

Indirect ELISA for diagnosis of *Brucella ovis* infection in rams

[ELISA indireto para diagnóstico da infecção por *Brucella ovis* em carneiros]

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ABSTRACT

Brucella ovis is a major cause of epididymitis in sexually mature rams, resulting in subfertility, infertility, and economic losses for the sheep industry worldwide. The aim of this study was to develop an indirect ELISA (iELISA) using recombinant proteins, namely rBoP59 and rBP26, as antigens for serological diagnosis of *B. ovis* infection. The BoP59 and BP26 recombinant proteins were expressed in *E. coli* and purified by affinity chromatography. Antigenicity was tested by Western blot and iELISA. Standardization of iELISA was performed with 500ng and 1µg BoP59 and rBP26 per well, testing serum from uninfected and experimentally infected rams. rBP26 was effective in distinguishing positive from negative rams. The rBP26 iELISA developed in this study is the first to use a completely purified rBP26 as antigen resulting in high sensitivity (100%) and specificity (90.2%), and an overall accuracy equal to 1.0.

Keywords: *Brucella ovis*, brucellosis, ELISAI, BP26, Western blot

RESUMO

Brucella ovis é uma das principais causas de epididimite em carneiros sexualmente maduros, resultando em subfertilidade e infertilidade e consequentes perdas econômicas para a ovinocultura em todo o mundo. O objetivo deste estudo foi desenvolver ELISA indireto (ELISAI), utilizando como antígeno proteínas recombinantes BoP59r e BP26r para diagnóstico da infecção por *B. ovis*. BoP59r e BP26r foram expressas em *E. coli* e purificadas por cromatografia de afinidade e a antigenicidade testada por Western blot e ELISAI. A padronização do ELISAI foi realizada testando 500 ng e 1 µg de BoP59r e BP26r por poço e soros de carneiros infectados e não infectados. A BP26r foi eficiente em diferenciar ovinos negativos de positivos. O ELISAI com BP26 desenvolvido neste estudo foi o primeiro a utilizar BP26 completamente purificada como antígeno, resultando em elevada sensibilidade (100%) e sensibilidade (90,2%), com acurácia igual a 1,0.

Palavras-chave: *Brucella ovis*, brucelose, ELISAI, BoP59, BP26, Western blot

INTRODUCTION

Epididymitis due to *Brucella ovis* infection is considered the most important cause of infectious reproductive disease in sheep that is associated with subfertility and infertility (Burgess *et al.*, 1982; Carvalho Júnior *et al.*, 2012). *Brucella ovis* is a Gram-negative

coccobacillus, with naturally rough LPS, which preferentially infects sheep causing chronic disease associated with epididymitis in sexually mature rams, abortion and birth of weak lambs (Xavier *et al.*, 2009).

Diagnosis of infection with *B. ovis* is accomplished by direct methods, such as bacteriology and PCR, and indirect or serological

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methods. Serological methods are the most commonly used tests for the diagnosis of *B. ovis* infection (Burgess, 1982), particularly immunodiffusion in agar gel (AGID), complement fixation (CF) (Xavier *et al.*, 2011), and indirect ELISA (enzyme-linked immunosorbent assay) (Nielsen *et al.*, 2007). Although there are several techniques available for serological diagnosis of *B. ovis* infection, none have high sensitivity and specificity, and therefore the combination of two diagnostic methods may be required for an accurate diagnosis (Costa *et al.*, 2012). Thus, the development of more efficient diagnostic tests is still a challenge for this particular disease.

