Talanta 195 (2019) 327–332

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Label-free electrochemical impedance immunosensor based on modified screen-printed gold electrodes for the diagnosis of canine visceral leishmaniasis

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ARTICLE INFO

Keywords:
Leishmania infantum
Visceral leishmaniasis
Immunosensor
Sensitivity
Biosensor
Electrochemical impedance spectroscopy

ABSTRACT

Leishmaniasis is a disease with high impact on public health in many countries. Visceral leishmaniasis (VL) is a vectorial zoonosis, with dogs as primary reservoirs in the domestic environment. VL presents high morbidity, mortality and importance in epidemiology in the American continent. In the present study, the first label-free electrochemical impedance immunosensor using screen-printed electrodes (SPEs) for the detection of anti-*Leishmania infantum* antibodies was developed. The soluble antigens of L. *infantum* were immobilized on an SPE by a 3-mercaptopropionic acid monolayer. Electrochemical impedance spectroscopy (EIS) was used for detecting bimolecular interactions occurring at the electrode surface. The addition of real samples consisting of canine and human sera positive and negative for VL presented high sensitivity and selectivity through EIS. Based on the results, a sensitive, specific, rapid and simple immunosensor was developed successfully with potential application for the serological diagnosis of leishmaniasis disease.

1. Introduction

Visceral leishmaniasis (VL) is a worrying infection with large impact in the public health system of many countries worldwide. The disease is caused by the *Leishmania infantum* protozoa, with significant incidence in Brazil, Ethiopia, India, Kenya, Somalia, South Sudan and Sudan [1,2]. The World Health Organization (WHO) estimates that 700,000–1 million new cases of VL and 20,000–30,000 deaths occur worldwide each year [3].

Although this zoonotic infection is considered a type of rural disease, there has been a notable increase in urban centers, with important impacts on public health and public economy in America, especially in South America [4,5]. Dogs represent the major reservoirs in the domestic environment, as main source of infection for shadflies, favoring the spread of the parasite between animals and humans [6–8]. Around 40–60% of infected canines have the asymptomatic form of the infection, making clinical diagnosis difficult [9]. In Brazil, serological screening is performed through chromatographic immunoassay Dual Path Platform (DPP) test and the reagent samples are confirmed by enzyme-linked immunosorbent assay (ELISA) [10–12]. However, several studies have shown that the DPP technique presents low sensitivity [13].

Although serological methods have a wide application in laboratory diagnostics, the cross-reaction with trypanosomatides, low sensitivity in detecting asymptomatic cases and difficult procedures to apply in endemic areas are the main limitations of these techniques. Moreover, the techniques require a long time of operation, which limits the application for a large number of serum samples, besides the cross-reaction with antibodies against other pathogens [14].

In this way, the possibility of a miniaturized system with low cost and fast application allowing control in remote and in field areas is desired, and in this work, we have evaluated the use of electrochemical impedance spectroscopy (EIS) as a tool for the development of a diagnostic method fulfilling these requirements [15,16]. The immunosensor

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https://doi.org/10.1016/j.talanta.2018.11.087
Received 5 August 2018; Received in revised form 22 November 2018; Accepted 22 November 2018
Available online 23 November 2018
0039-9140/

is a great system for the detection of positive serum for many infections described in the scientific literature. The potential application of electrochemical transducers for point-of-care testing (POCT) is well known because of its high selectivity, high sensitivity, the possibility of miniaturization, low cost and results from a simple operation interface [17].

With the recent development of miniaturized electrodes, such as screen-printed electrodes (SPEs), electrochemistry has become increasingly accessible for POCT applications [18]. In this context, the literature already presents some successful applications, such as: a label-free impedimetric immunosensor for detection of a biomarker of cancer [19]; EIS to detect *Escherichia coli* [20]; an electrochemical immunosensor for detection of avian leukosis virus subgroup J [21]; a platform for the impedimetric transduction for Chagas disease [22]; and a genosensor for Leishmania major [23].

