

Recombinant Outer Membrane Proteins (rOMPs) of *Brucella melitensis* as a Potential Serological Marker for Diagnosis of Caprine Brucellosis

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Abstract: The potential diagnostic ability of Recombinant Outer Membrane Proteins (rOMPs), a combination of equal concentrations of rOMP25, rOMP28 and rOMP31 of *Brucella melitensis* was investigated using Enzyme-Linked Immunosorbent Assay (ELISA) to differentiate the False Positive Serological Reactions (FPSR) in the serological diagnosis of caprine brucellosis. The rOMPs was tested using sera from three groups of goats with known *Brucella* exposure status which represent, naturally *B. melitensis* infected goats (infected), *Brucella* free goats (non-infected) and goats vaccinated with *B. melitensis* Rev. 1 vaccine strain (vaccinated). Additionally, all the sera were tested using the common serological tests which are Rose Bengal Plate Test (RBPT), BRUCELISA-400SG and Complement Fixation Test (CFT). When testing infected and non infected groups, the rOMPs I-ELISA recorded 94.44% (34/36) sensitivity and 100% (36/36) specificity and this almost agreed with the results obtained from testing the same serum samples using RBPT, BRUCELISA-400SG and CFT. However, when goats vaccinated with *B. melitensis* Rev. 1 vaccine strain were tested by the common serological tests, RBPT, BRUCELISA-400SG and CFT they wrongly recorded positive results for all the tested serum samples (26/26). While the developed rOMPs I-ELISA was able to differentiate the vaccinated from infected animals with 94.44 sensitivity and 84.62% specificity. The potential diagnostic ability of rOMPs would be of great importance as serologic marker to minimize the FPSR in eradication programs of caprine brucellosis.

Key words: *Brucella melitensis*, rOMPs, FPSR, ELISA, caprine brucellosis

INTRODUCTION

Brucellosis is one of the most important bacterial zoonoses worldwide (Garin-Bastuji *et al.*, 1998; Cutler *et al.*, 2005). The disease has important economic and public health consequences (Franco *et al.*, 2007). *Brucella melitensis* is the main etiological agent of caprine brucellosis (Alton, 1987; Corbel, 1997). The serological diagnosis of brucellosis is the most practical and economic means and recommended for large scale surveillance and/or eradication purposes (Garin-Bastuji *et al.*, 2006; OIE, 2009; Poester *et al.*, 2010). The most common serological tests for diagnosis of caprine brucellosis are Rose Bengal Plate Test (RBPT), Complement Fixation Test (CFT) and Enzyme Linked Immunosorbent Assay (ELISA) (Alton, 1987; Garin-Bastuji *et al.*, 2006; Nielsen and Yu, 2010). These tests use Smooth Lipopolysaccharide (S-LPS) as detecting antigen which could lead to False Positive Serological Reactions (FPSR) (Cutler *et al.*, 2005;

Poester *et al.*, 2010). The FPSR occurs because goats vaccinated against brucellosis with *B. melitensis* Rev. 1 strain induce serological response against S-LPS, similar to that induced due to infection with *B. melitensis* field strain (Diaz-Aparicio *et al.*, 1994; Cardoso *et al.*, 2006). These similarities in the immune response make the serological tests like RBPT, CFT and ELISA unable to differentiate between vaccinated and infected animals (Garin-Bastuji *et al.*, 2006; Poester *et al.*, 2010; Blasco and Molina-Flores, 2011). *Brucella* proteins especially Outer Membrane Proteins (OMPs) have been used as non-LPS antigen to minimize FPSR in the serodiagnosis of brucellosis (Cherwonogrodzky *et al.*, 1990; Letesson *et al.*, 1997). However, no individual rOMP has proven to be of sufficient diagnostic utility to replace the LPS-based tests especially in terms of sensitivity (Chaudhuri *et al.*, 2010; Liang *et al.*, 2010). Therefore, combination of more than one rOMP antigen in one test might increase the sensitivity of the immunoenzymatic assay (Moreno and Moriyon, 2006). Accordingly, the

development of ELISA based on the combination of rOMP25, rOMP28 and rOMP31 as one detecting antigen could be able to increase the sensitivity of the developed test. Therefore, this study aimed to evaluate the ability of the rOMPs I-ELISA to differentiate between vaccinated and infected goats and to compare the sensitivity and specificity of rOMPs I-ELISA with the RBPT and BRUCELISA-400SG and CFT.