BP26 is an outer membrane protein of *Brucella* spp. that has been shown to form a channel-like structure (Kim *et al.*, 2013). It is an antigenic and a serologic marker of infection by *B. melitensis* in humans (Liang *et al.*, 2010), sheep (Seco-Mediavilla *et al.*, 2003), and goats (Liang *et al.*, 2010), by *B. abortus* in cattle (Rossetti *et al.*, 1996) and *B. ovis* in sheep (Seco-Mediavilla *et al.*, 2003). BP26 is immunodominant in cattle, sheep, goats, and humans (Lindler *et al.*, 1996), and its gene (*BP26*) is conserved among classical species of *Brucella* (Seco-Mediavilla *et al.*, 2003; Liang *et al.*, 2010), with 100% identity between *B. melitensis*, *B. abortus*, *B. suis* (Liang *et al.*, 2010), and *B. ovis* (Seco-Mediavilla *et al.*, 2003). Indirect ELISA (iELISA) using recombinant BP26 (rBP26) as antigen have been developed for serodiagnosis of infections with *B. melitensis* in sheep (Cloeckert *et al.*, 2001) and goats (Gupta *et al.*, 2010), *B. abortus* in cattle (Tiwari *et al.*, 2011), *B. suis* in pigs (McGiven *et al.*, 2012), and *B. ovis* in sheep (Zygmunt *et al.*, 2002), in this later case using a partially purified BP26. However, purified rBP26 has not yet been tested for diagnosis of *B. ovis* infection. The VirB12 protein is a serum marker of infection by *B. abortus* in cattle and mice, and *B. melitensis* in goats (Rolán *et al.*, 2008). However, VirB12 has not been tested as a serological marker of *B. ovis* infection. This study also included a putative hemagglutinin that has been identified by Tsolis *et al.* (2009) as part of a recently characterized *B. ovis* pathogenicity island 1 – BOPI-1 (Silva *et al.*, 2011). A potential pathogenic role of this gene has been evaluated and it proved to play no role during *in vivo* infection in the mouse (Silva *et al.*, 2011), although its antigenic potential has not been evaluated.

The aim of this study was to develop an indirect ELISA (iELISA) using recombinant proteins, namely rBoP59 and rBP26, as antigens for serological diagnosis of *B. ovis* infection.

MATERIAL AND METHODS

The *hmg* gene (GenBank accession number NC_009504) for production of recombinant protein BoP59 (BoP59r) of *B. ovis* was amplified by PCR using the sense primer (5' - CACCATGAATTTGGAAGACAGTGGTGC - 3') and antisense (5' - TAGCTCAATGCCGTTGAAGGC - 3'), resulting in a fragment of 1808 bp. PCR product was cloned into TOPO pTrcHis[®] vector using the pTrcHis TOPO[®] TA Expression Kit (Invitrogen, USA) upon insertion of the *hmg* in the plasmid, containing the ampicillin resistance cassette. The *BMEI0536* gene (GenBank accession number NC_003317) for production of recombinant protein BP26 (BP26r) was amplified using gene specific primers and cloned into pXT7 vector (Liang *et al.*, 2010) using high-throughput PCR and recombination method as described previously (Davies *et al.*, 2005). The *VirB12* gene (GenBank accession number AF141604_1) was amplified using gene specific primers and cloned using high-throughput PCR and recombination method as described previously (Sun *et al.*, 2005). The cloned genes were sequenced and it was verified that the correct sequence was inserted.

Expression plasmids were transformed into electrocompetent *Escherichia coli* BL21 (Phoneutria, Brazil) by electroporation. Transformed *E. coli* BL21 containing rBoP59 expression plasmid were grown in Luria-Bertani (LB) broth (1% tryptone, 0.5% yeast extract, 1% NaCl) with 100 mg/mL ampicillin (Phoneutria, Brazil) and 1mM glucose, and incubated under agitation (200 rpm) at 37°C for 12 to 15 hours. Two aliquots of this culture were added to fresh LB with 100mg/mL ampicillin at the proportion of 1 to 10, these cultures were either induced with IPTG or non induced. The induced culture had 1mM IPTG (Invitrogen, USA), whereas 1mM glucose was added to the non induced culture. Both cultures were incubated under agitation (200 rpm) at 37°C for 5 hours. Then, 45 mL aliquots were centrifuged at 3.000 g at 4°C for 30 minutes. The supernatant was discarded and the pellet stored at -80°C until purification.

