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Development of improved enzyme-based and lateral flow immunoassays for rapid and accurate serodiagnosis of canine brucellosis

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ABSTRACT

Brucellosis is a widespread zoonotic disease caused by *Brucella spp. Brucella canis* is the etiological agent of canine brucellosis, a disease that can lead to sterility in bitches and dogs causing important economic losses in breeding kennels. Early and accurate diagnosis of canine brucellosis is central to control the disease and lower the risk of transmission to humans. Here, we develop and validate enzyme and lateral flow immunoassays for improved serodiagnosis of canine brucellosis using as antigen the *B. canis* rough lipopolysaccharide (rLPS). The method used to obtain the rLPS allowed us to produce more homogeneous batches of the antigen that facilitated the standardization of the assays. To validate the assays, 284 serum samples obtained from naturally infected dogs and healthy animals were analyzed. For the *B. canis*-iELISA and *B. canis*-LFIA the diagnostic sensitivity was of 98.6%, and the specificity 99.5% and 100%, respectively. We propose the implementation of the *B. canis*-LFIA as a screening test in combination with the highly accurate laboratory *g*-iELISA. The *B. canis*-LFIA is a rapid, accurate and easy to use test, characteristics that make it ideal for the serological surveillance of canine brucellosis in the field or veterinary laboratories. Finally, a blind study including 1040 serum samples obtained from urban dogs showed a prevalence higher than 5% highlighting the need of new diagnostic tools for a more effective control of the disease in dogs and therefore to reduce the risk of transmission of this zoonotic pathogen to humans.

1. Introduction

Brucellosis is a major zoonotic disease caused by members of the genus *Brucella* that affects animals and humans. *Brucella canis* is the etiological agent of brucellosis in dogs and also a human pathogen. Even though the first case of naturally acquired human infection with this organism was reported four years after the identification of this species (Swenson et al., 1972), few cases have been described so far, particularly in veterinarians after close contact with animals that recently aborted or laboratory workers exposed to large amounts (e.g., bacterial cultures) of the organism (Lucero et al., 2010, 2005; Wallach et al., 2004). For this reason, *B. canis* is considered a pathogen with low zoonotic potential. However, the clinical importance of human brucellosis by *B. canis* may be underestimated due to difficulties in diagnosing the disease. In most cases, this pathogen induces a subclinical infection that may remain undiagnosed for long periods of time. The infection should be always suspected in patients with compatible symptoms of brucellosis and negative serology by diagnostic tests for *Brucella* strains such as *B. abortus, B. melitensis* and *B. suis*, all of them smooth strains (Lucero et al., 2005).

B. canis causes abortions and stillbirths in pregnant bitches and epididymitis, orchitis and prostatitis in males (Carmichael and Kenney, 1968; Moore and Kakuk, 1969). These clinical manifestations can lead to sterility, a factor that produces important economic losses in dog breeding kennels. Dogs that have been neutered do not have reproductive signs, but they occasionally develop other conditions such as

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ocular disease and discospondylitis. Among dogs the infection is transmitted through contaminated aborted fetuses, urine, milk, vaginal secretions and semen. Additionally, *B. canis* can spread through fomites. It has been demonstrated that uninfected dogs living with infected animals of the same sex can acquire the infection within 6 months (Carmichael and Joubert, 1988).

Definitive diagnosis of canine brucellosis relies on the isolation of the bacteria from blood, semen/vaginal swab culture and/or detection of *B. canis* by the polymerase chain reaction (PCR). These direct tests allow the identification of dogs with an active infection but have low sensitivity to detect chronically infected animals. Additionally, they are time-consuming, particularly blood culture which can take at least 7 days, and costly to be used when a high number of animals must be screened (Flores-Castro and Segura, 1976; Wanke, 2004). For these reasons, routinely diagnosis of the disease is based on serological methods. Since *B. canis* is naturally rough, the assays used for the serodiagnosis of brucellosis caused by the smooth zoonotic species, which are based mostly on the detection of specific antibodies against the O polysaccharide section of the lipopolysaccharide (LPS), cannot be used for the detection of *B. canis* infection. Currently, the most commonly used tests for serodiagnosis of canine brucellosis are the rapid slide agglutination test (RSAT) (Carmichael and Joubert, 1987)and the agar gel immunodiffusion test (AGID) (Zoha and Carmichael, 1982). Both detect the presence of antibodies against surface antigens of *Brucella*, especially the rough LPS (rLPS), but suffer from false negative (low sensitivity) and false positive results (low specificity) (Carmichael and Joubert, 1987; Keid et al., 2009).

In this work, we present the development and validation of an indirect enzyme-linked immunosorbent assay (iELISA) and a lateral flow immunoassay (LFIA) for the diagnosis of canine brucellosis using as antigen a combination of the rLPS of *B. canis* and outer membrane proteins (Omps) including Omp16, Omp25 and Omp31. Our results demonstrate that both assays are highly sensitive for diagnosis of the disease in naturally infected dogs by *B. canis* and very specific. The implementation of a screening serological test that is rapid and simple to perform, such as the *B. canis*-LFIA, in combination with a highly accurate laboratory test like the *B. canis*-iELISA, can improve the detection and control the disease, especially in canine breeding kennels, and reduce the risk of transmission of brucellosis to humans.

