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Research paper

Development of plasmonic ELISA for the detection of anti-*Leishmania* sp. IgG antibodies

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ABSTRACT

Recently, a novel Enzyme-Linked Immunosorbent Assay (ELISA) strategy has emerged, known as "plasmonic ELISA" (pELISA), which enables the detection of disease biomarkers at low concentrations with the naked eye. For the first time, this research has developed a signal-generation mechanism for the detection of anti-*Leishmania* sp. IgG antibodies with the naked eye using pELISA. The immunoassay incorporates an indirect ELISA with successive growth of gold nanoparticles to obtain blue or red-colored solutions in the presence or absence of anti-*Leishmania* sp. IgG antibodies in canine serum, respectively. The technique we developed was successfully tested in canine serum positive and negative for canine leishmaniasis (CanL), and was shown to be an effective method that could be used as an additional tool for CanL diagnosis. It will be particularly useful in resource-constrained countries, because it does not require sophisticated instruments to read the results, increasing the practicality of CanL detection in these areas.

1. Introduction

Visceral leishmaniasis (VL) is a severe systemic disease that is fatal if not diagnosed and treated. In the Americas, VL is endemic in 12 countries with 59,769 new human cases reported from 2001 to 2017. Approximately 96% of these cases were reported by Brazil, however, southern countries like Argentina, Colombia, Paraguay and Venezuela are among those with the highest case records (WHO, 2019). In endemic areas for VL, infected dogs are the main reservoir for the zoonotic disease and play an important role in human transmission. Therefore, the diagnosis of canine leishmaniasis (CanL) is an essential measure for disease control (Solano-Gallego et al., 2009).

Various techniques are available for diagnosing CanL, which are typically classified into parasitological, immunological and molecular methods (Solano-Gallego et al., 2011). Indirect ELISA is one of the most commonly used immunoassay platforms in official VL control programs in Brazil (Brasil, 2014). However, recent research has shown the low sensitivity of this method compared with molecular tests, suggesting that one out of five seronegative dogs are infected by *Leishmania infantum* (Lopes et al., 2017).

The problem with current indirect ELISA strategies is the high detection limit (1 ng/mL), which means they are inaccurate at the clinical threshold of many biomarkers, especially in the initial phase of the disease (Ogiso et al., 2013; Wu et al., 2007). In addition, the need for an expensive instrument to read the results has limited its wide applicability, particularly in resource-constrained countries (Satija et al., 2016).

A novel immunoassay pELISA has emerged that enables the detection of a few molecules with the naked eye (de La Rica and Stevens, 2013). This technique is based on the optical properties (Localized Surface Plasmon Resonance, LSPR) of metal nanoparticles, especially gold, and the successive growth of these nanoparticles mediated by a biocatalytic cycle of the enzymes (Ogiso et al., 2013). LSPR may be defined as a special optical phenomenon conferred by the interaction of light with electrons on the metallic nanoparticle surfaces (Eustis and El-Sayed, 2006; Guo and Kim, 2012). Generally, following modulation in the growth (size/morphology/aggregation) of the gold nanoparticles, a prominent color change occurs (e.g. red to blue when there is a growth of gold nanoparticles in their aggregated state) that can be easily differentiated with the naked eye. In addition, minor changes in the state of gold nanoparticles leads to large optical changes in their properties (Satija et al., 2016).

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Although, pELISA has been used to investigate different diseases diagnoses, including cancer and HIV (de La Rica and Stevens, 2012), fungal (Sojinrin et al., 2017) and bacterial infections (Chen et al., 2015; Mohd Bakhori et al., 2018; Nie et al., 2014), in CanL, this method has not yet been investigated. Thus, for the first time, we developed a signal-generation mechanism to detect anti-*Leishmania* sp. IgG antibodies with the naked eye using pELISA.

