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# Evaluation of Diagnostic Potential of Recombinant Outer Membrane Protein (rOmp28) of *Brucella Melitensis* for Serodiagnosis of Ovine and Caprine Brucellosis

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## ABSTRACT

*Brucella melitensis* is the main causative agent of brucellosis in small ruminants. The diagnosis of brucellosis is mostly done by isolation of bacteria from aborted material, udder secretions or from tissues of infected animals. The presumptive diagnosis of Brucellosis is attempted by elucidating the serological responses to *Brucella* antigens. The present study was designed to evaluate the diagnostic potential of rOmp28 antigen of *Brucella melitensis* for ovine and caprine brucellosis. A total of 163 clinical sample (n=79 sample of ovine and n=84 sample of caprine) were tested in an indirect plate-ELISA format using rOmp28 antigen. Results of rOmp28 antigen based indirect ELISA were also compared with the native antigens [cell envelope antigen (CE) and whole cell sonicated antigen (SA)] based ELISA and with conventional Standard Tube Agglutination Test (STAT). Recombinant Omp28 antigen showed high sensitivity and specificity i.e., 71.4%, 97.7% for ovine samples and 74%, 87.8% for caprine samples as compared with CE antigen (40%, 75%) and (44%, 67.6%) and SA antigen (37.1%, 84%) and (42%, 70.5%) for ovine and caprine samples respectively. This study demonstrated that rOmp28 can be a good candidate antigen in the serodiagnosis of ovine and caprine brucellosis in India and also further in the development of rapid field-adaptable diagnostic assay for screening of ovine and caprine brucellosis.

**Keywords:** Outer membrane protein, rOmp28, *Brucella melitensis*, indirect ELISA, Ovine and caprine brucellosis

## 1. INTRODUCTION

The ovine and caprine brucellosis is caused by gram negative, coccobacilli, intracellular, facultative bacteria *Brucella melitensis.* This disease is characterised by severe economic loss caused by abortion in these small ruminants. The species B. melitensis consist of three biovars namely, 1, 2 and 3 and all three can cause disease in ovine and caprine. *B. melitensis* infection in sheep is endemic in Mediterranean region, Central Asia, Southern Arabian Peninsula, Eastern Mongolia, parts of Latin America, mainly Mexico, Peru and Northern Argentina, Africa and India. In India, the seroprevalence of brucellosis in small ruminants have been evaluated throughout the country[1,2]. The route of infection is through direct or indirect contact as animals become infected by aerosols or by intake of infected material. Alternatively the infection can also occur through grazing pastures healthy animals can be infected by contact with infected animals. The symptoms include abortion, retained placenta, orchitis, epididymitis and arthritis. The organism is excreted in urine, semen and in milk, shedding of bacterium in semen and milk can be for longer duration and possibly lifelong. In goats, the infection varies in duration from very short period and can be rapidly eliminated in vaccinated animals. In non vaccinated animals it is observed that the organism may be excreted up to two or more lactations. In sheep the susceptibility depends on the breed and is more resistant to reinfection[3]. The study of Werschilova & Striedter[4] showed strong resistance during pregnancy up to 8 to 9 month in sheep and the resistance declines after two year. Diagnosis of the disease depends on the isolation of bacteria from aborted material or udder secretions. The presumptive diagnosis of *Brucella* infection is mostly assessed by serological responses to *Brucella* specific antigens. The serological tests used in the diagnosis of brucellosis in sheep and goat are Standard Tube Agglutination Test (STAT), Rose Bengal Plate Agglutination test (RBPT), Buffered Plate Agglutination test (BPA), Complement Fixation test (CFT), and Enzyme Linked Immuno-sorbent Assay (ELISA). These tests have varied level of sensitivity and specificity and are used in the detection of infected animals. The removal of those infected animals so diagnosed employing the serological tests can contribute to disease control. The serological tests based on whole cell extract or LPS are not specific and not capable to distinguish *B. melitensis* from cross-reactions to other bacteria, particularly *Yersinia enterocolitica* O:9[5]. The recombinant proteins are better choice to overcome the problems associated with sensitivity and specificity. The rOmp28 of *Brucella melitensis* is one of the immunodominant antigens and its

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diagnostic potential for screening of human brucellosis was established earlier[6]. In the present study we have purified the recombinant protein (rOmp28) of *B. melitensis* and evaluated its diagnostic potential for the serodiagnosis of ovine and caprine brucellosis by indirect plate-ELISA.