Indirect ELISA for diagnosis...

The induction protocol was identical for rVirB12 and rBP26. Transformed *E. coli* BL21 was grown in LB broth with 100mg/mL kanamycin (Gibco, USA) under agitation (200 rpm) at 37°C for 12 to 15 hours. An aliquot was added to fresh LB broth with 100 µg/mL kanamycin at a ratio of 1:50, and incubated under agitation (200 rpm) at 37°C until reaching optical density at 600nm between 0.6 and 0.8. Then, 1mM IPTG (Invitrogen, USA) was added to culture (induced), which was then cultured for another 4 hours under agitation (200 rpm) at 37°C. The other culture (non induced) was cultured under the same conditions without IPTG. Both cultures (induced and non induced) were aliquoted, centrifuged at 3.000 g at 4°C for 30 minutes. The pellet was stored at -80°C until purification by affinity chromatography.

Purification of rBoP59, rBP26, and rVirB12 was performed with the same protocol. Bacterial pellets were resuspended in 6.0mL of lysis solution (20mM Tris, 1mM EDTA, 8 M urea, 5mM Imidazole, pH 8.0 - Sigma-Aldrich, Brazil) containing a protease inhibitors cocktail (Sigma-Aldrich, Brazil) at a ratio of 1 to 100. Protein extracts were sonicated with the Ultrasonic Vibra-Cell Processor apparatus (Sonics and Materials, USA) using 10 pulses of 30 seconds each with 40 degrees of amplitude and 1 minute intervals between each pulse. The protein extract was then centrifuged at 3.000 g at 4°C for 30 minutes. Columns were washed with 10 mL of sterile ultrapure water, then with 5 mL of lysing solution (20mM Tris, 1mM EDTA, 8 M urea, 5mM Imidazole, pH 8.0). Next, the supernatant was loaded onto a nickel chelate affinity chromatography column, GC (Glutathione Sepharose) TrapTM 4B columns (GE helthcare, USA). Then, the column was washed sequentially with solutions of imidazole in increasing concentrations, 5mM, 40mM, 80mM, 120mM, 250mM and 1 M. An aliquot of each wash, at different concentrations of Imidazole was collected and stored at -80°C, for subsequent analysis by SDS-PAGE. Protein quantification was performed using the modified Bradford method (Neuhoff *et al.*, 1988). Western blot using anti-His antibody was performed to confirm the expression of these recombinant proteins.

For Western blot analysis and evaluation of repeatability and reproducibility of iELISA using

rBoP59 and rBP26 antigens, serum samples from two rams were used, one negative and one positive for *B. ovis* based on AGID, urine PCR, and bacteriology. The antigen used in AGID tests was made from soluble extract of heat-inactivated *B. ovis* strain REO198 (TECPAR, Brazil).

iELISA with rBP26 were performed on 96 serum samples from 1-year-old cross-bred rams that were inoculated with a total of 3.6×10^9 CFU/ram of *B. ovis* (strain ATCC25840) intraconjunctivally and intrapreputially. Of the 96 samples, 41 were negative and 55 positive by AGID, urine PCR, and bacteriology, simultaneously. Additionally, rBP26 iELISA were performed using 82 AGID-negative and 42 AGID-positive serum samples collected under field conditions.

For Western blot analysis, aliquots containing 20µg of proteins from a total *B. ovis* lysate, as well as purified rBoP59, rBP26, and rVirB12, were submitted to SDS-PAGE and transferred electrophoretically onto PVDF membrane Immobilon^{®-P} (Millipore, USA) at 100 V for 1 hour using a Mini Trans-Blot system (Bio-Rad, USA) immersed in transfer buffer containing 48mM Tris base, 39mM glycine, 20% v/v methyl alcohol. The membrane was incubated in blocking solution (5% w/v skim milk in 0.1% TTBS [50mM Tris, 150mM NaCl, pH 7.5, and 0.1% v/v Tween 20]) for 1 hour under stirring at 25°C. The membrane was washed with TTBS three times, and incubated with the test serum (primary antibody) diluted at 1:100 in TTBS and 2% skimmed milk for 18 h under shaking at 4°C. The membrane was then washed three times with TTBS, and incubated with the conjugate (anti-sheep IgG conjugated rabbit peroxidase - Millipore, USA) at 1:2.000 dilution for 1 hour under shaking at 25°C, followed by three washes with TTBS. The reaction was developed using 3.3'-diaminobenzidine tetrahydrochloride (DAB, Millipore, Brazil) with H₂O₂ as a substrate, according to the manufacturer's instructions.