2. Materials and methods

2.1. Reagents and materials

Recombinant antigen rK28 (Infectious Disease Research Institute, Seattle, Washington, USA), streptavidin (Sigma-Aldrich, cat. no. S4762), catalase from bovine liver (Sigma-Aldrich, cat. no. C1345), 24-unit ethylene glycol functionalized with succinimidyl and maleimido ends (Thermo Scientific SM (PEG)24), bovine serum albumin (BSA; Sigma-Aldrich, cat. no. A7030), phosphate-buffered saline (PBS), Tween 20 (Sigma-Aldrich, cat. no. P9416), deionized water (resistivity ~18 MΩ cm), anti-dog IgG biotinylated antibodies and nonbiotinylated antibodies produced in goat (Sigma, St. Louis, MO, USA), MES (Sigma-Aldrich, cat. no. M3671), sodium hydroxide (NaOH; Sigma-Aldrich, cat. no. S8045), gold (III) chloride trihydrate (Sigma-Aldrich, cat. no. 520918), hydrogen peroxide (Sigma-Aldrich, cat. no. H1009), DMSO, carbonate buffer (0.05 M and pH 9.6), vacuum filtration systems (0.2 μm), disposable PD-10 desalting columns (GE Healthcare Life Sciences, cat. no. 17–0851-01) and polystyrene microtiter plates, 96 well (Greiner Bio-one, cat. no. 655081) were used.

2.2. Samples and dilutions

To determine the optimal concentration of canine serum for use in indirect pELISA, serial dilutions of serum positive and negative for canL (1/2, 1/50, 1/100, 1/200, 1/300 and 1/400) were analyzed by indirect pELISA. The concentration of the anti-dog IgG biotinylated antibodies was also tested by indirect pELISA in the following dilutions (1/50, 1/100, 1/200, 1/300 and 1/400). Blank wells without samples were included in all assays as control and the samples were analyzed in duplicate. After 15 min, the absorbance values at 550 nm were determined and photographs were taken showing the growth of the gold nanoparticles in different colors and intensities.

2.3. Preparation of solutions and conjugation of streptavidin to catalase

The preparation of gold-III and MES stock solution (100 nM), washing and blocking buffer and the procedure for conjugating streptavidin to catalase were performed as previously described (de La Rica and Stevens, 2013). To establish the success of the conjugation reaction and determine a suitable working dilution, microtiter plates were modified with 100 μl of anti-dog IgG biotinylated and nonbiotinylated antibodies diluted 1/200 in PBS-BSA (PBS and BSA to a 1 mg/mL, pH 7.2) and incubated overnight at 4 °C. After washing and blocking the plates, the conjugate (streptavidin-catalase) was added at different dilutions (1/20, 1/50, 1/100; 1/200, 1/300 and 1/400) and the plasmonic signal was generated.

2.4. Determination of the optimal concentration of hydrogen peroxide and gold ions

To test the impact of the concentration of hydrogen peroxide on morphology and optical properties of the growth of gold nanoparticles, 100 μl of different concentrations of hydrogen peroxide (500, 400, 300, 250, 200, 150, 125, 100, 75, 50, 25 μM) and gold ions (0.10, 0.20, 0.25 and 0.30 mM) were diluted in MES buffer (1 mM, pH 6.5) and added to the microtiter plates in triplicate. After 15 min, the absorbance values at 550 nm were determined and photographs were taken showing the growth of the gold nanoparticles in different colors and intensities.

2.5. Indirect pELISA protocol

The assay was performed as previously described (de La Rica and Stevens, 2013) with modifications for the detection of anti-*Leishmania* sp. IgG antibodies. A synthetic gene, K28, was generated by fusing multiple tandem repeat sequences of the L. *donovani* haspb1 and k39 kinesin genes to the complete open reading frame of haspb2, thereby increasing antigen epitope density, while providing complementing epitopes in the resulting recombinant protein (Pattabhi et al., 2010) and donated for use in the assay. Briefly, the microtiter plates were sensitized with 100 μl of recombinant antigen rK28 protein diluted in 0.05 M carbonate buffer, pH 9.6, at a concentration of 250 ng/mL, as described by (Venturin et al., 2015). The plates were then incubated overnight at 4 °C, washed three times in PBS containing 0.05% Tween 20, pH 7.4 (washing buffer), and saturated for 1 h with 300 μl per well of PBS-BSA at room temperature. Next, the plates were washed again three times with washing buffer and 100 μl of serum sample diluted in PBS-BSA (1/50) was added to each well and incubated at room temperature for 3 h, followed by three washes with washing buffer. Subsequently, 100 μl of biotinylated secondary antibody diluted 1/200 in PBS-BSA was added to each well of the microtiter plate, incubated at room temperature for 1 h and washed three times with washing buffer. After that, 100 μl of streptavidin-catalase conjugate diluted in PBS-BSA (1/20) was added to each well and incubated at room temperature for 1 h. The microtiter plate was then washed three times with washing buffer, twice with PBS and at least once with deionized water. This step was meant to remove remaining salts from buffers used in the previous steps. Next, 100 μl of hydrogen peroxide solution (250 μM) was added to each well of the microtiter plate and incubated at room temperature for 30 min. After homogenization, 100 μl of the 0.30 mM gold solution was added and incubated at room temperature for 15 to 30 min. When the process is performed correctly, positive controls present blue coloration, while negative controls present red or pink coloration.