2. Materials and methods

2.1. B. canis antigen

Production of the *B. canis* antigen was performed by a hot saline-based method (Myers et al., 1972) with several modifications. To produce the antigen the *B. canis* (M-) strain was grown in tryptic soy broth (TSB) (Difco/Becton-Dickinson, Sparks, MD) at 37 °C. Cells were harvested by centrifugation at 5,000 x *g* for 15 min at 4 °C and washed twice with 0.01 M phosphate-buffered saline pH 7.2 (PBS). The pellet was weighted, resuspended in PBS 10% p/v and autoclaved at 121 °C for 30 min. After centrifugation, the supernatant was collected and ultra-centrifuged at 100,000 x *g* for 6 h at 4 °C. The resulting pellet was resuspended in deionized water, dialyzed against 200 vols of PBS during 24 h at 4 °C and the antigen was precipitated adding 10 mM MgCl2. The precipitate was washed with deionized water and then lyophilized. The antigen concentration was determined by dry weight. All work with live *B. canis* was performed in a biosafety level 3 laboratory facility at Universidad Nacional de San Martín.

2.2. Sera

To validate the assays two different sample panels were analyzed. *Panel I.* Samples used to determine the diagnostic sensitivity and specificity of the tests. This panel included 71 serum samples obtained from culture-positive and serologically positive (RSAT and AGID positive) dogs (n = 38 from Argentina and n = 33 from Colombia), and 213 samples obtained from healthy dogs from Argentina with negative results by RSAT and AGID. The serum samples from Argentina were provided by the DILAB-SENASA and the sera from Colombia were provided by the Biogénesis group-Universidad de Antioquia. *Panel II.* Samples included in the double-blind study. A set of 1040 encoded serum samples obtained from animals coming from Villa Hidalgo, Buenos Aires province, Argentina, was analyzed. These samples were provided by the Programa Nacional de Tenencia Responsable y Sanidad de Perros y Gatos (ProTenencia, Ministerio de Salud de Nación, Argentina) and were taken from dogs who attended the veterinary consultation for anti-rabies vaccination and/or castration prior informed consent signed by their owners. These animals came from an urban area and were pets that were allowed to roam and be in contact with other dogs. All the samples were analyzed in parallel by RSAT (at SENASA) and the *B. canis*-iELISA (at UNSAM). Samples that resulted positive by RSAT and/or the *B. canis*-iELISA were additionally analyzed by AGID (at SENASA).

2.3. Ethics statement

The DILAB-SENASA, Biogénesis group (Universidad de Antioquia) and ProTenencia program provided all the samples analyzed in this study. All the studies with animals were done in accordance with the institutional animal guidelines and approved by the local regulatory agencies (CICUAE-UNSAM).

2.4. Positive- and negative-control sera

The positive-control serum was obtained from a *B. canis* culture-positive and serologically positive (RSAT and AGID positive) animal. The negative-control serum was obtained from a serologically negative (RSAT and AGID negative) animal belonging to a kennel without clinical, serological or bacteriological evidences of brucellosis. These controls were included in all iELISA assays. The same controls were used to evaluate each new lot of LFIA strips.

2.5. Silver-stained SDS-PAGE

Different quantities of *B. canis* antigen and *Escherichia coli* O111:B4 smooth lipopolysaccharide (Sigma #L4130) were analyzed by denaturing 15% SDS-PAGE followed by silver staining (Tsai and Frasch, 1982).

2.6. Western blotting

The *B. canis* antigen (10 μg) was subjected to 15% SDS-PAGE and transferred to nitrocellulose membranes by semidry electroblotting. Immunoblotting was performed with the indicated serum samples or the following monoclonal antibodies (MAbs) against *Brucella* rLPS and Omps, kindly provided by Cloeckaert (Cloeckaert et al., 1990, 1992): rLPS A68/24D08/G09 (IgG1), Omp10 A68/08E07/B11 (IgG2a), Omp16 A68/04G01/C06 (IgG2a), Omp19 A76/18B02/D06 (IgG2a), Omp25 A59/05F01/C09 (IgG2a) and Omp31 A59/10F09/G10 (IgG2a). Serum samples and MAbs were diluted in 0.05 M Tris-buffered saline pH 7.5 containing 0.1% Tween 20 (TBST) and 5% of bovine skim milk at 1:1000 and 1:2000 dilutions, respectively. Bound antibodies were visualized using horseradish peroxidase (HRP)-labeled rabbit anti-canine IgG (Jackson ImmunoResearch Laboratories) or HRP-labeled goat anti-mouse IgG (Sigma) secondary antibodies and enhanced chemiluminescence (SuperSignal West Pico chemiluminescent substrate detection reagents; Pierce Chemical Co.), according to the manufacturer's instructions.