MATERIALS AND METHODS

Study design: Serum samples from goats with known *Brucella* exposure status were used in the study. The samples were grouped as following: group 1 (infected); 36 serum samples from goats naturally infected with *B. melitensis* as showed by strong serological reactions using RBPT and CFT however, among the 36 samples tested, only six were confirmed by bacterial isolation and PCR. Group 2 (non-infected), 30 serum samples from *Brucella*-free goats (flock that had no previous record of brucellosis), all the animals were screened using RBPT in addition, random animals were selected for bacterial isolation. Both of RBPT and bacterial isolation results were negative. Group 3 (vaccinated), 26 serum samples from vaccinated goats generously provided by Jordan Bio-Industries Center (JOVAC, Jordan). These samples were obtained from goats vaccinated subcutaneously with *B. melitensis* Rev. 1 strain and were bled at day 24 post vaccination. The blood samples were collected from goats via jugular venipuncture. Serum was kept at -20°C until further use. Reference control sera were obtained from (VLA, UK).

Enzyme-Linked Immunosorbent Assay (ELISA) using rOMPs: The rOMPs used in this study was produced by combination of equal concentrations of rOMP25, rOMP28 and rOMP31 of *Brucella melitensis* strain 0331, Malaysian field isolate which has been confirmed as *B. melitensis* biovar 1 by Veterinary Laboratory Agency (VLA, Weybridge, UK). The rOMP25, rOMP28 and rOMP31 were obtained from earlier study of cloning and expression of omp25, omp28 and omp31 with Genbank Accession No. JX627633, JX627634 and JX627635, respectively. The Checker Board Titration (CBT) was used to optimize the working conditions of the newly developed rOMPs I-ELISA. The wells of immunoplates (Maxisorp, Nunc, Denmark) were coated with 100 µL of purified rOMPs at the final concentration of 0.39 µg mL⁻¹ of carbonate bicarbonate coating buffer (pH 9.6) and incubated overnight at 4°C. Following incubation, the wells were emptied and washed three times with Phosphate-Bufferedsaline containing 0.05% Tween 20 (PBST) and tapped on clean tissue to remove any

remaining washing buffer then blocked with 5% skim milk and incubated at 37°C in humidified chamber for 1 h. After three washings with PBST as mentioned above, a volume of 100 µL of goats sera diluted to 1:200 were added into the plates in duplicate wells and incubated at 37°C for 1 h. following incubation, the plates were washed as mentioned above and incubated with 100 µL of 1/5000 rabbit-anti goat IgG HRPO (H+L conjugate) (KPL, USA) diluted in diluting buffer for 1 h at 37°C. After washing with PBST three times, the wells were fill with 100 µL substrate solution containing TMB (3, 3', 5, 5'-tetramethyl benzidine) (KPL, USA). The color development was stopped by adding 100 µL 1N HCL, after 10 min of incubation of the plates in dark at room temperature. The optical density was measured at 450 nm wavelength using microplate reader (Bio-Rad, USA).

Rose Bengal Plate Test (RBPT): Rose bengal plate test was performed according to the standard method described by OIE (2009) using RBPT antigen procured by Veterinary Laboratory Agency (VLA-Weybridge, UK) in the presence of reference control sera.

Commercial I-ELISA (BRUCELISA-400SG): BRUCELISA-400SG (VLA-Weybridge, UK) is an indirect ELISA kit for the detection of circulating antibodies to *B. melitensis* in the serum of sheep and goat blood samples. The assay procedure was performed following the manufacturer protocol. A positive/negative cut-off was calculated as 10% of the mean of the OD of the 8 positive control wells. Any test sample that gave an OD equal to or above this value was considered positive.