Taking into account the need for advances in diagnosis of canine leishmaniasis, this work shows the development of a low-cost platform for the detection of anti-*Leishmania infantum* antibodies in canine clinical serum and real blood samples through a label-free electrochemical impedance method.

2. Materials and methods

2.1. Reagents and chemicals

3-Mercaptopropionic acid (MPA), *N*-hydroxysuccinimide (NHS), *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimidehydrochloride (EDC), 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES), potassium ferrocyanide and potassium ferricyanide (99%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium monohydrogen phosphate and sodium dihydrogen phosphate were purchased from Labsynth (São Paulo, Brazil). Ethylenediamine tetra-acetic acid (EDTA), KCl, NaCl and HCl were purchased from Isofar (Rio de Janeiro, Brazil). Tween 20 (surfactant P20) 0.005% and KOH were obtained from NEON (São Paulo, Brazil). Ethanol (99.5%) was purchased from Dinâmica® Química (São Paulo, Brazil). Sulfuric acid (ACS grade) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

HEPES buffer solution – (HBS-EP) pH 7.4 buffer solutions were prepared by mixing solutions of 10 mmol L^{-1} HEPES pH 7.4, 3.0 mmol L^{-1} EDTA pH 8.0, 150 mmol L^{-1} NaCl and 0.005% surfactant P20. The MPA ethanolic solution was prepared freshly before using. All aqueous solutions were prepared with water purified in a Milli-Q system and all chemicals were of analytical grade and used as received.

2.2. Apparatus

Cyclic voltammetry (CV) and EIS were performed with an AUTOLAB Potentiostat model PGSTAT204 equipped with impedance module FRA32M coupled to a PC microcomputer with NOVA 1.10 software.

The impedance data were analyzed by NOVA 1.10 software. A sinusoidal potential modulation of 10 mV amplitude was superimposed on the open circuit potential (OCP) of the system. The amplitude and phase angle of the resulting current were recorded at frequencies ranging from 100 kHz to 10 mHz.

Screen-printed gold electrodes (SPE-Au), model DRP-220AT (Lot. E0016560) were purchased from DropSens® (Llanera, Asturias, Spain). Each chip had a gold working electrode, a gold counter electrode and a silver reference electrode fixed onto a ceramic base.

2.3. Production of soluble antigens of Leishmania infantum

The soluble antigens used in this work were obtained from the Federal University of Ouro Preto (UFOP – Brazil), that used promastigotes from lysate "*in house*" method with protocol and patent deposited in the National Institute of Intellectual Property (INPI) PI0605889-2.

The promastigote forms were cultivated in a *Liver Infusion Tryptose* (LIT) medium in a stationary phase. Then, the parasites were washed three times with phosphate buffer solution (PBS) pH 7.20 using centrifugation 600*g* for 10 min. The parasites were lysed in a cold bath using a 40 W Sonifier® Cell Disruptor (Brason Sonic Power Co, EUA). Then, a new centrifugation was conducted in a Jouan BR4 multifunction refrigerated centrifuge (Thermo Electron Corporation) at 37,000*g* for 90 min at 4.0 °C. The supernatant was dialyzed using PBS pH 7.20 for 24 h at 4.0 °C under agitation. Finally, the rest of material in the dialysis bag was filtered in 0.22 μm disposable filters (Millipore co. Massachusetts, EUA), and one sample was taken to adjust protein concentration to the value of 1000 μg mL^{-1}. After the confirmation of positive and negative immunological reaction using the ELISA method, the serum was stored at − 80.0 °C.

