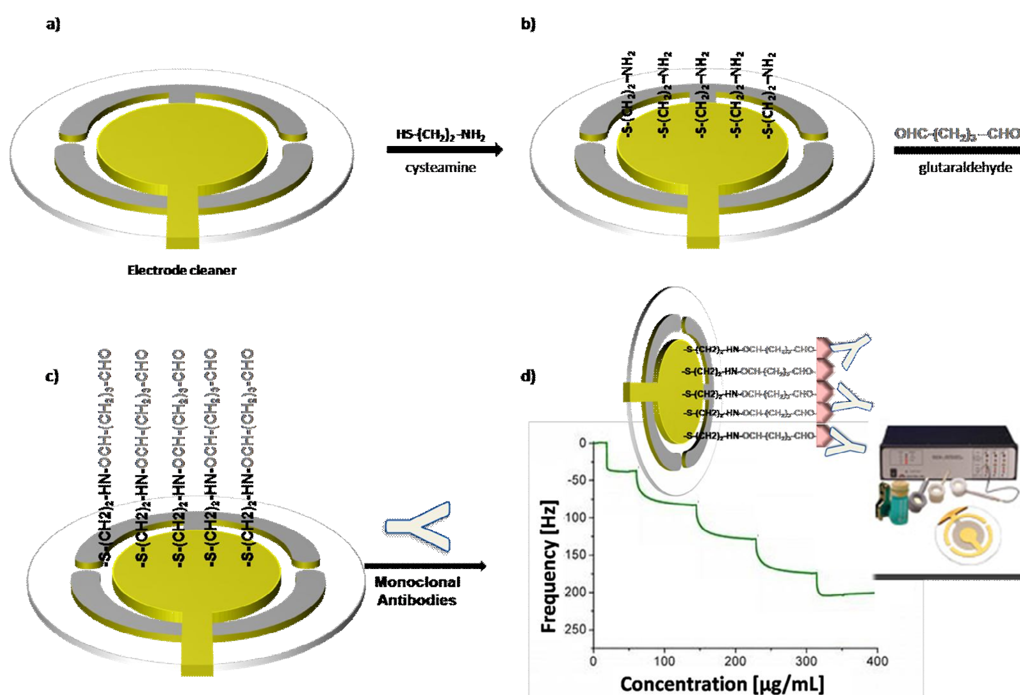

Figure 1. Schematic figure of the Christian arrangement of piezoelectric quartz and the process of antibody immobilization. (**a**) The quartz crystal electrode, a disk made of piezoelectric quartz crystal coated with a thin film of gold. To bind the antibodies on the electrode, the gold surface was covered by cysteamine (**b**), followed by covalent binding of glutaraldehyde (**c**). (**d**) The measurements of the frequency in real time permit to monitor the interfacial phenomena that occur by the antigen binding to specific antibodies immobilized on the electrode surface. Figure adapted from de Jesus et al., *in press*.