Standardization of iELISA with rBoP59 or rBP26 was performed with the same protocol. Polystyrene 96-well plates (Nunc, USA) were coated with 500ng or 1mg per well of purified recombinant proteins, diluted in carbonate buffer (bicarbonate pH 9.6 containing 0.015M sodium carbonate, 0.035M sodium bicarbonate). 100µL

of diluted rBoP59 or rBP26 were applied per well, and incubated at 4°C for 18 hours, then washed twice with 200µL of PBST per well, followed by incubation with 200µL of PBST containing 5% skim milk at 25°C for 1 hour, and then two washes with 200µL of PBST. After washings, 100mL of serum samples diluted in 1% PBSTL were applied and the plate incubated for 1 hour at 25°C.

Serum samples from rams that were either negative or positive by AGID, urine PCR and bacteriology were tested in duplicates at 1:5, 1:10, 1:20, 1:40, 1:80, 1:160, 1:320 and 1:640 dilutions. At the end of the incubation period three washes were performed with PBST. Then, 100µL of IgG of rabbit anti-sheep peroxidase conjugate, at a dilution of 1:2000 in 1% PBSTL were applied and the plate was incubated at 25°C for 1 hour. Three further washes with 200µL of PBST were performed, followed by incubation with 100 µL of 0.5 mg/mL of the substrate o-phenylenediamine (OPD, Invitrogen, Brazil) diluted in acid buffer pH 0.1 (5M anhydrous citric acid, 0.2M sodium phosphate and hydrogen peroxide PA 130 volumes) at 25°C for 10 minutes. The reaction was then stopped with 40 µL of 4 M sulfuric acid. Plates were read in an ELISA reader (MR-96A Microplate Reader) at 492nm. The cut-off point was calculated based on two standard deviations of the negative controls.

rBP26 iELISA data were analyzed using the STATA version 12 software for calculating overall accuracy based on the area under the ROC curve. Sensitivity, specificity, positive predictive value (PPV) and negative predictive value were calculated according to Florkowisk (2008). Repeatability (intra-plate variability) was assessed testing two samples, one positive and one negative, six times on the same plate, at the same time. Means and coefficient of variation (CV) were calculated using Excel version 2007 (Microsoft Corp, USA). Reproducibility (inter-plate variability) of the test was determined using the results of two samples, one positive and one negative, repeated 10 times. These repetitions were performed on different plates. The means and coefficients of variation (CV) were calculated using Excel version 2007 (Microsoft Corp, USA). Statistical analysis was performed using the non-parametric Mann-Whitney test (GraphPad Prism).

RESULTS AND DISCUSSION

Both rBoP59 and rBP26 were successfully expressed in transformed *E.coli* BL21, which was followed by a thorough purification as evidenced by a coomassie blue stained SDS-PAGE gel (Figure 1).