2.4. Canine clinical serum samples

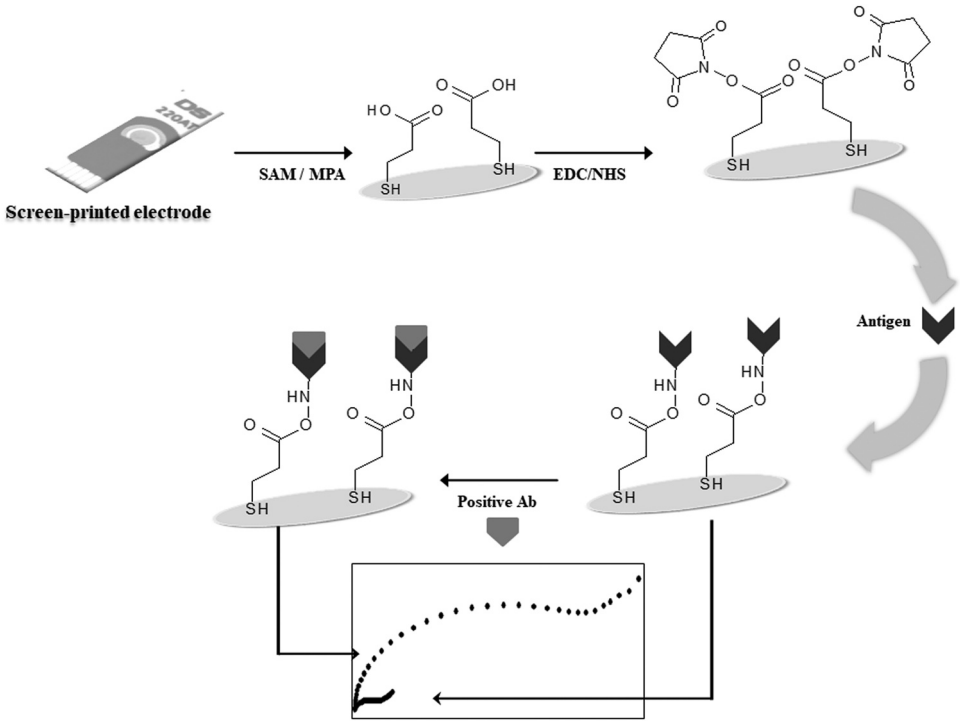
The positive canine clinical serum samples were obtained from infected dogs in endemic areas. The samples were tested through the ELISA and indirect immunofluorescent-antibody (IFA) methods. All samples testing positive were selected. The Secretary of Health in city Belo Horizonte, Minas Gerais (MG), Brazil, initially performed the diagnoses during a serological survey of canine VL. The samples were sent to the Laboratory of Immunopathology, Federal University of Ouro Preto (UFOP), Ouro Preto – MG, Brazil and the diagnoses were confirmed through the serological assays. In addition, clinical sera from healthy mongrel dogs housed in non-endemic regions (Animal Center of the UFOP) were used as control. The sample collection was approved by the ethics committee in research UFOP, Project no 2007/83.

2.5. The formation of MPA/SAM on SPE-Au surface and antigen immobilization

Immediately before use, an electrochemical pretreatment using sulfuric acid was performed on the SPE-Au. The electrode was washed copiously with ethanol, then immersed in an ethanolic solution of MPA (1.0 mmol L^{-1}) for 24 h. After SAM formation, the electrode was rinsed several times with ethanol and dried with a pure N_2 stream. The SAM was activated by introducing NHS–EDC reagent (aqueous solution of 150 mmol L^{-1} NHS and 100 mmol L^{-1} EDC) for approximately 10 min. At room temperature (25 ± 2 °C) in non-humid conditions, the activated surfaces were covered with a volume of 20 μL of L. *infantum* antigens in concentrations of 12.5, 25, 50 and 100 μg mL^{-1} for 30 min. Then, the immunosensor was thoroughly rinsed with HBS-EP buffer at pH 7.4 to remove the excess unbonded material. Saturation of the electrode surface was investigated using bovine serum albumin (BSA) in order to prevent nonspecific binding, but no significant changes were observed in the biosensor response, probably because the SAM itself acts as a protective layer. Therefore, BSA was not used.

