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Development of a simple and economical diagnostic test for canine leishmaniasis

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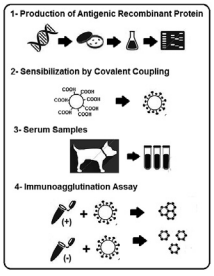
HIGHLIGHTS

- A rapid diagnostic tool was developed.
- Direct Agglutination Test for canine leishmaniasis was evaluated.
- Sera with high concentration of hemoglobin, lipids or bilirubin can be use in the test.
- The agglutination can be read after 5 min with 100% specificity.

GRAPHICAL ABSTRACT



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ABSTRACT

Visceral leishmaniasis is a public health problem worldwide. The early diagnosis in dogs is crucial, since they are an epidemiologically relevant reservoir of the disease. The aim of a field study is to early identify the disease allowing rapid intervention to reduce its effects. We propose an immunoagglutination test as a visual *in situ* method for diagnosis of canine visceral leishmaniasis. Latex-protein complexes were sensitized by covalent coupling of a chimeric recombinant antigen of *Leishmania* spp. onto polystyrene latex with carboxyl functionality. The reaction time and the antigen concentration under which the immunoagglutination assay shows greater discrimination between the responses of a positive control serum and a negative control serum were determined. Then, the latex-protein complexes were evaluated as a visual diagnostic tool with a panel of 170 sera. The test may be read between 2 and 5 min and can be performed even using sera with elevated concentration of lipids, bilirubin or with variable percentage of hemolysis. The sensitivity, the specificity and the diagnostic accuracy were 78%; 100% and >80%, respectively. The visual immunoagglutination test is of potential application as a method for field studies because it shows results in less than 5 min, it is easy to implement and does not require sophisticated equipment.

1. Introduction

Visceral leishmaniasis is a major public health problem worldwide. In America, this disease is caused by the protozoan parasite *Leishmania infantum*, which is transmitted to humans by the bite of

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sandflies from the genus *Lutzomyia* (Alvar et al., 2004, 2013). Dogs are considered the main reservoir of urban visceral leishmaniasis since: i) these animals harbor high parasitism in skin that offers a high capacity of parasite transmission to sandflies, ii) humans and dogs coexist in close proximity and iii) canine cases generally precede the occurrence of visceral leishmaniasis in humans (Albuquerque et al., 2009; Alvar et al., 2004, 2013; Salomon et al., 2008; Teixeira-Neto et al., 2014). The finding of leishmanian amastigotes in spotted spots, bone marrow or splenic aspirates is generally accepted as the gold standard for diagnosis, but the method is invasive, generally not feasible when it is most needed and has little sensitivity; whereas the *in vitro* culture of parasites from any of the above samples would improve sensitivity, though requires sophisticated laboratory facilities. Serological tests for the detection of antileishmanial antibodies, including indirect immunofluorescence (Badaro et al., 1983), ELISA (Hommel et al., 1978) and the direct agglutination assay (Akhoundi et al., 2013; Attar et al., 2001; Babakhan et al., 2009; El et al., 1988, 1989; Harith et al., 1987; Hatam et al., 2009; Sundar et al., 1998; Oliveira et al., 2016) have also been described. However, sensitivity and specificity of each of the systems are variable in different regions; there are cross-reactions in case of co-infection with other pathogens, including other *Leishmania* spp. (responsible for cutaneous or mucocutaneous infections), *Trypanosoma cruzi* and mycobacteria (Barbosa-De-Deus et al., 2002; da Costa et al., 1991; el Amin et al., 1986; Harith et al., 1987). Most of the agglutination tests developed in recent times are based on antigens partially purified from promastigotes or amastigotes cells. The use of recombinant leishmanial antigens (Houghton et al., 1998; Sundar et al., 2005) or synthetic peptide antigens (Fargeas et al., 1996) has recently been introduced for serology in both ELISA and strip test format. At present, no satisfactory antigen detection assays are available in the market and attempts to develop such assays have not been convincing (Kohanteb et al., 1987; Senaldi et al., 1996). To address this latter drawback, the use of chimera polypeptides or proteins expressing several unrelated antigenic determinants has been proposed.