2.7. B. canis-iELISA development and optimization

Microtiter plates (Corning® #3591) were coated with 100 µl of *B. canis* antigen at 50 µg/ml. The antigen was diluted in 0.05 M carbonate buffer, pH 9.6 and incubated for 18 h at 4 °C. The plates were blocked with 5% bovine skim milk in 0.01 M phosphate-buffered saline pH 7.2 (PBS) containing 0.2% Tween 20 (PBST) for 1 h at room temperature (RT). Serum samples were diluted in PBST, added in duplicate and incubated for 1 h at RT. Positive- and negative-control samples were included in each plate. Subsequently, horseradish peroxidase (HRP)-labeled rabbit anti-canine IgG (Jackson ImmunoResearch Laboratories) antibodies diluted in 0.4% BSA PBST was added and incubated for 1 h at RT. Between each reaction step, the plates were washed five times with PBST. After incubating with the substrate (0.015% H_2O_2 and 0.01% 3,3',5,5'-Tetramethylbenzidine [TMB] in citrate-phosphate buffer, pH 5.0) for 10 min at RT, the reaction was stopped with 1% HCl and the absorbance was determined at 450 nanometers using a plate reader (FilterMax F5Multi-Mode Microplate Reader, Molecular Devices). Optimization of the assay was performed as previously described (Ciocchini et al., 2014). Based on these analyses, the optimal antigen concentration was 50 µg/ml (5 µg/well) and the optimal dilution of the samples and conjugate were 1:400 and 1:16,000, respectively. These established parameters were used to test all the samples.

2.8. B. canis-LFIA development and optimization

The *B. canis*-LFIA strips consist of a backing card (BC) to which the conjugate pad (CP), nitrocellulose membrane (M) and absorbent pad (wick) were laminated. The colloidal gold-labeled goat anti-canine IgG antibodies were dispensed at a speed of 10 µl per cm into the pretreated CP and dried. The membrane was stripped with the *B. canis* antigen (test line, TL) and donkey anti-goat IgG antibodies (control line, CL) at a speed of 1 µl per cm and a concentration of 1.0 mg/ml and 0.5 mg/ml, respectively. After all the reagents were dispensed and dried, the materials were laminated over the BC. Two plastic cover tapes were placed manually over the laminated sheet; one in the upper part of the sheet covering the wick and the overlap of the wick with the M, and the other in the lower part covering the overlap of the CP with the M. Finally, laminated sheets were cut into strips of 4.5 mm width and stored in a moisture-resistant pouch made of plastic-coated aluminum foil containing a silica gel packet. Optimization of the amounts of antigen, antibodies and detection reagents as well as the selection to the different materials was performed in a step-by-step procedure with a panel of positive and negative control sera. The flow assay was performed placing the strip into a test tube containing 100 µl of running buffer and 5 µl of serum. The result of the test was read after 10 min by visual inspection for staining of the TL and CL. The CL should stain in all cases; no staining of the CL invalidated the test. The assay was scored negative when no staining of the TL occurred, and positive when a distinct staining of the TL was observed.

2.9. Other tests

The rapid slide agglutination test (RSAT) and agar gel immunodiffusion test (AGID) were performed by the National Brucellosis Reference Laboratory (DILAB-SENASA) of Argentina as previously described (Carmichael and Joubert, 1987; Myers et al., 1974).

2.10. Data analysis

B. canis-iELISA reactivity values were expressed as percentage of reactivity of the mean absorbance at 450 nm (Abs450) of the positive-control serum included in each assay. Percentage of reactivity (PR) was calculated as follows: PR = (mean Abs450 of the test sample/mean Abs450 of the positive control) x 100. Dotplot, receiver-operating characteristic (ROC) (Greiner and Gardner, 2000; Swets, 1988), two graph-ROC (TG-ROC) (a plot of the diagnostic sensitivity and specificity of the assay as a function of the cut-off values) (Greiner et al., 1995), and Mann-Whitney test analyses were performed using the GraphPad Prism software (version 5.01 for Windows; GraphPad, San Diego, CA, USA). Inter-rater agreement for qualitative (categorical) items was measured by Coheńs kappa statistic (κ) (Viera and Garrett, 2005).

3. Results

3.1. Production and characterization of the antigen

It has been previously demonstrated that the rLPS is the immunodominant antigen in *B. canis* infections, and serological assays based on the detection of antibodies against surface antigens are the most sensitive tests (Barrouin-Melo et al., 2007; Keid et al., 2009; Lucero et al., 2002). In this work, we produced the *B. canis* antigen using a hot-saline improved method with several modifications (see Materials and methods) that allowed us to obtain greater reproducibility between different batches of the antigen. After lyophilization, the antigen was quantified by weighing the dried product and characterized by SDS-PAGE and silver staining, in comparison with the LPS from *E. coli* O111:B4 (Fig. 1A), and immunoblotting with anti-rLPS and anti-Omps monoclonal antibodies (MAbs) (Fig. 1B). Silver staining showed a band that migrated above the 10 kDa marker that reacted with the MAb against the rLPS (Fig. 1B). Additionally, bands of approximately 16, 25 and 31 kDa were detected with the MAbs directed to Omp16, Omp25 and Omp31, respectively (Fig. 1B). With the anti Omp31 MAb a double band was detected; a similar reactivity pattern was previously observed using this MAb by Cloeckaert et al. (Cloeckaert et al., 1990, 1992). No reactivity was observed with anti Omp10 and Omp19 MAbs (Fig. 1B). To evaluate the potential use of this antigen preparation for serodiagnosis of canine brucellosis, an immunoblotting was performed with serum samples obtained from culture-positive and serologically positive (RSAT and AGID positive) dogs, and serum samples from healthy dogs with negative results by RSAT and AGID (Fig. 1C). A strong reactivity against the rLPS was observed with the positive samples but not with the serum samples from healthy dogs. Bands compatible with the presence of antibodies against Omp16 and Omp25/Omp31, and a band of strong reactivity between the 10 and 15 kDa markers were observed with the most reactive samples (Fig. 1C). This low molecular weight band was also faintly observed by silver stained SDS-PAGE and immunoblotting with the anti rLPS MAb (Fig. 1A and 1B). These results demonstrate that the *B. canis* antigen is mainly composed of rLPS and probably several Omps that copurify with it, and that it could be useful for the development of new diagnostic tools for canine brucellosis.