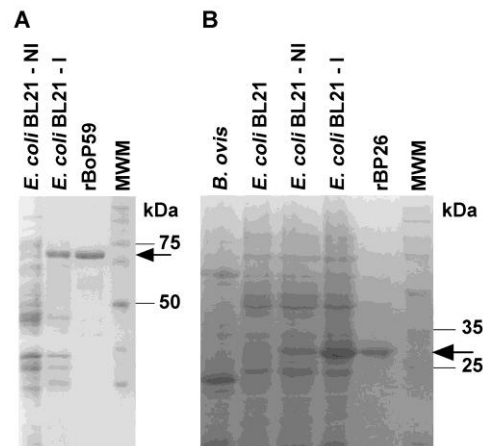


Figure 1. Coomassie blue-stained SDS-PAGE. (A) Expression of rBoP59, and (B) expression of rBP26 in transformed *Escherichia coli* BL21. Whole lysates of *Brucella ovis*, *E. coli* BL21 (induced - I or non induced - NI), purified rBoP59 or BP26, and molecular weight marker (MWM). Lateral bars indicate molecular weight and the arrows indicate the expected band for rBoP59 and BP26.

Western blot analysis with serum sample from a ram that was negative for *B. ovis* resulted in very faint bands corresponding to rBP26 and rBoP59. In contrast, on the membrane incubated with serum from an infected ram, a strong signal was observed with the whole *B. ovis* lysate as well as with rVirB12 and more strongly with BP26. Labeling of rBoP59 with serum from infected ram was somewhat similar to that observed with serum from an uninfected ram (Figure 2). Previous reports indicate that Western blot may have sensitivity and specificity as high as 98.6% (Kittelberger and Reichel, 1998) and 100% (Cerri et al., 2000), respectively. This method, employing BP26 as an antigen, has been applied for serological diagnosis of *B. melitensis* infection in sheep (Seco-Mediavilla et al., 2003) and humans (Lindler et al., 1996). VirB12 is known to be antigenic and a suitable serologic marker for *Brucella* spp. infections (Rolán et al., 2008). However, this is the first time that BoP59 and VirB12 have been tested as serological markers for *B. ovis* infection by Western blot.

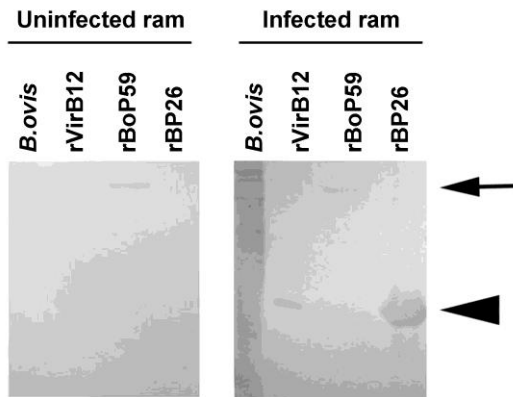


Figure 2. Western blot. Total *Brucella ovis* lysate, purified rVirB12, rBoP59, and rBP26. Membranes were incubated with serum from uninfected or *B. ovis* infected rams. The arrow indicates the expected position of the rBoP59 band and the arrow head indicates the expected position of the rBP26 and rVirB12 bands.

While assessing reproducibility of the iELISA using either 500ng or 1µg of purified rBoP59 per well, it was clearly demonstrated that the assay did not discriminate positive from negative samples, and therefore rBoP59 was not considered a suitable marker for diagnosis of *B. ovis* infection in rams (data not shown). These results are unfortunate since BoP59 is specific of *B. ovis* (Tsolis *et al.*, 2009), and should it be a suitable marker it would allow discrimination between *B. ovis* and *B. melitensis* infections in sheep, which is extremely relevant from a public health point of view since *B. melitensis* has the highest zoonotic potential among *Brucella* spp.

(Xavier *et al.*, 2009). Conversely, rBP26 proved to be consistently antigenic and clearly differentiated sera from uninfected or *B. ovis*-infected ram with a better discrimination with 500 ng/well (Figure 3). Antigenicity of BP26 has been previously reported in infections with *B. melitensis* (Lindler *et al.*, 1996; Liang *et al.*, 2010) and *B. abortus* (Connolly *et al.*, 2006). Based on these results, 500ng/well of rBP26 was selected for further characterization of the iELISA, testing serum samples from rams experimentally infected with *B. ovis* in a previous study (Xavier *et al.*, 2010; Carvalho Júnior *et al.*, 2012) and uninfected controls.