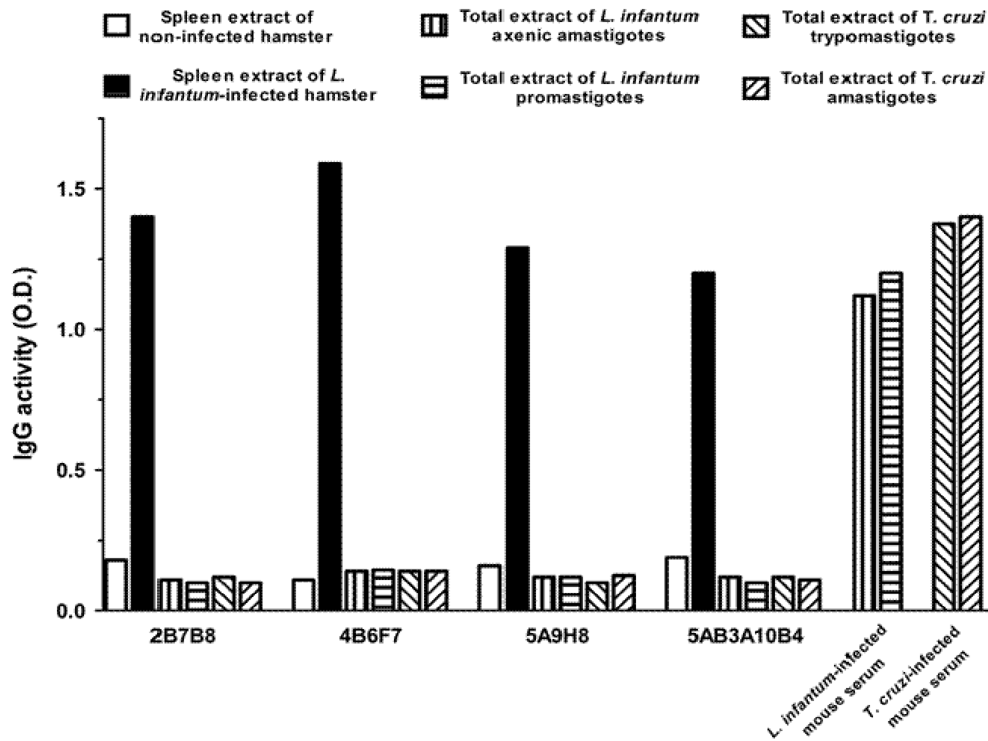

Figure 2. Reactivity of anti-*Leishmania infantum* amastigotes monoclonal antibodies against non-specific antigens by ELISA. To analyze the specific reaction against *L. infantum* tecidual amastigotes, the wells were coated with the 4B6F7, 5A9H8, 2B7B8, or 5AB3A10B4 monoclonal antibodies. The black columns represent the results obtained from hamster spleen extracts containing *L. infantum* amastigotes (prepared from the spleen of infected hamsters), and the white columns represent the results obtained from a control extract prepared from a non-infected hamster spleen. The reaction was developed using *L. infantum*-infected dog antibodies, an anti-dog immunoglobulin - peroxidase conjugate, and a chromogen-substrate mixture, as explained in the Material and Methods. To analyze the reactions against non-specific antigens, the wells were sensitized with: *L. infantum* axenic amastigotes or promastigotes; *Trypanosoma cruzi* amastigotes or trypomastigotes. The reaction was developed using 2B7B8, 4B6F7, 5A9H8 or 5AB3A10B4. *L. infantum*- or T. cruzi-infected mouse sera were used as inner controls.

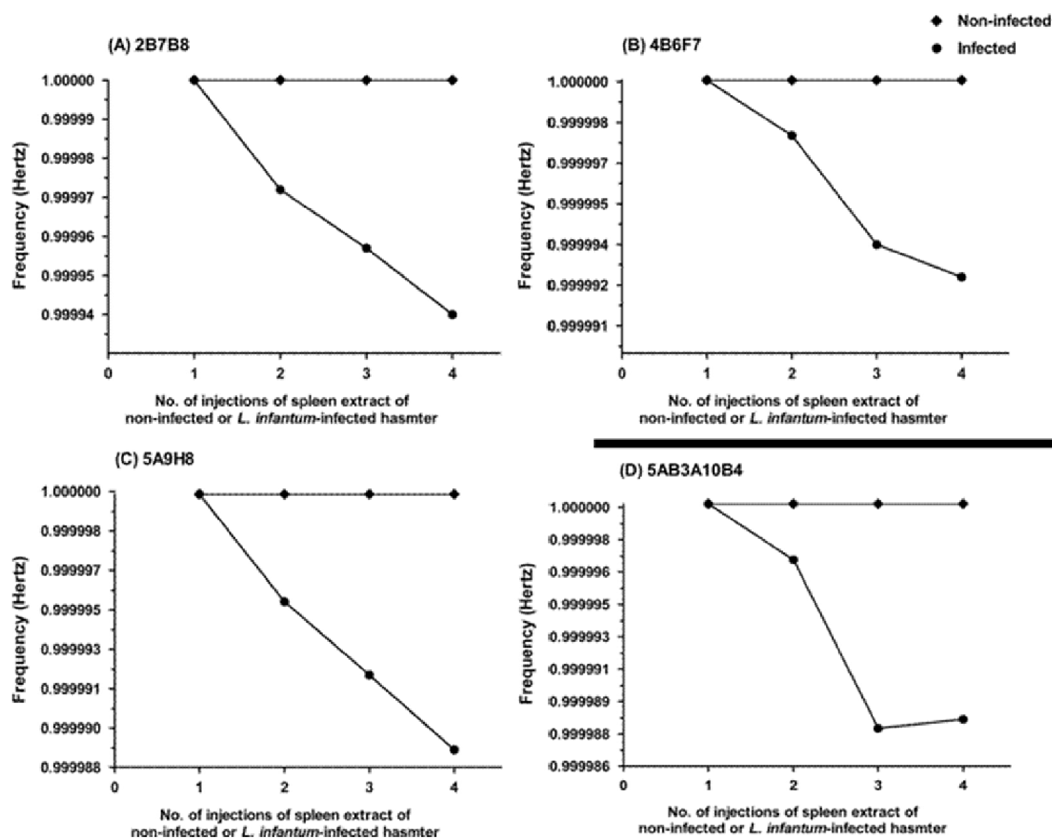

Figure 3. Reaction of anti-*Leishmania infantum* monoclonal antibodies (αLiMAb) against extracts of spleens from *L. infantum*-infected or non-infected hamsters in piezoelectric technology-based immunosensor. Four injections of 1/1000 dilutions of spleen extracts were inoculated on the immunosensor surface pre-coated with the 17 2B7B8 (**A**), 4B6F7 (**B**), 5A9H8 492 (**C**), and 5AB3A10B4 (**D**) **(αLiMAb)** until the immunosensor frequency was stabilized.