## 2. MATERIALS AND METHODS

### 2.1 Serum Samples

The sera samples of ovine (n= 79) and caprine (n= 84) brucellosis tested in the study were obtained from Animal Disease Monitoring and Surveillance (ADMAS) Collaborating Unit, Western Regional Disease Diagnostic Laboratory (WRDDL), Pune, Maharashtra, India. Samples collected from Nagpur and Nasik region of Maharashtra State, India and were stored at -20 °C till use in STAT and indirect ELISA.

### 2.2 Preparation of Recombinant Omp28 Antigen

The rOmp28 antigen of *Brucella melitensis* used in the study was prepared as described previously[6]. The omp28 gene clone was grown in 10 mL LB media with kanamycin (25 µg/mL) and this was used for the expression and purification of rOmp28 antigen. This clone was further scaled up in 250 mL media with antibiotic in shake flask and incubated at 37°C until the OD of culture reached to 0.5 for induction. After induction with 1 mM IPTG and incubation of 5 h, the cells were harvested and used for the purification of rOmp28 by Ni-NTA affinity chromatography protocol (Qiagen) under native conditions. The purified protein was dialysed with 1X PBS (pH 7.2) with 3 changes and stored at -20 °C in small fractions. The protein concentration was estimated by Lowry's method and used as antigen.

### 2.3 Preparation of Native Antigens

#### *2.3.1 Whole Cell Sonicated Antigen*

Overnight grown culture of *Brucella melitensis* 16 M was inactivated with 1 % formaldehyde for 1 hr and centrifuged at 8000 rpm for 15 min at 4 °C. The inactivated preparation was checked for viability by spreading on *Brucella* selective agar. Cell pellet was washed thrice with 1X PBS and resuspended in PBS for sonication. Cell suspension was sonicated for 3 cycle of 5 min each at 40 W amplitude and 8 s pulse. The sonicated sample was then centrifuged at 10,000 rpm for 10 min and supernatant was used as SA antigen.

#### *2.3.2 Cell Envelop Antigen*

The CE antigen was prepared according to the previously described protocol[7] with modifications. The inactivated 100 mL of bacterial culture was centrifuged at 8000 rpm for 15 min at 4 °C and pellet was washed twice with 1X PBS and resuspended in 100 mL of buffer 1 (15 mM Tris-HCl pH 8.0, 0.45 M sucrose, 8 mM EDTA and 0.4 mg/mL lysozyme) and incubated at 47 °C for 15 min. The samples was centrifuged and pellet was dissolved in 10 mL of buffer 2 (50 mM Tris-HCl pH 7.6, 5 mM $M_gCl_2$, 2 mM PMSF). The suspension was sonicated for 3 cycle of 5 min each at 40 W amplitude and 8 sec pulse and centrifuged at 6000 rpm for 15 min to remove unbroken cells. The supernatant was ultracentrifuge at 43,500 rpm for 90 min and pellet was dissolved in 1 mL of buffer 3 (50 mM Tris-HCl pH 7.6, 2 mM PMSF) and stored at -20°C. The concentration of both the native antigens was estimated by Lowry's method and used for ELISA.