2.6. Instrumental analysis

The growth of the gold nanoparticles determined with the naked eye was compared with absorbance obtained by a Tecan microplate reader (Sunrise model ref. 16039400) using a wavelength of 550 nm.

3. Results

3.1. Optimization of streptavidin-catalase conjugate, hydrogen peroxide and gold ion concentrations

In this experiment, the streptavidin-catalase conjugate was tested at different dilutions (1/20 to 1/400) and the indirect pELISA signals were recorded (Fig. 1). We verified that the solution changed blue in all wells that were sensitized with anti-dog IgG biotinylated antibodies, regardless of conjugate dilution. However, as the dilution factor increased, the color of the solutions in wells sensitized with anti-dog IgG nonbiotinylated antibodies changed from pink to purple at dilutions of less than 1/100. Therefore, to obtain a good balance between low

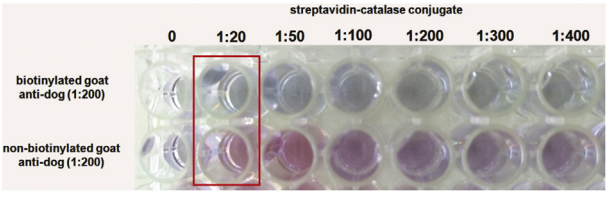

Fig. 1. The growth of the gold nanoparticles in different dilutions of streptavidin-catalase conjugate.

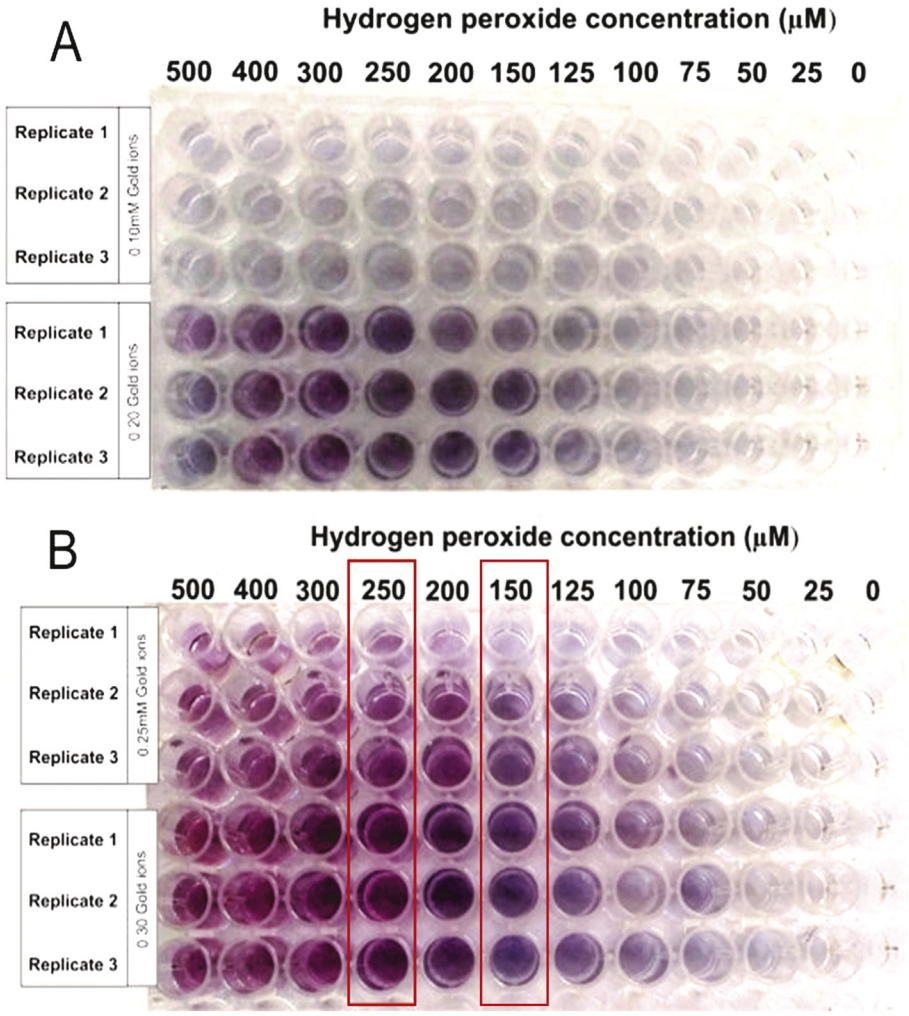

Fig. 2. The growth of the gold nanoparticles depends on the concentration of hydrogen peroxide. Gold ion concentrations of 0.10 and 0.20 mM (A) and 0.25 mM and 0.3 mM (B) at different hydrogen peroxide concentrations.

nonspecific signal and high detection sensitivity, the optimal dilution of the conjugate was set at 1/20 for the assay.