This article describes the development of a latex agglutination test to detect antibodies in serum from dogs with visceral leishmaniasis by means of latex particles sensitized with a chimeric recombinant antigen of *Leishmania* spp. To do this, we have focused first on the rational selection of antigenic peptides from those that have already proven to be useful for diagnosis. A novel chimerical multiepitope construct (LeQuiDi) was synthesized by fusion of highly conserved antigenic sequences in different *Leishmania* spp. Latex-protein complexes were then sensitized by covalent coupling of the recombinant multiepitope protein onto carboxyl external groups of functionalized polymer latexes. Then, complexes were evaluated as a diagnostic tool with a panel of 170 sera (between positives and negatives). The main advantages of this method are its rapidness and ease of implementation in endemic areas, together with the fact that it does not require sophisticated equipment, agglutination being visualized directly (Garcia et al., 2013; Gonzalez et al., 2008a, 2008b; Lucas et al., 2006; Peula-Garcia et al., 2002; Polpanich et al., 2007; Santos and Forcada, 2001). Thus, in our region, a technique for visual detection of immunoagglutination could be of great help for field studies where the scientists do not have instrumental support for the diagnosis.

2. Materials and methods

2.1. Production and purification of antigenic recombinant protein

2.1.1. Gene construction

The *lequidi* gene was synthesized at Integrated DNA Technologies (IDT, US). The synthetic gene encodes three 14-aa repeats present in *L. infantum* K26 protein (amino acids 128–173, GenBank accession XP_001465795) and two 39-aa repeats present in *L. infantum* K39 kinesin (amino acids 782–859, GenBank accession P46865). The 399 base pair (bp) gene was subcloned into the *Bam*HI/*Xho*I sites of pET28a (Novagen, US); the resulting plasmid was named pET28a/*lequidi*. The coding sequence of the Red Fluorescent Protein (*rfp*) was released from pNUS-mRFPnD (Tetaud et al., 2002) by *Nde*I and *Bgl*II digestion, and cloned into *Nde*I and *BamH*I sites of pET28a/*lequidi* to create the pET28a/*rfp-lequidi* plasmid.

2.1.2. Expression and purification

Competent *E. coli* BL21 (DE3) cells were transformed with pET28a/*rfp-lequidi* plasmid. Protein production was carried out using 500 mL of Luria-Bertani medium (LB) supplemented with 50 μg/mL kanamycin. Cells were grown at 37 °C and 200 rpm until OD_{600} reached ~0.6 and induced for 16 h at 25 °C with 0.5 mM IPTG. Cells were harvested by centrifugation at 5000 × g for 10 min and stored at − 20 °C until use. Cells were resuspended in buffer A (20 mM Tris–HCl, pH 7.5, 400 mM NaCl and 10 mM imidazole) and disrupted by sonication on ice. The suspension was centrifuged at 15,000 × g for 20 min and the supernatant (crude extract) was loaded on a 1 mL HisTrap column (GE Healthcare) previously equilibrated with buffer A. The recombinant protein was eluted with a linear gradient from 10 to 300 mM imidazole. Red colored fractions were pooled, concentrated and dialyzed against 10% (v/v) glycerol/PBS. In these conditions, the protein was stored at − 80 °C until use, without loss of antigenicity for at least a year.

2.1.3. Westernblotting assay for testing inmunoreactivity of LeQuiDi

Purified RFP-LeQuiDi and RFP were analyzed electrophoretically by SDS–PAGE according to Laemmli (1970). Western blotting was performed after standard techniques. Proteins in the gel were blotted onto nitrocellulose membranes using a Biometra-Fastblot apparatus. The membrane was blocked with 3% w/v skim milk solution (in PBS) during 2 h, subsequently incubated with primary antibody at room temperature for 1 h and then incubated with a HRP-conjugated anti-rabbit secondary antibody for 1 h. Detection was carried out with 3, 3′-diaminobenzidine and hydrogen peroxide (Sigma) in 50 mM Tris–HCl, pH 8.0, 150 mM NaCl.