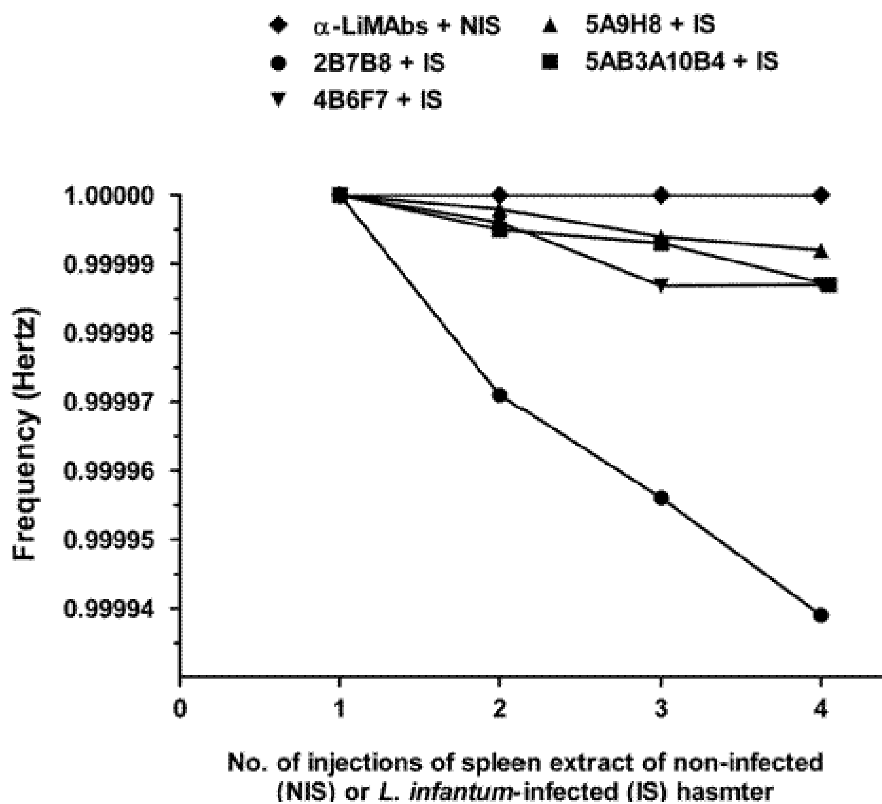

Figure 4. Signal strengths conferred by different anti-*Leishmania infantum* monoclonal antibodies (αLiMAbs) in piezoelectric technology-based immunosensors. The lines correspond to data obtained by (**A**) 2B7B8, (**B**) 4B6F7, (**C**) 5A9H8, and (**D**) 5AB3A10B4 **αLiMAbs**, against extracts of spleens from *L. infantum* infected (IS) or non-infected (NIS) hamsters. The data were plotted using the software OriginPro8.

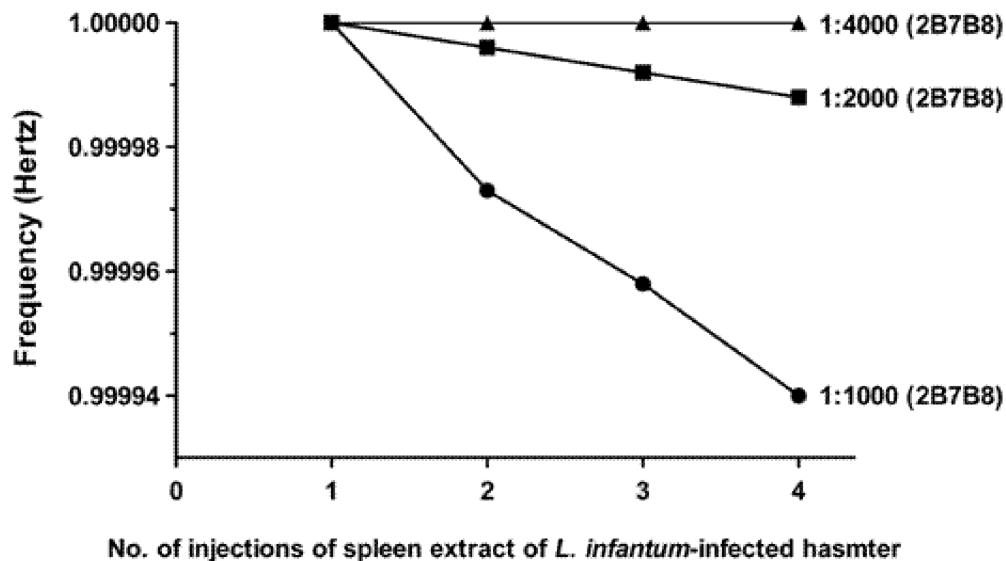

Figure 5. Dilution curve of the hamster-infected spleen extract used to react against 2B7B8 αLiMAbs. The hamster-infected spleen extract was diluted to 1:1000, 1:2000 and 1:4000 and applied four times on the immunosensor surface pre-coated with the 2B7B8 α**LiMAbs**. The data were plotted using the software OriginPro8.