3.2. B. canis-iELISA development and validation

An indirect ELISA coupling the *B. canis* antigen to microtiter plates (*B. canis*-iELISA) was developed and optimized as indicated in Materials and Methods. To validate the *B. canis*-iELISA for the serodiagnosis of canine brucellosis, a total of 284 serum samples were analyzed. This panel included 71 positive reference samples (POS) obtained from dogs in which the infection was confirmed by bacteriological culture and serology (RSAT and AGID positive), and 213 samples obtained from healthy animals with negative results by RSAT and AGID (NEG). The results of the analysis were outlined in a dotplot diagram and showed a very good discrimination power with minimal overlapping between POS and NEG groups (Fig. 2A).

In order to evaluate the diagnostic performance of the *B. canis*-iELISA, a ROC analysis was performed calculating the rate of true-positive (Se) and false-positive (1-Sp) results for all the possible reactivity values of the assay. Based on this analysis, the area under the ROC curve (AUC) was of 0.9989 (0.9971 to 1.000, 95% confidence interval) indicating that assay is highly accurate. Selection of the cut-off values

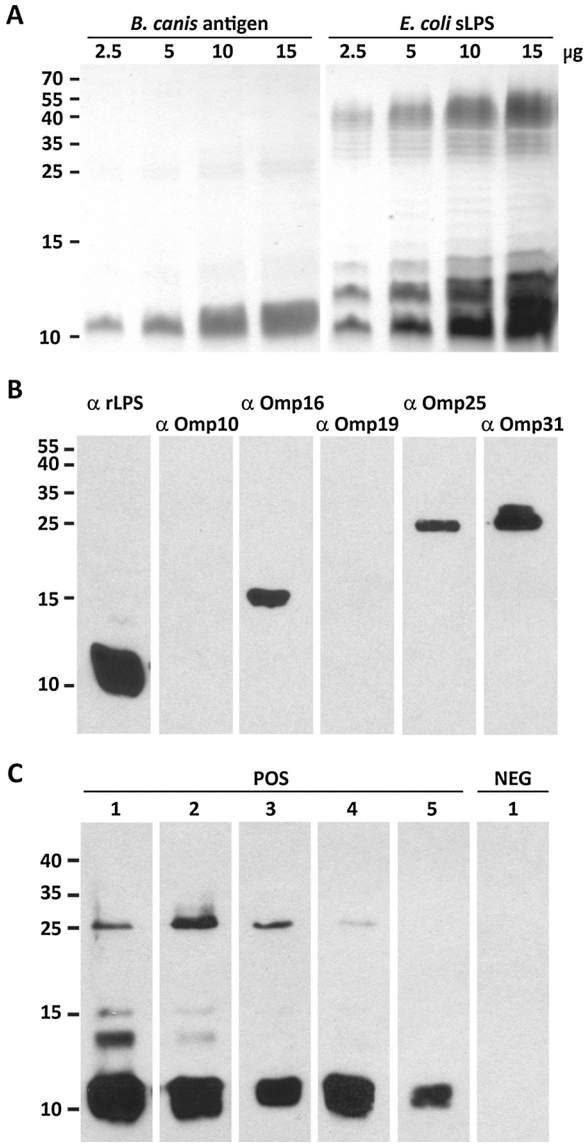


Fig. 1. Silver-stained SDS-PAGE and Western blot analysis of the *B. canis* antigen. (A) 2.5 to 15 µg of the *B. canis* antigen and *E. coli* O111:B4 smooth lipopolysaccharide (sLPS) were analyzed by denaturing 15% SDS-PAGE followed by silver staining. (B) Immunoblotting with monoclonal antibodies against *Brucella* rLPS and the indicated outer membrane proteins (Omps). (C) Immunoblotting with serum samples obtained from culture-positive and serologically positive (RSAT and AGID positive) dogs, and a serum sample from a healthy dog with negative results by RSAT and AGID (only the analysis of representative samples is shown). The position of the molecular weight standards (in kDa) is indicated on the left.