### 2.4 Indirect Plate-ELISA

In the present study we standardised an indirect plate-ELISA for screening of sera samples using purified rOmp28 protein. The protein was diluted to 25 µg/mL in 0.05 M carbonate buffer and 100 µL of diluted antigen was coated per well in ELISA plates (Nunc, Denmark). After coating the ELISA plate was incubated at 37°C for 1 hr and after incubation washed thrice with 1X phosphate buffer having 0.05 % Tween-20 (PBS-T). Plates were then blocked with 200 µL of 1 % BSA at 37 °C for 1 h and after washing with PBS-T, serum samples were added to individual wells at 1:100 dilutions in sterile 1X phosphate buffer and incubated for 1 hr at 37 °C. Again plates were washed with PBS-T and incubated with 100 µL of respective conjugate at 1:1000 dilutions in 1X PBS for 1 hr at 37 °C. Polyclonal anti sheep-HRP conjugate (Dako, Denmark) was used for sera samples of sheep whereas polyclonal anti goat-HRP conjugate was used with goat samples. After washing with PBS-T, reaction was developed by the addition of 100 µL of developing solution consisting of o-phenylenediamine and $H_2O_2$, plate was incubated for 5-10 min in dark for colour development and reaction was stopped by 10 µL of 1 N $H_2SO_4$ per well. The absorbance was read at 495 nm in an ELISA reader. A culture confirmed positive and negative control was included in each run to ensure the accuracy of the test. ELISA was also performed following the same procedure using sonicated antigen and cell envelop antigen for comparison.

### 2.5 Standard Tube Agglutination Test

All 163 sera samples of brucellosis were screened with standard tube agglutination test (STAT), the conventional serological agglutination assay primarily used for the diagnosis of brucellosis. To detect the antibodies against *Brucella* in serum samples, antigen of *Brucella abortus* S99 was used as per the method of Alton[8], *et al.* The two fold serial dilutions (1:20 to 1:640) of the sera was prepared in phenol saline and 0.5 mL of antigen was added to each tube. All the tubes were incubated at 37 °C for 24 h. The control tube with the antigen was compared with the test and tubes showing 50 % agglutination were marked. The titer of 1:40 or above was considered as positive. Agglutination reaction was also performed with culture confirmed positive and negative sample in every run.

### 2.6 Comparison of ELISA with STAT Test

Results of ELISA using recombinant as well as native antigens were compared with STAT considering it as gold standard and sensitivity and specificity of ELISA was calculated as per the following formulas; Sensitivity = [true positives/ (true positives + false negatives)] X100, Specificity = [true negatives/ (true negative + false positives)] X100, Positive Predictive Value= [true positive/ (true positive + false positive)] X100, Negative Predictive Value = [true negative/ (true negative + false negative)] X100, Correlation= [(true positive + true negative)/ total number of samples] X100.

A true positive was defined as a sample positive by the agglutination test as well as by the ELISA and a sample was consider true negative if it was found negative by both the tests evaluated. False negative sample was classified as it was positive by agglutination tests but negative in ELISA and false positive sample was that which was negative by agglutination tests but positive by ELISA. Results of ELISA using rOmp28 antigen were also compared with CE-ELISA and SA-ELISA to evaluate the diagnostic potential of recombinant antigen over the native antigens.

## 3. RESULTS

### 3.1 Preparation of Recombinant Omp28 and Native Antigens

The rOmp28 protein was purified from shake flask culture by Ni-NTA affinity chromatography under native conditions and different fractions of purification were analysed in SDS-PAGE as shown in Fig. 1. The band of 32 kDa in size for rOmp28 protein was observed in gel and protein was purified with greater than 95 % purity. Finally the yield of purified protein was estimated as 16.5 mg/L of bacterial culture. The concentration of sonicated and cell envelop antigen was estimated as 60 mg/L and 42 mg/L of bacterial culture respectively. The antigens were stored at -20 °C in small aliquots for their use in plate-ELISA.