2.6. Detection of the immunoreaction between soluble antigens of L. infantum and anti-L. infantum antibodies

The diagnostic capability of the developed immunosensor was evaluated with 12 canine sera: half negative and half positive for VL. All dogs were diagnosed based on parasitological and serological results. Canine sera were used for preparing a pool of animals both positive and negative to VL. Sera were prepared with different dilutions (1:40, 1:80, 1:160, 1:320, 1:640 and 1:1280) in HBS-EP buffer at pH 7.4. For each dilution factor mentioned, 20 μL was dropped over the immunosensor surface and all measurements obtained in triplicate. The immunoreactions between immobilized antigen and antibody (the association phase) was studied, at room temperature (25 ± 2 °C), for different periods (10, 20, 30 and 60 min). The fabrication process of the immunosensor is presented in Scheme 1.



Scheme 1. Fabrication protocol of the impedimetric immunosensor.

3. Results and discussion

3.1. Electrochemical characterization of the modified electrode

Modification of the electrode surface is an important step that determines the future immunosensor performance. CV and EIS techniques were used to characterize the SAM formed by the absorption on the gold substrate. The redox couple $Fe(CN)_6^{3-/4-}$ was used as anionic electrochemical probe for examining the integrity of SAM on gold [24].

Fig. 1A shows the voltammograms for the SPE-Au (curve 1) and SAM/SPE-Au (curve 2). There are two well-defined peaks characteristic for the redox couple observed for the bare gold electrode, indicating that the electron transfer reaction is completely diffusion controlled. On the other hand, the modified electrode resulted in a decrease in the peak current and an increase in the separation of the peak potentials (curve 2).

EIS is one of the very sensitive techniques used for characterization of surfaces and also for detecting bimolecular interactions occurring at the surface of an electrode [25–27]. Fig. 1B shows the Nyquist plots for the unmodified and modified SPE-Au electrodes in the presence of the redox probe. The data was simulated by two different equivalent circuits (Fig. 1B, insert), depending on the extent of surface modification. A Randles circuit (Fig. 1B, insert 1) was successfully applied to fit the initial modification of the bare SPE-Au. After SAM formation, the layer became thicker and the reaction slowed, so another equivalent circuit was proposed (Fig. 1B, insert 2).

The SPE-Au electrode exhibits a very small semicircular domain. The obtained simulation shows low resistance of charge transfer (R_{ct} = 4.8 ± 0.8 Ω) suggesting a fast charge transfer between redox probe and electrode. After immobilization of the SAM, the value of R_{ct} increased to 19.9 ± 2.0 Ω, which indicated the successful SPE modification by blocking of the interfacial charge transfer. Solid lines were obtained by fitting, using the equivalent circuit insert in Fig. 1B. The obtained results of EIS and CV showed strong evidence of the formation of MPA/SAM on the surface of the SPE-Au.

3.2. Evaluation and optimization of L. infantum antigen immobilization

Fig. 2A shows the Nyquist plots of the SAM/SPE-Au and the L. *infantum* antigen immobilized on the modified electrode (antigen/MPA/SPE-Au) in the presence of the redox probe. The EIS data were simulated with a modified Randles circuit (2) shown in the insert of Fig. 1B.

EIS conducted with the electrochemical solution $Fe(CN)_6^{3-/4-}$ provides high sensitivity. Therefore, we used the changes in the charge