Complement Fixation Test (CFT): The complement fixation test was performed by the Serology Laboratory, Veterinary Research Institute (VRI), Ipoh, Malaysia using the warm fixation method as described by OIE (2009).

Calculations and statistical analysis: The statistical analysis was performed using IBM SPSS Statistics 19 Software (IBM Corp., Armonk, NY, USA). The Sensitivity (Se), Specificity (Sp) of the rOMPs I-ELISA were calculated according to Crowther (2009) using serum samples from infected, non-infected and vaccinated goats. The results obtained were compared to RBPT, BRUCELISA-400SG and CFT using the same serum samples. The p<0.05 was considered statistically significant at 95% Confidence Interval (CI). Calculation was performed using the equation:

$$Se = \left(\frac{TP}{(TP+FN)} \right) \times 100$$

$$Sp = \left(\frac{TN}{(TN+FP)} \right) \times 100$$

K is poor if $K \leq 0.20$, fair if $K \leq 0.40$, moderate if $K \leq 0.60$, substantial if $0.61 \leq K \leq 0.80$, good if $K > 0.80$.

RESULTS AND DISCUSSION

To the best of the knowledge an ELISA using rOMPs which is combination of OMP25, OMP28 and OMP3 as one antigen for serological diagnosis of caprine brucellosis had not been presented before. The determination of cut-off point for trade-off between sensitivity and specificity for the rOMPs I-ELISA was done using the Receiver Operating Characteristic (ROC) analysis. The OD cut-off value of 0.497 was chosen in order to differentiate between vaccinated and infected goats. Accordingly, any serum sample with $OD > 0.497$ was considered positive for *Brucella* infection. The rOMPs I-ELISA was able to detect the anti-*Brucella* antibodies in (34/36) serum samples obtained from naturally infected goats and recorded 94.44% Se. While the Sp was 100% as none of the *Brucella* free goats found positive (30/30) (Table 1). The sensitivity of the developed rOMPs I-ELISA was higher than recorded by previous studies of 85.7 and 87.50%, respectively (Gupta *et al.*, 2007, 2010) using single recombinant OMP to test sera obtained from naturally *B. melitensis* infected goats. Additionally, the rOMP I-ELISA has agreement of 0.94K and covered 0.988 Area Under the Curve (AUC) (Fig. 1). The two samples from naturally infected goats that the rOMPs I-ELISA was unable to detect as infected may be as a result of individual variability of the humoral immune response against OMPs. The specificity of rOMPs I-ELISA was the same as that reported by Gupta *et al.* (2007) who found 90-100% Sp when testing, single recombinant protein, rOMP31 using sera from naturally infected goats with *B. melitensis* (infected) and *Brucella*-free goats (non-infected). When samples from vaccinated goats were tested, RBPT, BRUCELISA-400SG and CFT categorized these samples as positive. This implies that in the field situation where vaccine is used for prevention of *B. melitensis* infection, the common serological tests are not able to differentiate those truly infected from those vaccinated. Therefore, this will create confusion during serosurveillance activities which may lead to erroneous

culling of uninfected but vaccinated animals. The study found that the developed rOMPs I-ELISA was able to differentiate vaccinated from infected animal with 94.44% Se and 84.62% Sp (Table 1) and agreement 0.80 K and covered 0.904 AUC (Fig. 2). This clearly indicates that the rOMPs I-ELISA, unlike RBPT, BRUCELISA-400SG and CFT has the ability to differentiate the antibodies from the