2.2. Particles sensitization by covalent coupling

Recombinant proteins of increasing concentrations (0.3–1.2 mg/mL) were incubated with carboxylated latex particles (0.2 m^2 of latex particles surface) in phosphate buffer (pH 5, 2 mM) under stirring along 5 h at room temperature. The activation of carboxyl groups was carried out simultaneously with the covalent coupling to minimize the hydrolysis of the acylurea intermediate. To this effect, the concentration of carbodiimide was selected to be 100-fold greater than the concentration of carboxyl groups, in order to ensure the complete transformation of all the carboxyl groups into acylurea and to produce a recommended surface density of 10 mg N-N-(3-dimethylamine propyl) N′-ethyl carbodiimide (EDC)/m^2 latex. The resulting latex–protein complexes were first isolated from the solution by ultracentrifugation during 30 min at 10,000 rpm and then they were resuspended in Triton X-100 1% for 24 h to elute proteins not covalently attached to the particles. Finally, the sensitized particles were resuspended in borate buffer (pH 8) and kept at 4 °C (Gonzalez et al., 2008a, 2008b). The total-linked protein (i.e., both physically adsorbed and covalently bound) and the covalently-coupled protein (i.e., the protein that remains on the particle surface after desorption with Triton X-100) were indirectly determined by measuring the concentrations of

Table 1
Test score for the latex agglutination test.

Test score	Size of the agglutination beads	Appearance of the background	Rapidity of the agglutination
1+	Small clumps	Cloudy	>2 min
2+	Small/Large clumps	Cloudy	>1 min
3+	Large clumps	Clear	>30 s

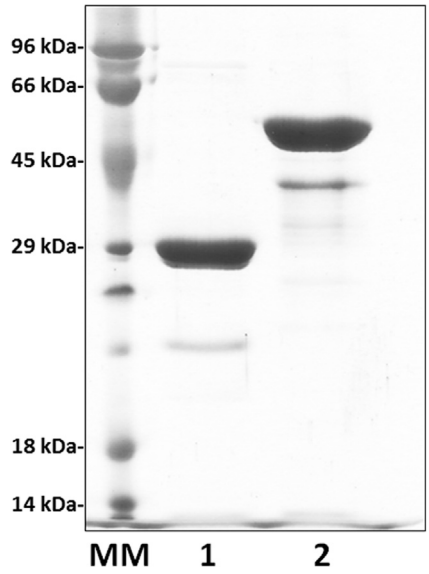

Fig. 1. Fifteen percent SDS-PAGE of purified RFP-LeQuiDi. The purified recombinant proteins were defined by electrophoretic migration under reducing and denaturing conditions, and subsequent staining with Coomassie blue. Lane 1: Molecular mass marker; lane 2: RFP (2 μg); lane 3: RFP-LeQuiDi (2 μg).

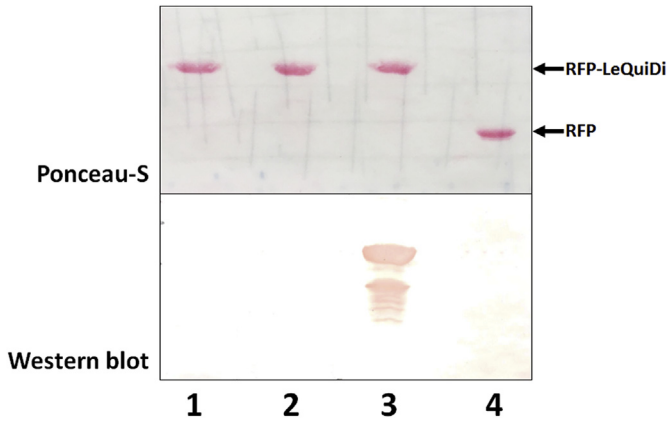

Fig. 2. Ponceau-S staining of RFP-LeQuiDi (2 μg) and RFP (2 μg) transferred onto nitrocellulose membrane. Western blotting assays using different controls. Lane 1: pool of sera from healthy dogs; lane 2: pool of sera from dogs infected with Leptospira interrogans, Trypanosoma cruzi or Ehrlichia spp.; lane 3 and lane 4: pool of sera from dogs with leishmaniasis.

dissolved protein through the copper reduction/bicinchoninic acid (BCA) method (Smith et al., 1985). A blank without proteins was also prepared.

2.3. Serum samples

The positive serum samples were obtained from Corrientes city (Argentina), an endemic region located in northeast Argentina. The infection status was determined by using two different conventional laboratorial diagnosis methods: i) parasitological method (detection of the parasite) by Giemsa stain and ii) serological method (detection of anti-*Leishmania* antibodies) using immunochromatographic strip assay identified commercially as Kalazar Detect Rapid Test Kit (Canine). The sera were considered positive when they showed immunoreactivity in immunochromatography, and amastigote nests were found in bone marrow stained by Giemsa technique. Negative sera (from no endemic areas) did not show signs of immunoreactivity (nor amastigotes in the bone marrows).