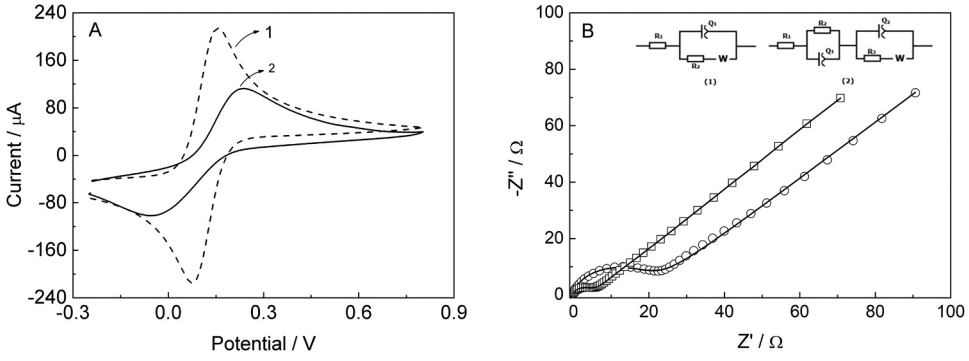


Fig. 1. (A) Cyclic voltammograms obtained for bare SPE-Au (curve 1) and MPA/SPE-Au (curve 2) in aqueous solution containing $Fe(CN)_6^{3-/4-}$ (1.0 mmol L^{-1}) and KCl (0.1 mol L^{-1}) at scan rate of 50 mV s^{-1}. (B) Nyquist plot of impedance spectra for SPE-Au (□) and MPA/SPE-Au (○). Insert: Equivalent circuits used for simulation of the experimental data, where (1) was used for SPE-Au and (2) for MPA/SPE-Au.

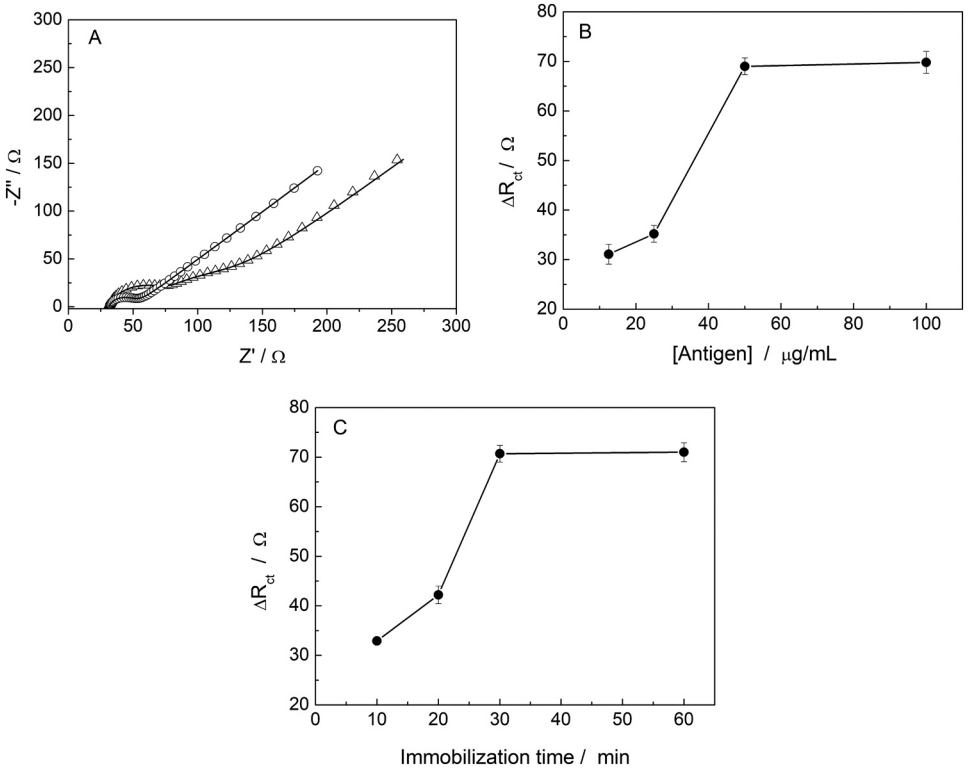


Fig. 2. (A) Nyquist plots (−Z" *vs* Z′) of SAM/SPE-Au (○) and Antigen/SAM/SPE-Au (Δ). (B) The dependence of ΔR_{ct} of the fabricated immunosensor on the concentration of antigen (immobilization time of 30 min). (C) The dependence of ΔR_{ct} as a function of the immobilization time using 50 μg mL^{-1} of the antigen over the SAM/SPE-Au. All measurements were carried in KCl solution (0.1 mol L^{-1}) containing $Fe(CN)_6^{3-/4-}$ (1.0 mmol L^{-1}).

transfer resistance (R_{ct}) to detect immobilization of the antigen on the electrode surface. The simulated curve for the antigen/SAM/SPE-Au exhibits a moderate resistance and the successive assembly of the L. *infantum* antigen on SAM increased the R_{ct} to 72.9 ± 1.9 Ω, which indicated successful immobilization of the antigen.