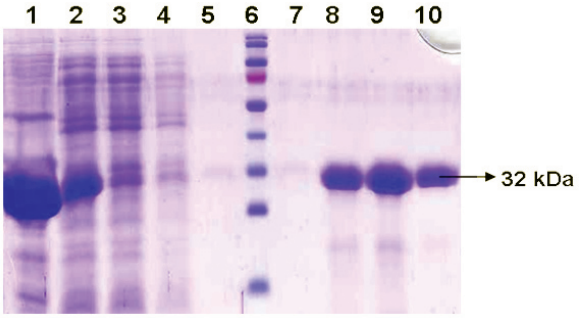


**Figure 1. SDS-PAGE analysis of purification of rOmp28 protein, Lane 1: clone induced for 5 h with 1 mM IPTG, 2: lysed clear lysate, 3: Flow-through, 4-5: Wash 1 and Wash 2, 6: Prestained protein marker (Fermentas, #SM0671), 7: Wash 3, 8-10: protein eluates (E1, E2, and E3).**

### 3.2 Indirect plate-ELISA with Clinical Samples of Ovine and Caprine Brucellosis

All the 163 samples were tested by indirect plate-ELISA using recombinant (rOmp28) as well as native (CE and SA) antigens and a cutoff OD value of 0.22, 0.45, 0.46 for ovine samples as shown in Fig. 2 and 0.25, 0.5, 0.24 for caprine samples as shown in Fig. 3 was determined for rOmp28-ELISA, CE-ELISA and SA-ELISA respectively. Out of 79 ovine samples tested, 26 (32.9 %) were found positive by rOmp28-ELISA, whereas 25 (31.6 %) and 20 (25.3 %) samples were found positive by CE-ELISA and SA-ELISA. 53, 54 and 59 sample were found negative by rOmp28-ELISA, CE-ELISA and SA-ELISA respectively. In case of caprine brucellosis, out of 84 sample tested 42 (50 %), 33 (39.3 %) and 31 (36.9 %) were found positive whereas 42, 51 and 53 sample were found negative by rOmp28-ELISA, CE-ELISA and SA-ELISA respectively.

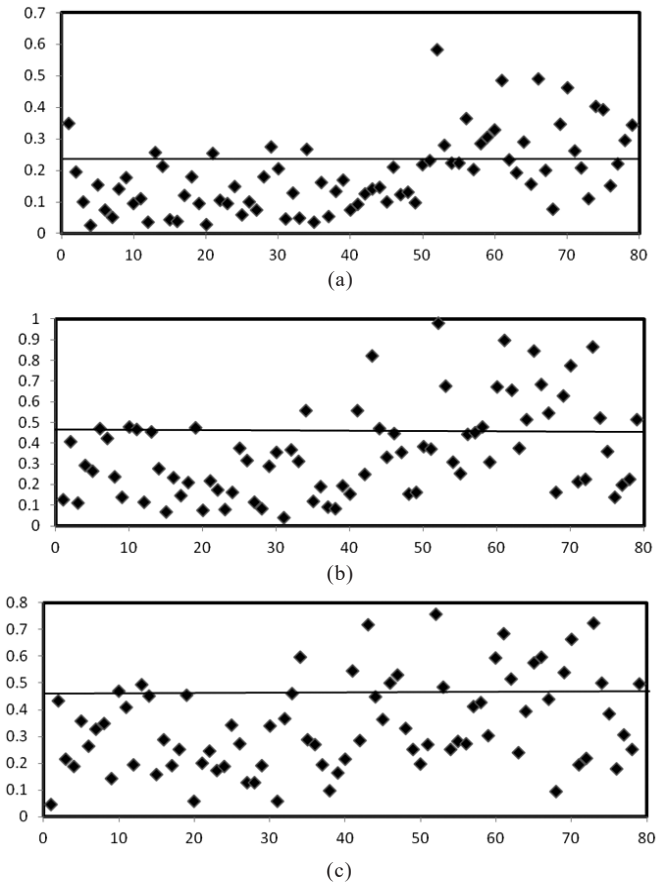


**Figure 2. Distribution of positive and negative samples of ovine Brucellosis according to their absorbance values in indirect plate-ELISA (A: with rOmp28 antigen, B: CE antigen, C: SA antigen) (a) Recombinant Omp28, (b) Cell Envelope antigen and (c) Sonicated Antigen.**