2.4. Immunoagglutination assays

On a slide of black background, 25 μL of latex-protein complex and 25 μL of serum (positive or negative) were mixed. The agglutination reaction was observed by visual inspection. Initially, using 2 positive and negative control sera, the degree of immunoagglutination was determined as a function of i) time, registering the time at which the process of agglutination was visualized and ii) the concentration of Ag (amount of bound protein per unit of particle surface). Then, under such optimal conditions (time and Ag concentration), a panel of 170 sera was studied. The result was reported as negative or positive. The absence of agglutination was reported as negative and visible agglutination or hardly visible was reported as positive. For a positive result, the degree of agglutination was scored from 1 + to 3 + based on the appearance and the rapidity of the agglutination development as shown in Table 1. The relative sensitivity, specificity and diagnostic accuracy (in percent) of the assay were determined in comparison to the reference techniques as described below:

$$\text{Sensitivity} = a/(a+c) \times 100,$$

Where “a” is the number of serum samples positive by the test and reference techniques; “c” the number of serum samples positive by reference techniques, but negative by test.

$$\text{Specificity} = d/(b + d) \times 100$$

Where “d” is the number of serum samples negative by test and reference techniques; “b” the number of serum samples negative by reference techniques, but positive by test.

$$\text{Diagnostic accuracy} = (a+d)/(a+b + c + d) \times 100$$

3. Results

3.1. Production and purification of antigenic recombinant protein

The recombinant chimera LeQuiDi was obtained by means of technologies like synthesis *de novo* of genes and elemental molecular biology methods. The protein was obtained fused to the RFP (Fig. 1) in a prokaryotic expression system (*E. coli* BL21 (DE3)). The protein was purified chromatographically and assayed

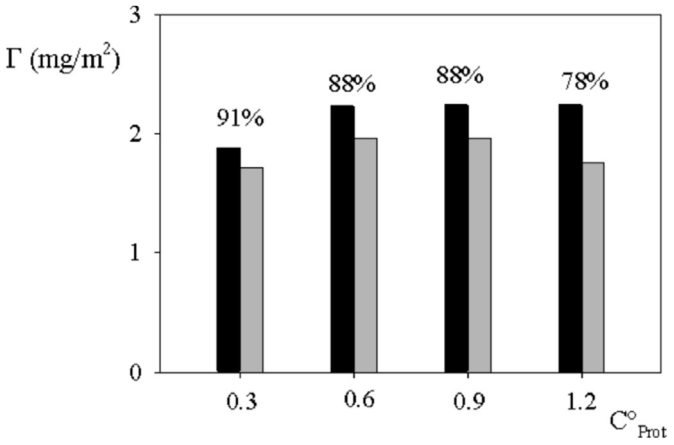

Fig. 3. Chemical coupling of AgL onto the carboxylated latex at pH 5 vs the initial protein concentration. The surface density of total-linked protein (black) is compared to the covalently-bound protein (grey) after desorption with Triton X-100. The percentages on the grey bars indicate the fractions of covalently-coupled protein.

immunologically to test its usefulness as antigen in the diagnostic of canine leishmaniasis. One hundred and seventy serum samples were collected. One hundred and twenty-four positive controls and 46 negative controls. Thirty sera out of these 46 were obtained from healthy dogs, 6 of the negative sera were seropositive for infection with *Trypanosoma cruzi*, 8 for infection with *Leptospira interrogans* and 2 for infection with *Ehrlichia* spp. Three different pools of sera were prepared and used in a western blotting assay (Fig. 2). A pool of sera from healthy dogs (negative control) and other from dogs infected with *Leptospira interrogans*, *Trypanosoma cruzi* or *Ehrlichia* spp. did not show reactivity against RFP-LeQuiDi (Fig. 2, lane 1 and lane 2, respectively). The pool of sera from dogs with visceral leishmaniasis recognized RFP-LeQuiDi (Fig. 2, lane 3), while RFP was not recognized by antibodies in the pool of positive sera (Fig. 2, lane 4).

3.2. Particles sensitization by covalent coupling

In Fig. 3, the surface densities of total-linked and covalently-coupled protein (Γ_{Prot}) onto the carboxylated latex are represented as a function of the initial protein concentration ($C°_{Prot}$) at pH 5. In all cases, the amount of total-linked protein increases with the concentration of added protein. However, the ratio between the total-linked protein and the total added protein decreases when increasing the amount of added protein, and the same tendency is observed with the ratio between the covalently-bound protein and the total added protein. These results could be due to the saturation of the particle surface by the protein. This may be related to steric hindrance generated between neighboring protein molecules and the shielding of reactive groups attached to the surface.