The L. *infantum* antigen concentration was optimized. Fig. 2B shows the dependence of the ΔR_{ct} values with an antigen concentration increase from 12.5 to 100 μg mL^{-1}. The sensor presented increased response with the concentration increase until 50 μg mL^{-1}, after which no major changes were observed. This observation suggests that the surface coverage is saturated when the concentration of antigen is higher than this value. Moreover, each immobilization step was followed by washing to remove the non-adsorbed antigen, thus ensuring reproducibility. As the procedure used the activated carboxylic acid to react specifically with the antigen, the antigen immobilization is limited to the availability of this activated functional group. Therefore, a 50 μg mL^{-1} antigen concentration in HBS-EP was chosen for further experiments. Optimization of the system was conducted by the evaluation of different immobilization times (10, 20, 30 and 60 min). Fig. 2C shows that the ΔR_{ct} increased with an increase in incubation time to 30 min. This suggests that the binding reaction proceeds quickly and the binding reaction is completed within 30 min. To ensure the binding efficiency and to use a short incubation time, an incubation time of 30 min was selected for the subsequent experiments.

3.3. Immunosensor response to anti-L. infantum antibodies and optimization

Fig. 3A shows the Nyquist plots obtained of the Antigen/SAM/SPE-Au (curve 1) in the presence of canine serum samples containing negative (Ab^-, curve 2) and positive (Ab^+, curve 3) antibodies in ferrocyanide/ferricyanide solution.

The association due to the antigen/antibody interaction resulted in an increase in R_{ct}. The immunosensor response to anti-L. *Infantum* positive antibodies (Ab^+/Antigen/MPA/SPE-Au) showed a high value (R_{ct} = 518 ± 6.9 Ω), which represents a high interaction with the antibodies in the positive serum samples. The results obtained after interaction with negative serum samples (Ab^-/Antigen/MPA/SPE-Au) showed an R_{ct} value of 302 ± 4.2 Ω. The use of crude antigens forced the binding of some nonspecific antibodies in the negative serum samples tested, but the R_{ct} difference clearly reinforces the possibility of detection and differentiation of positive and negative serum samples.

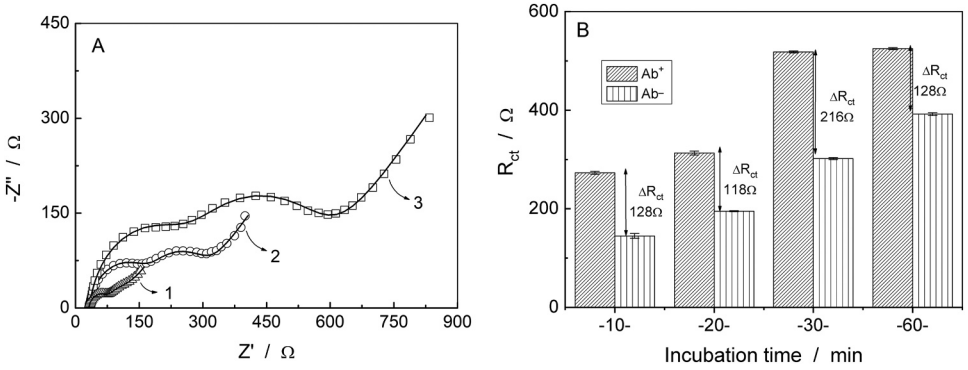


Fig. 3. (A) Nyquist plots (−Z" *vs* Z′) of Antigen/MPA/SPE-Au (Δ, curve 1), Ab^-/Antigen/MPA/SPE-Au (o, curve 2) and Ab^+/Antigen/MPA/SPE-Au (□, curve 3). (B) R_{ct} values in function of the incubation time for immobilization of the positive and negative antibody over immunosensor. All measurements were carried in KCl solution (0.1 mol L^{-1}) containing 1.0 mmol L^{-1} $Fe(CN)_6^{3-/4-}$.