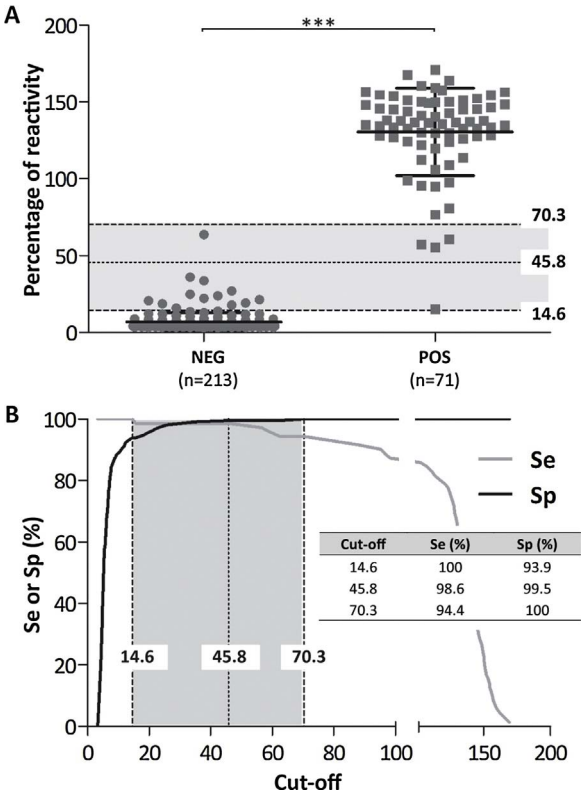


Fig. 2. Dotplot and ROC analysis of *B. canis*-iELISA results. (A) Dotplot analysis. Serum samples from positive (POS) and negative (NEG) animals were tested with the *B. canis*-iELISA as indicated in Materials and Methods. The POS group included serum samples obtained from culture-positive and serologically positive (by RSAT and AGID) dogs. The NEG group included samples obtained from healthy animals with negative results by RSAT and AGID. The mean and standard deviation for each group are indicated: POS, 130.5 ± 28.34; NEG, 6.93 ± 6.17. ***, $P < 0.0001$; Mann-Whitney test. (B) TG-ROC plot of the results. ROC analysis was carried out using as reference samples the POS and NEG groups described for the dotplot in panel A. The dashed lines indicate the cut-off values for which maximal diagnostic Se or Sp were achieved (14.6% and 70.3% cut-off values). These two cut-off values represent the bounds of an intermediate range (IR) of reactivity values (shaded areas). The dotted line indicates the cut-off value that concurrently optimizes Se and Sp (cut-off 45.8%). Inset: Se and Sp values obtained for the three indicated cut-off values.

that optimize the diagnostic sensitivity (Se) and/or specificity (Sp) of the *B. canis*-iELISA was performed by a TG-ROC plot (Fig. 2B). The cut-off values for maximal Se (100%) and Sp (100%) were 14.6% and 70.3%, respectively. The cut-off value that concurrently optimized the Se and Sp was 45.8% (Se = 98.6%, Sp = 99.5%).

Only one sample of the POS group (sample number 205) gave a striking low reactivity value by *B. canis*-iELISA (15.3%) although this value was above the lowest selected cut-off (14.6%) (Fig. 2A). This sample was also negative by Western blot using as antigen a total extract of *B. canis* or the purified *B. canis* antigen (data not shown). On the other hand, the serum sample number 657 from the NEG group showed a reactivity value of 68.2% (Fig. 2A) and when this sample was analyzed by Western blot against the *B. canis* antigen or a total extract of *B. canis*, a clear positive reactivity was observed (data not shown). Based on these results a second ROC analysis was performed not including these two serum samples. Selecting a cut-off value of 45.8%, the same value that concurrently optimized the Se and Sp in the first ROC analysis including all the samples, the Se and Sp of the test were of 100%. For these reasons, 45.8% was selected as the cut-off value for the interpretation of the upcoming results.

Taken together, these results demonstrate that the *B. canis*-iELISA is a highly accurate test for the serodiagnosis of canine brucellosis.

3.3. Double-blind analysis

To further validate the assay, a double-blind study was carried out with 1040 encoded serum samples obtained from dogs coming from Villa Hidalgo, Province of Buenos Aires, Argentina, and collected during the period 2014–2016. The serum samples included in this study were taken from dogs who attended the veterinary consultation for anti-rabies vaccination and/or castration prior informed consent signed by their owners. These animals comes from an urban area and were pets that were allowed to roam and be in contact with other dogs. All samples were analyzed blindly by RSAT (performed by DiLab-SENASA) as well as with the *B. canis*-iELISA, and classified as positive or negative based on RSAT results (Fig. 3). Of the 1040 samples, 984 were negative and 56 positive by RSAT. Considering a cut-off value of 45.8%, 975 samples (975/984, 99.1%) of the RSAT NEG group were also negative

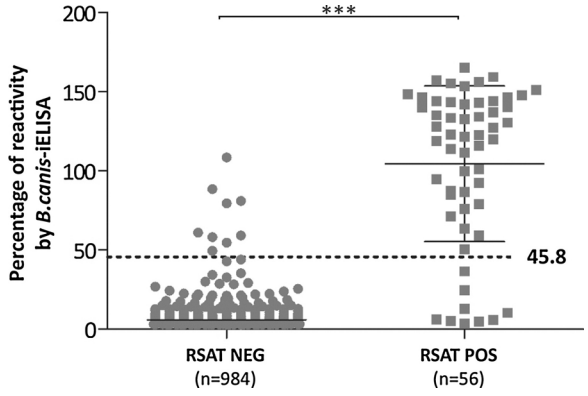


Fig. 3. Double-blind analysis. Dotplot of the results obtained with the *B. canis*-iELISA performed with RSAT positive (POS) and negative (NEG) samples from animals of Villa Hidalgo, Province of Buenos Aires, Argentina. The mean and standard deviation for each group are indicated: RSAT POS, 104.6 ± 49.1; RSAT NEG, 5.9 ± 7.7. ***, P < 0.0001; Mann-Whitney test.