3.3. Immunoagglutination assays

Agglutination was not seen when latex-protein complex alone

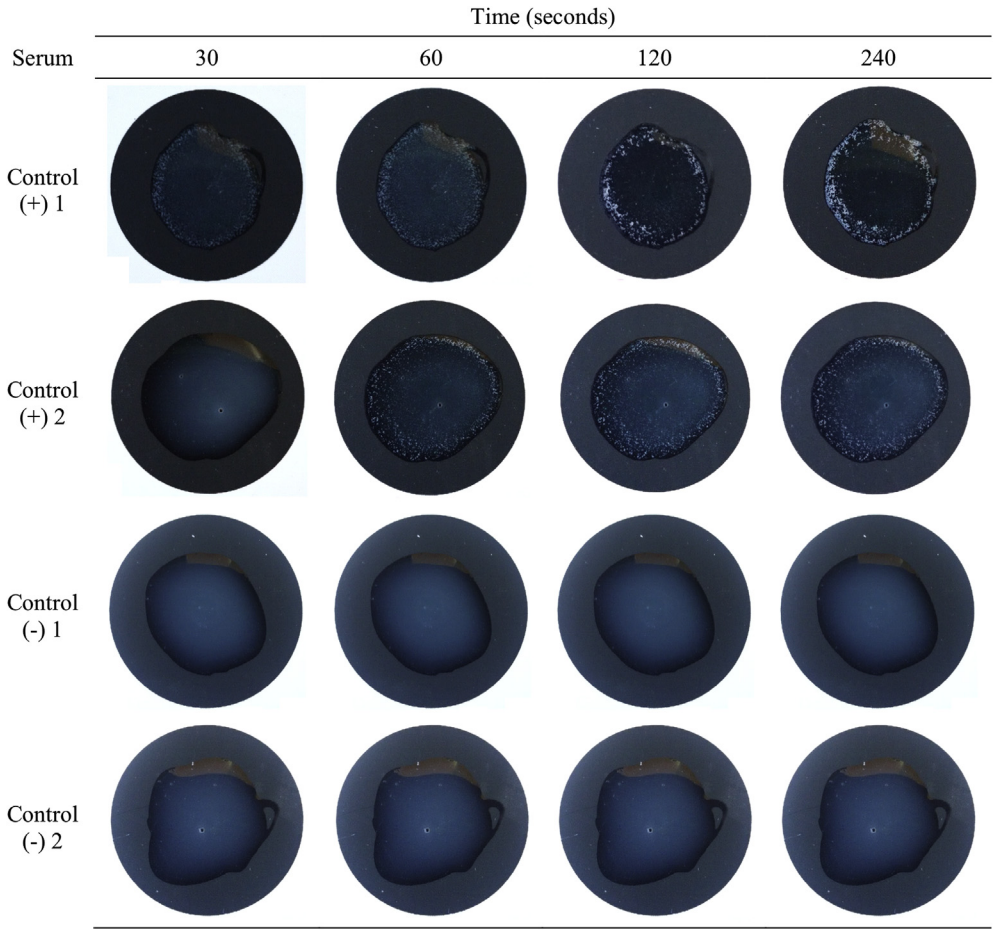

Fig. 4. Influence of reaction time on the immunoagglutination assay using a concentration of covalently coupled protein equal to 1.96 mg/m2 and undiluted serum.