Table 1
R_{ct} values for the positive and negative dilutions of serum and the values of ΔR_{ct}.

Dilution of antibodies	Ab^+ / R_{ct} (Ω)	Ab^- / R_{ct} (Ω)	ΔR_{ct}
1:40	998 ± 4	684 ± 5	314 ± 6
1:80	826 ± 6	532 ± 3	294 ± 7
1:160	678 ± 2	318 ± 2	360 ± 3
1:320	518 ± 7	302 ± 4	216 ± 8
1:640	347 ± 3	288 ± 3	59 ± 4
1:1280	206 ± 5	183 ± 3	23 ± 6

With a dilution (1:320), an optimization of the system immunoreactions was conducted (Fig. 3B) through the variation of interaction time (10, 20, 30 and 60 min). A minimum time of 10 min was chosen to ensure interaction between the antigen and the antibody. Times greater than 60 min were not tested, since the goal is to obtain a higher ΔR_{ct} of differentiation and a shorter response time. The increase in the positive Ab response was accompanied by a less evident increase in the negative Ab response. Because of the increase in the immobilization time, more antibodies were immobilized on the sensor surface. After 30 min, the positive antibodies immobilized on surface reaches saturation, which results in no change of R_{ct}. These results suggest that 30 min is the optimum time for immobilization of positive and negative antibodies.

3.4. Immunosensor performance

After optimization, the performance of the immunosensor was evaluated in the detection of anti-L. *infantum* antibodies in canine samples by the addition of a pool of sera positive for *Leishmania* infection (Ab^+). Table 1 shows the results obtained for R_{ct} values for the positive and negative dilutions of the serum pool (from 1:40–1:1280) and the variation between them (ΔR_{ct}).

The detection of antibodies was observed in all dilutions (1:40–1:1280), although the R_{ct} values decreased as the dilution increased, probably because of a lower antibody concentration in the sample (Fig. 4A). Fig. 4B presents the calibration curve, which shows a linear relation between R_{ct} and diluted canine sera with a correlation coefficient value of 0.9997. Immunosensor response against negative pool sera for L. *infantum* infection was also evaluated as control under the same conditions. In general, the values of R_{ct} detected were lower than those obtained for the positive pool in all concentrations, but the difference was more marked at 1:160 dilution, which should be selected as the probable dilution in the immunoassay test.

The results revealed that label-free electrochemical impedance immunosensors for anti-*Leishmania infantum* antibodies using modified SPEs represent a great diagnostic strategy, with the advantage of being a cheaper and faster method than other platforms so far used in the construction of immunosensors for the detection of canine leishmaniasis [28–31].

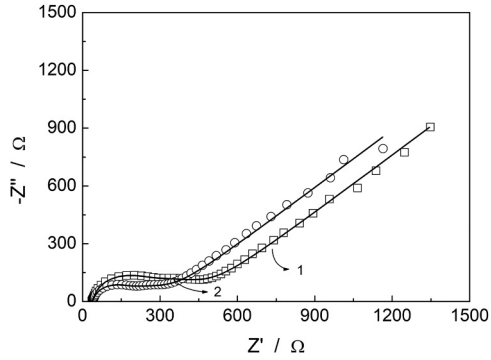


Fig. 5. Immunosensor response regarding the immobilization of positive (curve 1) and negative (curve 2) blood samples. All measurements were processed in KCl solution (0.1 mol L^{-1}) containing 1.0 mmol L^{-1} $Fe(CN)_6^{3-/4-}$.