Table 1
Double-blind analysis. Serum samples that were positive by RSAT but negative by *B. canis*-iELISA, and negative by RSAT but positive by *B. canis*-iELISA.

Sample no.	RSAT[a]	AGID[a]	*B. canis*-iELISA[b]
RSAT positive and B. canis-iELISA negative			
B025	POS	NEG	6.4
B127	POS	POS	10.5
B141	POS	POS	5.8
B406	POS	POS	5.5
B532	POS	NEG	4.9
B790	Weak POS	POS	36.8
B960	POS	POS	24.9
B962	Weak POS	NEG	3.7
B965	POS	NEG	13.1
RSAT negative and B. canis-iELISA positive			
B079	NEG	NEG	88.7
B136	NEG	NEG	79.5
B264	NEG	NEG	54.7
B421	NEG	NEG	81.1
B422	NEG	NEG	108.6
B508	NEG	NEG	61.1
B639	NEG	NEG	58.2
B849	NEG	NEG	59.3
B1018	NEG	NEG	49.5

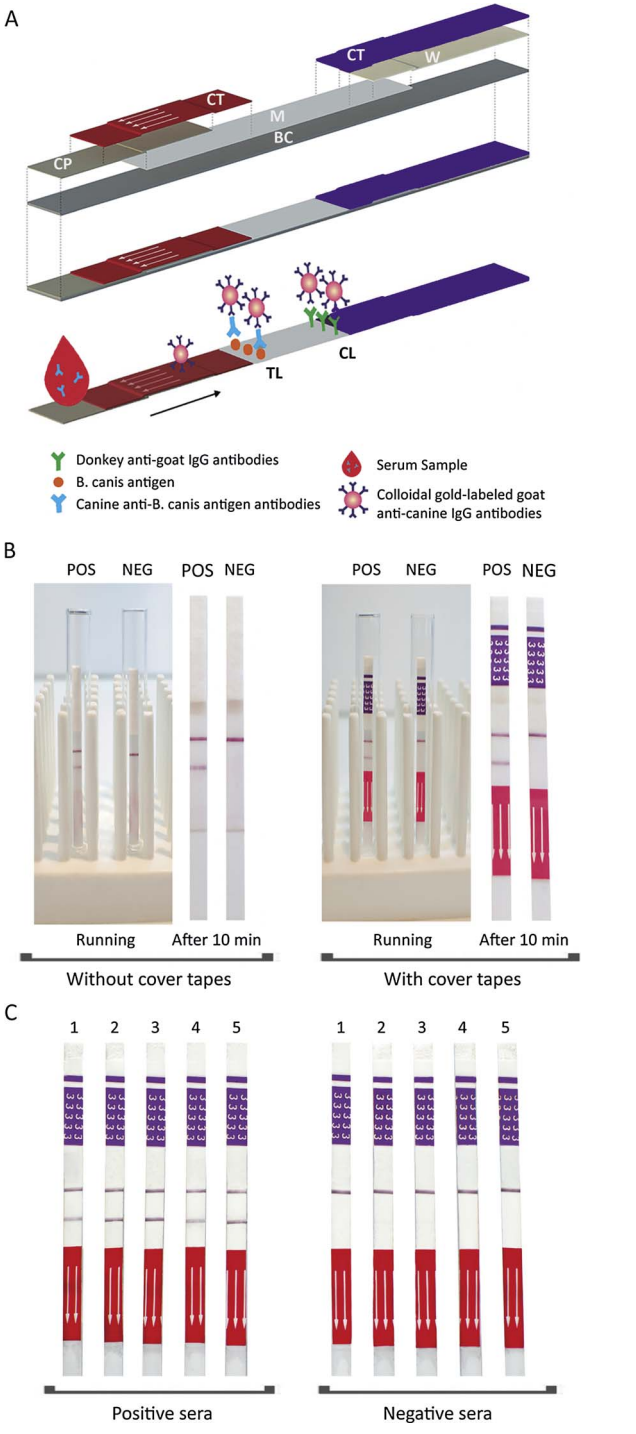
[a] POS, positive; NEG, negative.
[b] *B. canis*-iELISA cut-off value, > 45.8%.

with the *B. canis*-iELISA, and 47 samples (47/56, 83.9%) of the RSAT POS group were positive by iELISA (Fig. 3). Nine samples of the RSAT NEG group were positive by the *B. canis*-iELISA and 9 samples of the RSAT POS group were negative by the *B. canis*-iELISA (Fig. 3 and Table 1). Correlation between RSAT and *B. canis*-iELISA was measured using Cohen's kappa statistic (κ) given a kappa index of 0.835 (κ = 0.835). It is considered that a kappa value higher than 0.810 corresponds to an almost perfect agreement between the two tests under analysis (Viera and Garrett, 2005).