### 3.3 Indirect Plate-ELISA Vs Standard Tube Agglutination Test

Samples were screened with conventional tube agglutination method and out of 79 ovine sample tested by STAT, 35 (44.3 %) were found positive whereas 44 sample were negative, in case of 84 caprine sample, 50 (59.5 %) were positive and 34 sample were found negative. It was noted that 10 sheep and 13 goat sample that were found positive by STAT were detected negative by rOmp28-ELISA. In same way 21 sheep and 28 goat sample in case of CE antigen and 22 sheep and 29 goat sample in case of SA antigen were found positive by STAT but negative by respective ELISA. When the sensitivity of all the three ELISAs (rOmp28, CE and SA) were compared with STAT considering it as gold standard test, it was found 71.42 %, 40 %, 37.14 % for ovine and 74 %, 44 %, 42 % for caprine by rOmp28, CE and SA ELISA respectively. The specificity of these three ELISA assays were found as 97.72 %, 75 %, 84.09 % for ovine and 87.8 %, 67.64 %, 70.58 % for caprine respectively as shown in Table 1. Positive and negative predictive values of the ELISA were also determined and only rOmp28-ELISA showed

**Table 1. Comparison between indirect plate-ELISA using recombinant (rOmp28) and native antigens cell envelope (CE) and sonicated antigen (SA) with standard tube agglutination test (STAT)**

| | Ovine samples | | | Caprine samples | | |
|---|---|---|---|---|---|---|
| | rOmp28-ELISA Vs STAT | CE-ELISA Vs STAT | SA-ELISA Vs STAT | rOmp28-ELISA Vs STAT | CE-ELISA Vs STAT | SA-ELISA Vs STAT |
| Sensitivity (%) | 71.42 | 40.0 | 37.14 | 74 | 44 | 42 |
| Specificity (%) | 97.72 | 75.0 | 84.09 | 87.8 | 67.64 | 70.58 |
| Positive predictive value (%) | 96.15 | 56.0 | 65.0 | 88.09 | 66.67 | 67.74 |
| Negative predictive value (%) | 81.13 | 61.11 | 62.71 | 69.04 | 45.09 | 45.28 |
| Correlation (%) | 79.43 | 55.77 | 63.29 | 78.57 | 53.57 | 53.57 |