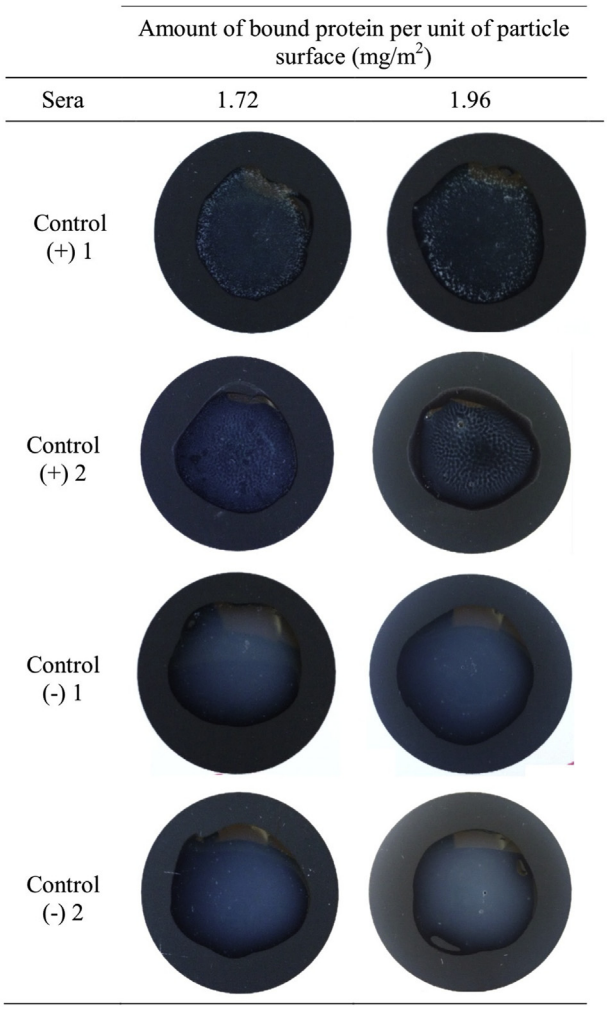

Fig. 5. Influence of the antigen concentrations on the immunoagglutination assay. Photographs taken a reaction time = 240 s.

was rotated for 3 min. No agglutination was seen when the latex (i.e., reagent without adsorbed antigen) was tested on serum sample. Negative reaction was consistently observed when the latex-protein complex was tested on normal saline and buffers throughout this study.

3.3.1. Evaluation of reaction time

Agglutination was visible after 30 s in the case of sera positive controls. However, to obtain a noteworthy differentiation between sera positive and negative, the test should be read after 2 min and may be extended even 4 min. Extending the time beyond 4 min resulted in reagent drying that might give false-positive result. No agglutination was seen when the control sera negative was tested (Fig. 4).

3.3.2. Evaluation of the concentration of Ag

During the immunoassays, Ag and Ab react specifically causing the agglutination of the complexes. The degree of immunoagglutination was determined varying the concentration of Ag (or amount of bound protein per unit of particle surface). Fig. 5 shows the influence of the Ag concentration on the immunoagglutination assay using undiluted serum. In all cases, the generation of large immune complexes was observed and this could be due to the fact that a similar amount of Ag was attached to the latex beads (1.72–1.96 mg/m^2) after the sensitization step.

3.3.3. Aspect serum

In the spectrophotometric analysis, results are often influenced by interfering factors related to the presence of hemoglobin, bilirubin and lipemia, which may alter the correct value of the result producing a false one; which does not reflect the *in vivo* situation. Unlike instrumental methods, the visual test can be performed using either serum normal, hemolyzed, lipemic or icteric (Fig. 6).

3.3.4. Comparison with the reference method: application of latex-protein complex to a sera panel

The sensitivity of the immunoagglutination assay calculated from the panel of serum samples (170) was 78.0% (95% CI, 71.3–84.7%). However, the majority of the positive samples showed strong (2+) to very strong (3+) agglutination and only a few samples reacted weakly (1+). The false-negative results were 33 samples tested (Table 2). False-negative results can be obtained from dogs with persistent infections showing low Ab levels. The latex agglutination assay was highly specific (100%) and showed a diagnostic accuracy greater than 80.6% (95% CI, 74.6–86.6%). An example of the classification of samples, according to the score, is shown in Fig. 7.

Table 2
Results of sera panel analyzed by visual immunoagglutination.

	Total Number of Samples: 170	
Reference Techniques	150 +[a]	20 –[d]
Visual Immunoagglutination	117 +[a]	0 +[b]
	33 –[c]	20 –[d]

[a] True positives.
[b] False positives.
[c] False negatives.
[d] True negatives.

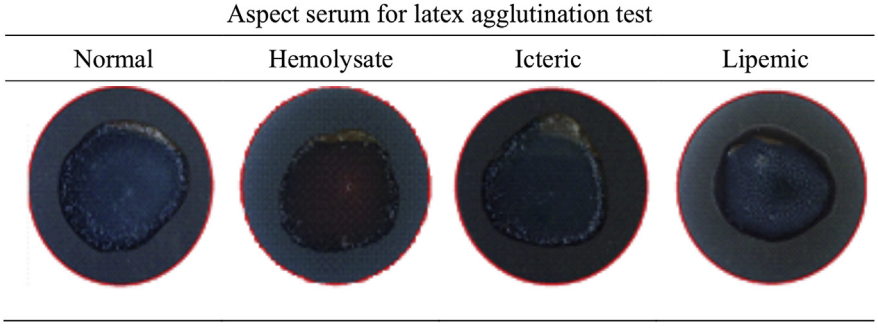

Fig. 6. Latex agglutination test with different aspect sera.