Those samples that were positive by RSAT and/or the *B. canis*-iELISA were also analyzed by AGID (performed by DiLab-SENASA) (Table 1 and Table S1, in the supplemental material). Four of the 9 samples that were RSAT positive and iELISA negative were negative by AGID (Table 1). Of the 9 samples that were RSAT negative but iELISA positive, all resulted negative by AGID. Finally, of the 47 samples that were positive by RSAT and iELISA, 17 (36.2%) were negative by AGID, indicating a lower sensitivity of the AGID test in comparison with RSAT or *B. canis*-iELISA (Table S1, in the supplemental material).

3.4. *B. canis*-LFIA development and validation

With the aim of implementing a rapid, simple to perform and low cost serological screening test for canine brucellosis, a lateral flow immunoassay in dipstick format (*B. canis*-LFIA) was developed and optimized as indicated in Materials and Methods. A scheme of the structure and the principle of the assay are shown in Fig. 4A. The *B. canis*-LFIA is based on the detection of IgG antibodies against the *B. canis* antigen immobilized at the test line (TL) in the membrane. The assay is performed by placing the strip into a test tube containing the serum sample diluted in running buffer and the result is read in 10 min (Fig. 4B). No differences in the performance of the assay were observed



(caption on next page)

Fig. 4. *B. canis*-LFIA. (A) Schematic diagram of the lateral flow strip structure in dipstick format and principle of the assay. The device consists of a backing card (BC) to which the conjugate pad (CP), nitrocellulose membrane (M), absorbent pad (wick, W) and cover tapes (CT) are laminated. Colloidal gold (Au)-labeled goat anti-canine IgG antibodies are dispensed into the CP and the nitrocellulose membrane is stripped with the *B. canis* antigen and donkey anti-goat IgG antibodies at the test line (TL) and control line (CL), respectively. The arrow indicates the direction of liquid flow during the assay that is started by adding the serum sample (diluted in running buffer) at the proximal end of the strip in the CP. The sample migrates to the zone where the dried colloidal gold conjugate was dispensed and re-mobilizes it. The dog anti-*B. canis* antigen IgG antibodies react with the Au-labeled goat anti-dog IgG conjugate to form a complex that migrates into the membrane. In the membrane, the specific antibody-Au complexes are captured at the TL and the Au-conjugate at the CL. Excess reagents are entrapped by the absorbent pad. (B) Pictures showing lateral flow strips in dipstick format for the positive and negative control sera with and without cover tapes. The assay is performed by placing the strip into a test tube containing 100 µl of running buffer and 5 µl of serum. The result of the test was read after 10 min by visual inspection for staining of the TL and CL. No reaction at the CL invalidates the test. The assay is scored negative when no staining of the TL occurred and positive when a distinct staining of the TL is observed. POS, control positive serum sample; NEG, control negative serum sample. (C) Pictures of strips in dipstick format with cover tapes showing the results for five representative positive and negative samples of the panel used to validate the assay.

using the *B. canis*-LFIA with or without the cover tapes in the upper and lower part of the strips (Fig. 4B). The cover tapes protect the strip, indicate how the strip should be handled and placed in the test tube and ensure the correct overlapping of the conjugate pad with the membrane as well as of the wick with the membrane.

To validate the *B. canis*-LFIA, the same sample panel (71 positive reference samples and 213 negative reference samples) used to evaluate the *B. canis*-iELISA was analyzed. Of the 71 positive samples, all were positive with the *B. canis*-LFIA except sample number 205, the same sample that resulted negative in the *B. canis*-iELISA, giving a diagnostic sensitivity of 98.6%. All the negative reference samples (n = 213) were negative with the *B. canis*-LFIA giving a diagnostic specificity of 100%. Five representative strips assayed with positive and negative samples are shown in Fig. 4C.

Finally, 203 samples coming from the double-blind study were also analyzed with the *B. canis*-LFIA (RSAT positive samples, n = 56; RSAT negative samples, n = 147). Five of the RSAT positive samples were negative with the *B. canis*-LFIA, and 9 of the RSAT negative samples were positive (Table 2). The correlation analysis resulted in a Cohen's statistic value of 0.831 (κ = 0.831) indicating an almost perfect correlation between RSAT and the *B. canis*-LFIA.

Overall, our results demonstrate that the *B. canis*-LFIA is a rapid, simple and accurate test that could be used as a screening test for serodiagnosis of brucellosis in dogs.

4. Discussion

Early and accurate diagnosis of canine brucellosis is of central importance to detect and control the disease in dogs and lowering the risk of transmission to humans. Unfortunately, the current tests for diagnosing canine brucellosis do not meet the need of simple, rapid and accurate assays for field and laboratory applications. Considering that the rLPS is the immunodominant molecule during *B. canis* infections and serological assays based on the detection of antibodies against this antigen proved to be the most sensitive tests, we developed new tools for laboratory and field serodiagnosis of the disease based on rLPS antigen in the ELISA and lateral flow platforms.

Table 2
Analysis by *B. canis*-LFIA of a subset of serum samples coming from the double-blind study.