Repeatability assessment (variation between replicates) of the rBP26 iELISA indicated that the mean OD value of the negative control serum at 1:20 dilution was 0.647 (range 0.407-0.887; i.e. mean±2 standard deviations) with a CV of 18.5%. Mean OD value for the positive control serum at 1:20 dilution was 1.560 (range 1.452-1.668; i.e. mean±2 standard deviations), with a CV of 3.4%. Assessment of reproducibility (variation between assays) of the rBP26 iELISA indicated that the mean OD value for the negative control serum at 1:20 dilution was 0.789 (range 0.595-0.983; i.e. mean±2 standard deviations), with a CV of 12.3%. Mean OD value for the positive control serum at 1:20 dilution was 1.915 (range 1.517-2.313; i.e. mean ± 2 standard deviations), with a CV of 10.1%.

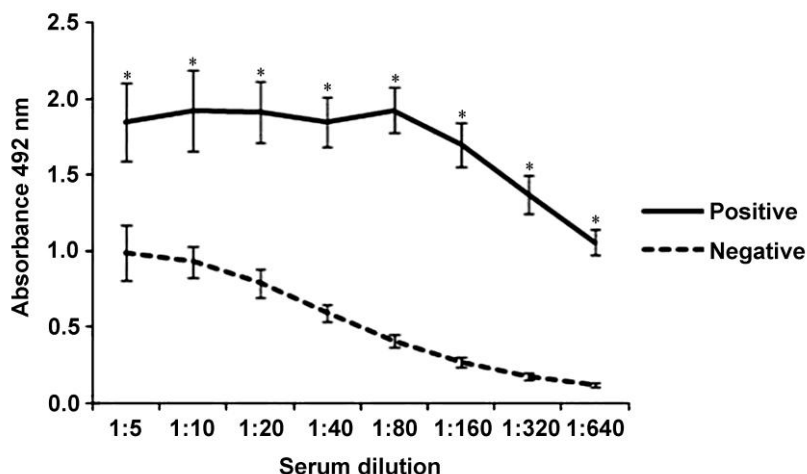


Figure 3. Reproducibility test of iELISA using 500 ng of purified rBP26 per well with samples from one positive (infected) and one negative (uninfected) ram. Means and standard deviation of 10 replicates. (*) indicates statistically significant differences with P < 0.05.

Discrimination between negative and positive serum samples by rBP26 iELISA was highly efficient using samples from rams that were experimentally infected with *B. ovis* (Figure 4). Sensitivity with serum dilutions of 1:20, 1:40, and 1:80 was 100% for all dilutions; whereas specificity was 90.2%, 85.4%, and 80.5%, respectively. As detailed in Tab. 1, accuracy was 1.0, 0.997, and 0.989, considering that good accuracy values are above 0.9 and it is ideally equal to 1.0. Furthermore, the positive predictive value was 93.2; 90.2; and 87.3 for dilutions 1:20, 1:40; and 1:80, while the negative predictive value as 100% for all dilutions.