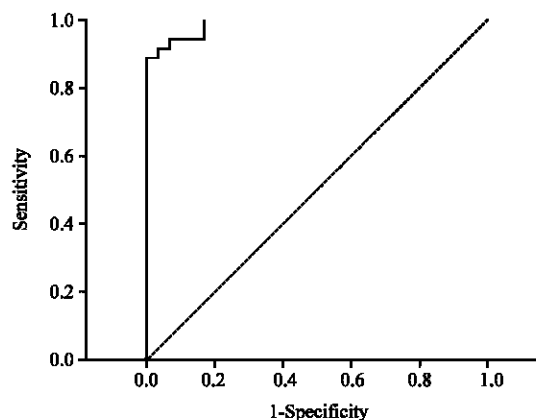


Fig. 1: Receiver Operating Characteristics (ROC) curve to determine OD cut-off value of rOMPs I-ELISA using naturally infected goats (infected) and *Brucella* free goats (non-infected)

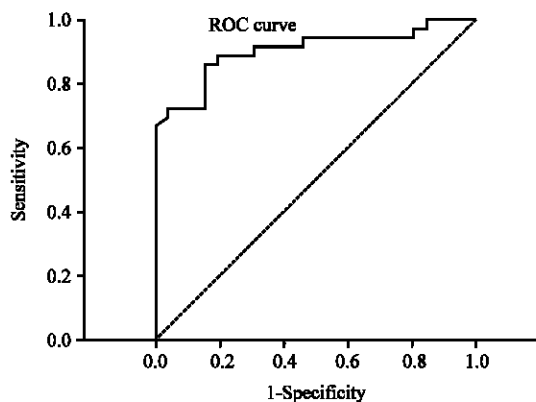


Fig. 2: Receiver Operating Characteristics (ROC) curve to determine OD cut-off value of rOMPs I-ELISA using naturally infected goats (infected) and goats vaccinated with *B. melitensis* Rev. 1 vaccine strain (vaccinated)

Table 1: Sensitivity and specificity of rOMPs I-ELISA, RBPT, CFT and BRUCELISA-400SG using three groups of goats (infected, non-infected and vaccinated)

Goats	rOMPs I-ELISA		RBPT		CFT		BRUCELISA-400SG	
	Se	Sp	Se	Sp	Se	Sp	Se	Sp
Naturally infected goats with <i>B. melitensis</i> (infected)	94.44	100.00	100	100	100	100	100	100
<i>Brucella</i> free goats (non-infected)	100.00	-	100	-	100	-	100	-
Goats vaccinated with <i>B. melitensis</i> Rev. 1 strain (vaccinated)	94.44	84.62	100	0	100	0	100	0

vaccinated goats. AntiOMP25, anti-OMP28 and anti-OMP31 antibodies were reported to be induced following an infection with *B. melitensis* rather than following vaccination using *B. melitensis* Rev. 1 vaccine strain. However, 4/26 goats were able to induce these anti-OMPs and therefore were detected using the developed rOMPs I-ELISA. In a comparative proteome analysis of *B. melitensis* Rev. 1 vaccine strain and virulent strain 16 M, it was found that certain metabolic pathways may be deregulated and altered in *B. melitensis* Rev. 1 vaccine strain especially expression of OMP31 and proteins involved in iron metabolism, sugar transport, lipid metabolism and protein synthesis (Eschenbrenner *et al.*, 2002). *Brucella melitensis* Rev. 1 vaccine remain the only acceptable vaccine in national control programs of brucellosis in small ruminants (Blasco, 1997; Banai, 2002; Minas, 2006). However, the interference of post vaccinal immune response in the serological diagnosis of brucellosis is still challenging due to FPSR. Additionally, factors like the age of the animal, vaccine dose and route of administration could have significant effect on post vaccination immune response which in turn increases the numbers of FPSR due to persistence of antibody titers for a prolonged period in a small proportion in the vaccinated animals (Corbel, 2006; Garin-Bastuji *et al.*, 2006; Nielsen and Yu, 2010).

CONCLUSION

Further, evaluation studies using large numbers of bacteriologically positive and vaccinated goats at different periods of time and administration routes should be performed to achieve a definitive conclusion regarding the diagnostic ability of the developed rOMPs I-ELISA for serological diagnosis of caprine brucellosis.

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