Different concentrations of hydrogen peroxide (500 to 25 µM) were tested at 0.10, 0.20, 0.25 and 0.30 mM of gold ion concentrations. The color tonality of solutions changed from pink to blue with the decrease in hydrogen peroxide concentration (Fig. 2). Gold ions at concentrations of 0.10 and 0.20 mM were insufficient to clearly distinguish the color of the solutions with the naked eye (Fig. 2A); however, gold ion concentrations of 0.20 and 0.30 µM showed adequate growth of nanoparticles to differentiate the color of the solutions, which changed from pink to blue at a hydrogen peroxide concentration between 250 and 150 µM (Fig. 2B).

Furthermore, the growth of gold nanoparticles was monitored by measuring the absorbance at 550 nm with different gold ion concentrations (Fig. 3A), taken together, these results indicated that the optimal negative control condition of hydrogen peroxide was 250 µM with gold ions at 0.30 mM for indirect pELISA. In the Fig. 3B, the growth of gold nanoparticles was monitored at different wavelengths (450–700 nm) and a peak shift of absorption spectra with gold nanoparticles growth in an aggregated state when compared to growth of gold nanoparticles in a non-aggregated state was observed.

3.2. Assay performance

The performance of the indirect pELISA developed was tested at different dilutions (1/50 to 1/400) of anti-dog IgG biotinylated antibodies (Fig. 4, A and B) and at different dilutions (1/2 to 1/400) of a canine serum positive and negative for canL (Fig. 5, A and B). The results were confirmed after visual reading in the laboratory by three observers properly trained.

The concentration of anti-dog IgG biotinylated antibodies was optimized to detect a suitable dilution that yielded the maximum signal with minimal nonspecific interactions. However, no differences between the dilutions were observed with the naked eye or in absorbance measurements (Fig. 4, A and B). Therefore, the dilution of anti-dog IgG biotinylated antibodies was set at 1/200 for the assay.

In addition, our technique was successfully tested on canine serum positive and negative for canL. We clearly observed the appearance of a blue colored solution only in the positive samples, while the pink coloration was observed in the negative samples. To test the possibility of nonspecific reactions with other proteins present in the solutions with the plate, we included controls (blank wells without samples) in the microtiter plates that also showed pink colored solutions (Fig. 5A). Although we were unable to verify any differences among serum dilutions with the naked eye, the absorbance values at 550 nm determined that 1/50 dilution optimized the assay. In addition, high mean absorbance values (0.340) were determined for negative samples, while positive samples showed low values (0.217) (Fig. 5B).