Furthermore, the developed immunosensor was able to distinguish between positive and negative canine sera for L. *infantum* infection at lower dilution rates, in contrast to studies for leishmaniasis [28,29] and for Chagas disease [32].

Considering this promising result of the use of an immunosensor to differentiate positive and negative sera at lower dilutions of the sample and the difficulty of processing samples, especially in epidemiological surveys when a large number of canine samples are analyzed, the sensor sensitivity in a complex biological sample was checked. However, a preliminary detection of anti-*Leishmania infantum* antibodies was carried out directly on blood samples without any pretreatment. Thus, to test sensor impedance response a small blood volume (20 µL) was used, which can easily be obtained from dogs' ears or from the digital pulp in humans. The blood samples were dropped on the immunosensor surface for 30 min, and the impedance spectra obtained for positive and negative samples are reported in Fig. 5.

The observed increase in the R_{ct} values of the positive sample (141 ± 3.0 Ω) is larger than that observed with the negative sample (88.1 ± 0.9 Ω). The comparison between the two values indicates the excellent selectivity of anti- L. *infantum* even when using crude antigen and unprocessed samples, since the addition of negative serum was accompanied by a smaller response. However, the study and optimization of the biosensor with this type of matrix was not the subject of this work.

Finally, aiming to evaluate the applicability of the immunosensor in obtaining a future method of diagnosis, the stability of the immunosensor was also investigated. After proper antigen immobilization, the sensor was stored at 4.0 °C for three weeks. The immunosensor for the detection of anti-*Leishmania infantum* antibodies retained about 92% of its initial response value. These data reveal the potential of the label-free electrochemical impedance immunosensor as an anti-*Leishmania infantum* antibody platform for the development of a new diagnostic method to be applied to the reality of the endemic areas of

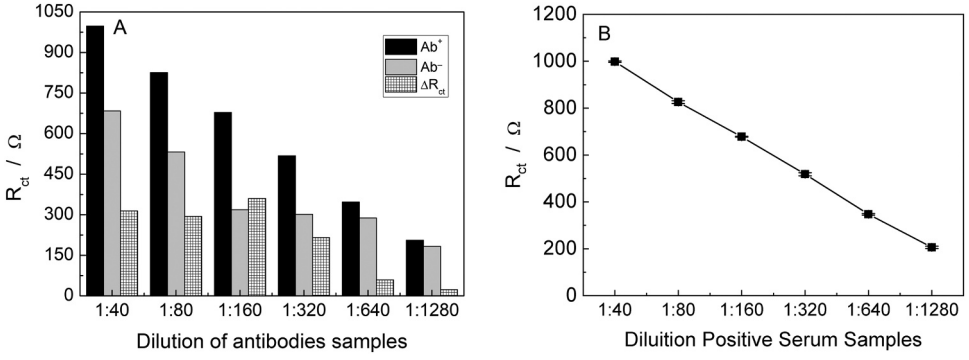


Fig. 4. (A) R_{ct} values obtained by immunosensor for positive and negative canine sera, and (B) R_{ct} in function of dilution factor of the positive canine sera.

canine VL, where there is a need for the analysis of a large number of samples, in a short time and with low cost.

4. Conclusions

The immunosensor development was based on impedance measurements for anti-*L-infantum* antibodies using for the first time a L. *infantum* antigen modified screen-printed gold electrode. The detection time for antibodies of L. *infantum* in canine serum through EIS was found to be less than 30 min. The developed EIS immunosensor demonstrated high sensitivity and specificity for detection of L. *infantum* antibodies in sera, illustrating its suitability for clinical and epidemiological applications. Thus, the biosensor proposed can effectively be used to diagnose dogs with VL. The sensor showed good response, small size, was marker-free and capable of real-time analysis. It shows great promise as a sensing system in VL-endemic regions.

Acknowledgments

The authors are grateful for financial support from Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), Brazil, (Project: APQ-03097-15) and wish to thank the LMMA sponsored by FAPEMIG (CEX-112-10) and SECTES/MG, Brazil, for their support.

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