		B. canis-LFIA		
		POS	NEG	
RSAT	POS	51	5	56
	NEG	9	138	147
		60	142	

In this work, we developed and validated the *B. canis*-iELISA and the *B. canis*-LFIA assays. The improved method used to produce the *B. canis* antigen allowed us to produce more homogeneous batches of the antigen that facilitated the standardization of the assays. Instead, using the technique of Galanos et al. (Galanos et al., 1969) and the hot phenol-water method (Westphal and Jann, 1965) with different modifications to produce the antigen we were unable to obtain reproducible results. The antigen is mainly composed of *B. canis* rLPS which co-purifies with a group of Omps that includes Omp16, Omp25 and Omp31 and probably some others yet undetermined. Omps are antigenic proteins of *Brucella* that can improve the diagnostic performance of the assays (Cassataro et al., 2004; Simborio et al., 2015). In this sense, all the analyzed positive sera mainly reacted against the rLPS and most of them also recognized Omps, probably Omp25/Omp31 and Omp16 as judged by the Western blot profile (Fig. 1C). However, there were several positive samples that showed reactivity only against the rLPS confirming that this molecule is the immunodominant antigen in *B. canis* infections. *B. canis*-iELISA and *B. canis*-LFIA were validated using a well characterized panel of 71 positive and 213 negative samples. For the *B. canis*-iELISA, selecting a cut-off value of 45.8%, the diagnostic Se was of 98.6% and the Sp 99.5%. Se and Sp reached a value of 100% when samples 205 and 657, whose status could not be confirmed by immunoblotting, were not considered in the ROC analysis. In comparison with other validated and reported iELISAs for diagnosis of canine brucellosis (Barrouin-Melo et al., 2007; de Oliveira et al., 2011), the *B. canis*-iELISA developed in this work showed a better diagnostic performance that could be explained by the differences in the antigen preparation. The *B. canis*-LFIA showed a Se and Sp of 98.6% and 100%, respectively. Considering that usually LFIAs have lower Se than other diagnostic assays (Keid et al., 2015), the excellent performance in terms of diagnostic Se and Sp obtained with the *B. canis*-LFIA in this work could represent a real improvement in canine brucellosis diagnostics specially for its application in the field and laboratories with no or minimal infrastructure.

Further validation of the *B. canis*-iELISA was performed by a serological blind study including more than 1000 serum samples obtained from pets that were allowed to roam. Excellent agreement between the results obtained by *B. canis*-ELISA and RSAT (κ = 0.835) was observed. For the *B. canis*-LFIA, the analysis of a subset of these samples showed also an excellent correlation (κ = 0.831) with RSAT. Additionally, we observed a lower diagnostic sensitivity of the AGID test in comparison with the ELISA and LFIA developed in this work, and with the RSAT, as it was previously reported (Keid et al., 2009). In this study, the estimated serological prevalence of *B. canis* was 5.4% (56/1040) based on RSAT and *B. canis*-iELISA results. This observation highlight the need of performing a widespread serological survey to determine the magnitude of *B. canis* circulation among the urban dog population in Argentina. This is relevant due to the risk of transmission of this zoonotic pathogen from dogs to humans.

Control of canine brucellosis depends on the early and accurate detection of infected dogs. The disease is usually introduced in a breeding kennel by an infected dog or by contaminated semen used to inseminate females. New animals should be tested before their introduction to the general population as well as before their release from quarantine in order to detect those in the early stage of infection that resulted seronegative upon arrival. It is also recommended to test domestic and kennel dogs either annually or before breeding. Due to the lack of highly accurate and quick test, most of the infected dogs are unnoticed. This determines that the magnitude of this urban zoonosis remains neglected. For these reasons, we propose the implementation of the *B. canis*-LFIA as a screening serological test in combination with the highly accurate laboratory test *B. canis*-iELISA. The *B. canis*-LFIA is a rapid, easy to use and high sensitive and specific test, characteristics

that make it an ideal test for the serological surveillance of canine brucellosis in the field or in low complexity veterinary laboratories. In this regard, the *B. canis*-iELISA and *B. canis*-LFIA immunoassays developed and validated in this work could facilitate the effective control of the disease in dogs reducing the risk of transmission to humans.

5. Conclusion

Canine brucellosis has been considered as an infection of low zoonotic potential. This misconception influenced the paucity of scientific advance in this subject, which is reflected in the scarce high precision diagnostic tests that exist today. In recent years, several reports of human brucellosis cases with confirmed isolation of *B. canis* raised a red flag on this neglected urban zoonosis. This fact, coupled to the underestimation of the clinical relevance of *B. canis* both in humans and dogs, impose the need to develop new high-performance diagnostic tests. Here, we describes the development and validation of enzyme-based (*B. canis*-iELISA) and lateral flow (*B. canis*-LFIA) immunoassays for improved serodiagnosis of canine brucellosis using as antigen the *B. canis* rLPS. We have demonstrated that *B. canis*-iELISA and *B. canis*-LFIA have an excellent diagnostic performance in terms of sensitivity and specificity. In order to improve the detection and control of the disease in dogs and reduce the risk of transmission of brucellosis to humans, we propose the implementation of the *B. canis*-LFIA as a rapid, easy to use screening test and the *B. canis*-iELISA as a confirmation test. Additionally, these tests were evaluated in a serological double-blind study including 1040 serum samples obtained from urban dogs showing a serological prevalence higher than 5%. These results reveal the need of implementing more effective measures to control the disease in dogs which would also lead to a lower risk of transmission to humans.

Conflict of interest statement

No conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetmic.2017.08.005.

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