4. Discussion

We adapted the analytical principle of the pELISA (de La Rica and

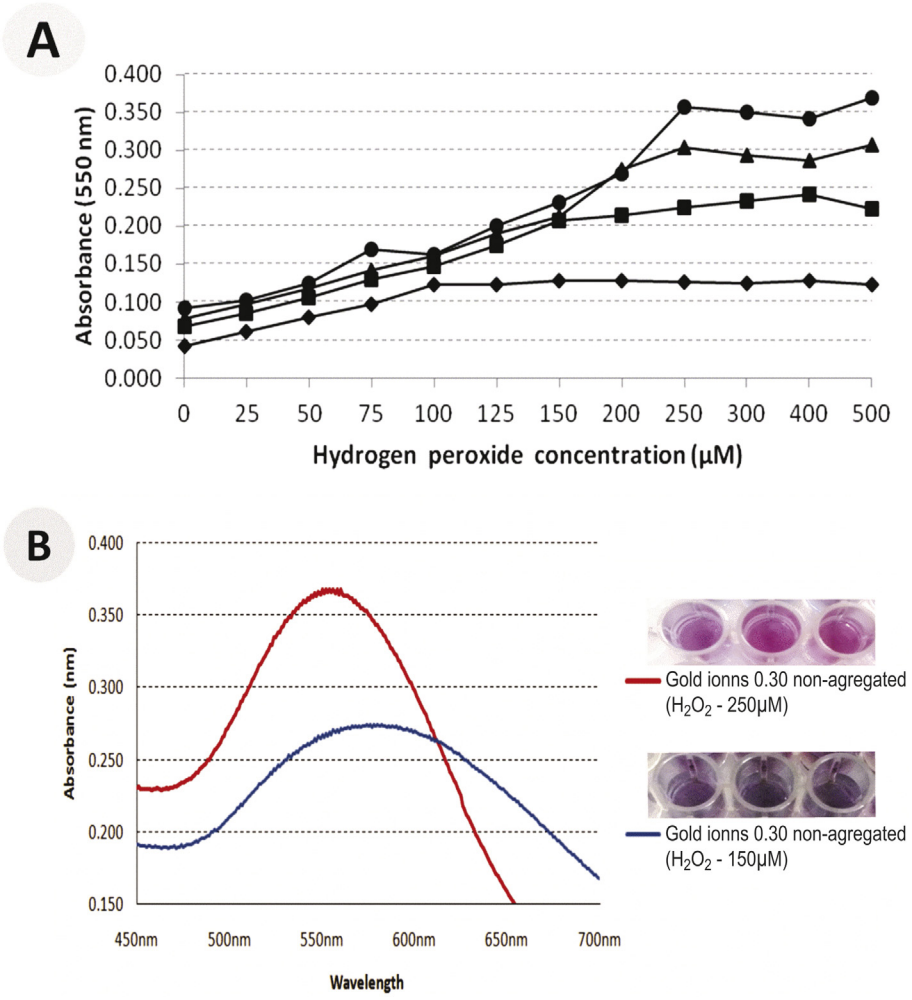

Fig. 3. Absorbance curve of gold nanoparticles according to the hydrogen peroxide concentration. (A) Absorbance values at 550 nm. (B) Absorption spectra at different wavelengths (450–700 nm) for the growth of nanoparticles gold in the non-aggregated (red line) and aggregated (blue line) state. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

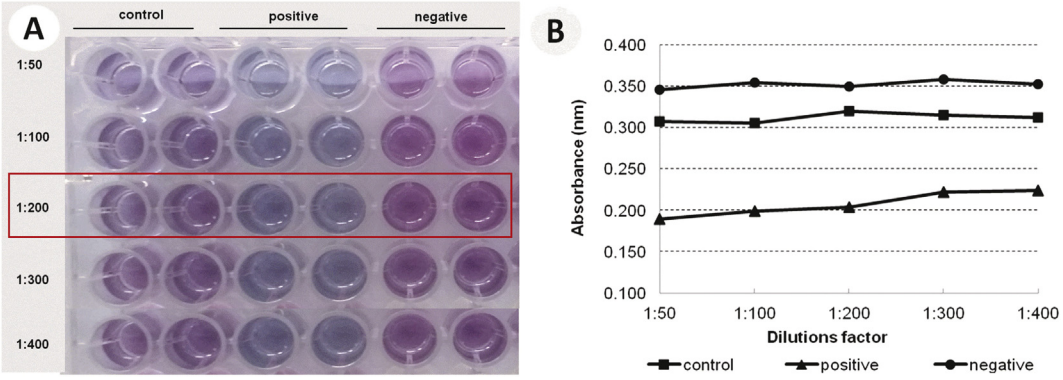

Fig. 4. Optimization of the concentration of biotinylated secondary antibody for the assay. (A) The growth of the gold nanoparticles in different biotinylated secondary antibody dilutions using canine serum positive and negative for canL. (B) Mean absorbance values at 550 nm for measurements performed in duplicate.

Stevens, 2013) to an indirect format (S1 Figure). In the method proposed in this research, the signal generation mechanism is associated with the successive growth of gold nanoparticles that produce blue or red colored solutions in the presence or absence of the anti-*Leishmania* sp. IgG antibodies in canine serum. Briefly, in the absence of the antibodies, the reduction in gold ions due to hydrogen peroxide occurs at a rapid rate, and under these conditions, the solution is expected to present the growth of gold nanoparticles in a non-aggregated state, which produces a pink or red solution. Conversely, in the presence of the antibodies, the catalase enzyme consumes the hydrogen peroxide. This slows down the kinetics of gold nanoparticle growth, which results in an aggregated state of gold nanoparticles, producing a blue solution. These blue and red colored solutions are easily distinguishable at a glance, therefore facilitating the detection of positive and negative results with the naked eye.