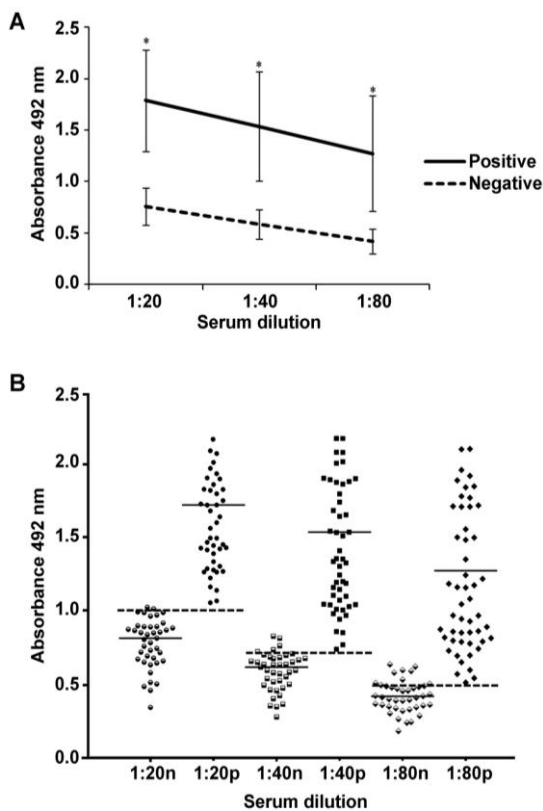


Figure 4. rBP26 iELISA for diagnosis of *Brucella ovis* infection in experimentally infected (positive, n=55) or uninfected rams (negative, n=41). Positive samples were from rams that tested positive by AGID, urine PCR, and bacteriology. Sera were tested in duplicates at 1:20, 1:40, and 1:80 dilutions. (A) 500ng of purified rBP26 were used per well. Data points represent mean and standard deviation of 10 replicates. (*) indicates statistically significant differences (P<0.05). (B) Scatter plot of rBP26 iELISA absorbance values at 492 nm of sample serum from infected (solid symbols) or uninfected rams (open symbols). The continuous black line indicates the mean and dark gray dotted line represents the cut-off for each serum dilution.

The rBP26 iELISA was applied to ovine serum samples collected under field conditions that were either negative or positive by AGID. When comparing to AGID the rBP26 iELISA generated less clear results (Figure 5). These results are likely to be a consequence of the unreliable performance of AGID (Xavier *et al.*, 2011), which does not correlate well with actual infection since rams that eliminate *B. ovis* in the semen can be AGID negative either in experimental (Nozaki *et al.*, 2011) or natural infections (Costa *et al.*, 2012).

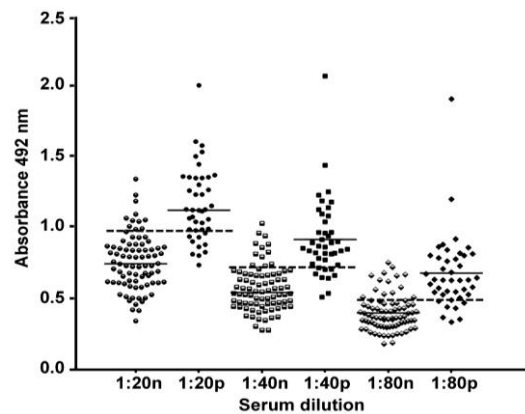


Figure 5. rBP26 iELISA for diagnosis of *Brucella ovis* infection with AGID-negative (n=82) and AGID-positive (n=42) ovine serum samples. Sera were tested at 1:20, 1:40, and 1:80 dilutions. The continuous black line represents the mean and dark gray dotted line represents the cut-off for each serum dilution.

The rBP26 iELISA developed in this study had a sensitivity of 100% with serum from experimentally infected rams, which contrasts with the rBP26 iELISA previously developed by Zygmunt *et al.* (2002) that employed a partially purified rBP26 as antigen, and had a sensitivity of 81.8%. iELISA using rBP26 as antigen was originally developed for the diagnosis of *B. melitensis* infection in sheep, with a sensitivity of 100% and specificity of 93% when testing serum samples positive by bacteriology and complement fixation (Cloeckart *et al.*, 2001). Other examples of the application of iELISA for diagnosis of brucellosis include the study by Jacques *et al.* (2007) that developed an iELISA capable of distinguishing sheep infected with *B. melitensis* from animals vaccinated with Rev 1 strain, with 100% specificity. Gupta *et al.* (2010) standardized an iELISA for diagnosing infection by *B. melitensis* in goats, with a sensitivity of 87.5% and specificity of 90%.

CONCLUSIONS

In conclusion, the rBP26 iELISA developed in this study is the first to use a completely purified rBP26 as antigen resulting in high sensitivity (100%) and specificity (90.2%), and an overall accuracy equal to 1.0, which is the highest possible value for a diagnostic test.

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