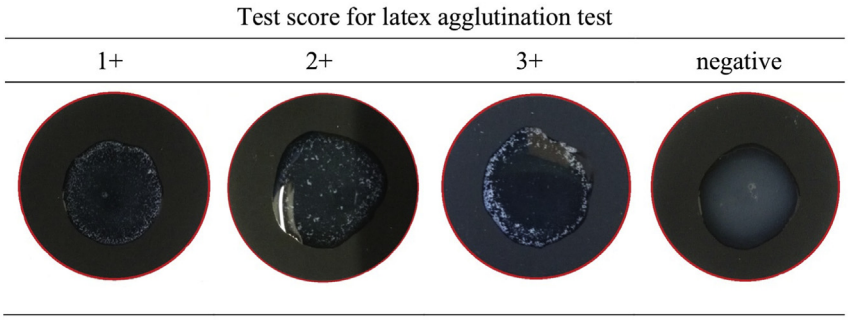

Fig. 7. Classification of samples according to the score.

4. Discussion

In a systematic review (Romero and Boelaert, 2010), the majority of studies on serological assays for the diagnosis of *L. infantum* infection in dogs found sensitivities higher than 75%. Nonetheless, parasitological culture, PCR and serological assays present disadvantages that prevent their use alone for the routine diagnosis of *L. infantum* infection in dogs: i) time consuming, on average 15 days to be completed in parasitological culture and several h to PCR and serologic assay; ii) difficult to perform, require special equipment and technical expertise; iii) susceptible to microbiologic contamination, which in many cases prevents its use in samples collected in the field, where proper storage and sterile conditions may be difficult to attain; iv) high cost; v) may yield false-positive results due to cross-reactivity and vi) difficult to differentiate between past and present infection (Barros et al., 2012; de Almeida et al., 2011a; de Almeida et al., 2011b; Gomes et al., 2008; Leite et al., 2011; Maia and Campino, 2008; Otranto et al., 2009; Troncarelli et al., 2009; World Health Organization, 2010; Xavier et al., 2006).

Latex-protein complexes were obtained by covalent coupling of a new chimeric recombinant antigenic protein of *Leishmania* spp. onto carboxylated particles. High levels of covalent coupling of the antigenic proteins were observed on the particles surface. The results of the current study confirm immunoagglutination (based on the observation of visible clumps that are formed from the reaction of Ag − Ab) as an *in situ* method for the diagnosis of *L. infantum* infection in dogs. While it is less sensitive than parasitological culture, PCR and some other serological assays previously reported by other groups (de Fatima et al., 2006; de Queiroz et al., 2011; Madeira et al., 2009; Romero and Boelaert, 2010; Xavier et al., 2006), the visual immunoagglutination test has a potential clinical application as a field study method because: i) it is simple to perform; ii) the results can be obtained within 2–4 min, making it suitable to be used in areas with limited laboratory facilities; iii) it has a low cost; iv) it is easy to interpret; v) it looks stable under extreme conditions; vi) it requires little or no processing of the sample; vii) it is 100% specific and viii) it does not demand sophisticated equipment. Visual immunoagglutination assay to detect canine visceral leishmaniasis was evaluated on the basis of positive and negative sera panel as a rapid method for visceral leishmaniasis diagnostic. The negative sera response was clearly different from that of the positive sera. The immunoagglutination test showed a sensitivity of 78% and a specificity of 100%. The diagnostic accuracy was greater than 80%, with a reaction time of 2 min proven to be adequate for a reading test. Although it is fast, easy to implement and could be used in field conditions, it is known that a sensitivity >95% is more adequate for a diagnostic test (Akhoundi et al., 2013; Babakhan et al., 2009; Hatam et al., 2009; Oliveira et al., 2016). For this reason, further work must be performed to improve the quality of this simple test that appears as an interesting screening method for the diagnosis of visceral leishmaniasis in dogs in our region.

Conflict of interest

No conflict of interest declared.

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