The optimal concentration of hydrogen peroxide was 250 µM with

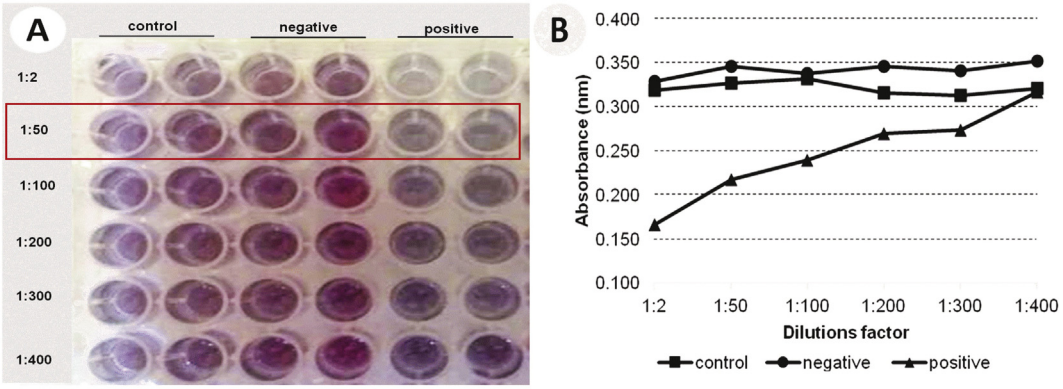

Fig. 5. Assay performance and optimization of the concentration of canine serum the assay. (A) The growth of the gold nanoparticles in different dilutions of positive and negative serum for canL. (B) Mean absorbance values at 550 nm for measurements performed in duplicate (B).

gold ions at 0.30 mM for pELISA. These concentrations were chosen because together they produced the lowest concentration of hydrogen peroxide without the growth of gold nanoparticles in the aggregated state and, consequently, promoted a change in the color of the solution. The essential step for the success of pELISA is a variation in the concentration of hydrogen peroxide to control the growth and state of aggregation of gold nanoparticles (de La Rica and Stevens, 2013). Therefore, during an actual case assay using indirect pELISA to diagnose CanL, the biocatalytic action of catalase should reduce the hydrogen peroxide concentration to less than 200 µM, producing blue-colored nanoparticle solutions.

The optimal streptavidin-catalase conjugate dilution was 1/20, which was chosen because it presented a good balance between a low nonspecific signal and high detection sensitivity. This is key factor that directly affects the pELISA sensitivity (de La Rica and Stevens, 2013).

We used hydrogen peroxide, gold ions and streptavidin-catalase conjugate at different concentrations than those determined previously (de La Rica and Stevens, 2012; Han et al., 2018; Mohd Bakhori et al., 2018). These discrepancies in the concentrations can be explained by environmental factors, including humidity and temperature, that can significantly affect the stability of the nanoparticles. Systematic experiments to test the ideal hydrogen peroxide and gold ions concentration should be performed and modifications must be adopted in the protocol, where necessary, to ensure the success of the immunoassay (Satija et al., 2016). Hence, the reasoning that supports the standardization carried out in our report.

The indirect pELISA was successfully tested on canine sera positive and negative for canL, in which positive serum yielded aggregated gold nanoparticles growth and solutions clearly distinguished by its blue coloration, whereas negative serum presented non-aggregated gold nanoparticles growth and red-colored solution. Similarly, the pELISA was effective at detecting the disease biomarkers with the naked eye. This makes the test particularly attractive because it provides good sensitivity, without requiring expensive equipment to read the results (Chen et al., 2015; de La Rica and Stevens, 2012, 2013; Liang et al., 2015; Mohd Bakhori et al., 2018; Nie et al., 2014; Sojinrin et al., 2017).

Considering that the human VL is associated with high morbidity and mortality, especially in resource-constrained countries (WHO, 2015), and that one of the strategies to reduce the disease in these areas is the monitoring and control of CanL cases (Brasil, 2014), indirect pELISA could be a useful additional tool in its diagnosis, dispensing the use of sophisticated instruments determine the results, while increasing the practicality of CanL detection in these areas.

5. Conclusion

In conclusion, we have demonstrated the detection of anti-*Leishmania* sp. IgG antibodies by indirect pELISA for the first time. The new immunoassay was shown to be an effective method that can be used as an additional tool for the diagnosis of CanL. We hope this report leads to future studies that lead to the validation of pELISA for CanL.

Declaration of Competing Interest

The authors declare that they have no conflicts of interest.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jim.2019.112664.

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