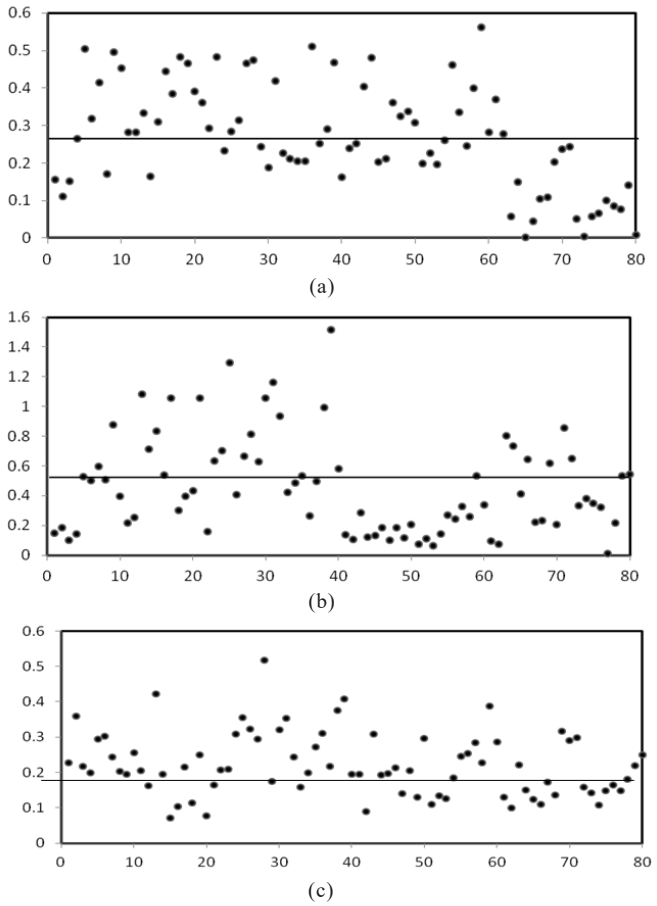


**Figure 3. Distribution of positive and negative samples of caprine Brucellosis according to their absorbance values in indirect plate-ELISA (A: with rOmp28 antigen, B: CE antigen, C: SA antigen) (a) Recombinant Omp28, (b) Cell Envelope antigen and (c) Sonicated antigen.**

maximum positive predictive value of 96.15 % and negative predictive value of 81.13 %. The rOmp28-ELISA also showed highest correlation of 79.43 % with STAT when compared with ELISA using native antigens as shown in Table 1. The rOmp28 ELISA correlated well in all the statistical parameters with that of STAT with high sensitivity, specificity, PPV, NPV and correlation.

### 3.4 Comparison of rOmp28 Antigen with Native Antigens in Indirect-ELISA

The OD cut off was calculated earlier with a set of calibrator serum samples for all the three antigens tested were calculated and was fixed as 0.22 for rOmp28 antigen, 0.45 for cell envelope antigen and 0.46 for whole cell sonicated antigen for ovine and 0.25, 0.5 and 0.24 respectively for caprine samples. In the indirect ELISA with the rOmp28 antigen in the 54 negative samples the OD ranged from 0.026 to 0.21 with a mean of 0.12±0.05, whereas in the positive samples the OD ranged from 0.22 to 0.584 with a mean of 0.33±0.09.

## 4. DISCUSSION

Brucellosis is a major threat to most small ruminants in Mediterranean basin, Middle East and Central Asia[9] and infection in sheep and goat occurs naturally with *Brucella melitensis*. Symptoms of the disease mimic other infectious diseases and making diagnosis of the disease challenging[10]. For diagnosis of brucellosis in in sheep and goat, the RBPT and CFT are the most favored and officially recognised tests for international trade. However, other studies demonstrate the value of BPA, SAT, skin delayed- type hypersensitivity (SDTH)[11,12] and more recently ELISA[13-15] assays for the detection of brucellosis in sheep. The current serological tests are efficient enough to detect brucellosis on a flock basis but detection in low prevalence area is a problem as the titre of infection in animals decline sharply. The isolation of *Brucella* is considered as gold standard test to prove the infection. The bacteriological examination is not relied upon to prove the presence or absence of infection in individual animals[8,17] as facilities for isolation is not available in endemic regions. Due to above reasons the serological tests with crude or whole antigens, LPS are usually accepted for diagnosis of brucellosis. The antibodies against LPS are also induced in vaccine animals, hence, it is required to identify the proteins which elicit the antibody response during infection but are not essential for protective immunity. Thus ELISA using recombinant antigens can provide better sensitivity than RBPT and STAT also reported in latent infections could be detected earlier by ELISA[18]. In this order several studies have been evaluated the use of OMPs, inner cytoplasmic proteins or cytosoluble protein extract (CPE)[14,19-22] in ELISA for diagnosis of brucellosis in sheep and goat. In the present study we have evaluated the diagnostic potential of an outer membrane protein of *Brucella melitensis* (rOmp28) in indirect plate-ELISA format for the diagnosis of ovine and caprine brucellosis. BP26, a periplasmic protein is evaluated as diagnostic antigen for sheep brucellosis that is caused by *Brucella melitensis* or *B. ovis*[13,14,23]. In this study, rOmp28 was prepared as per the standard protocols and concentration of the protein was found similar to our previous studies[6]. This recombinant antigen was

used in indirect plate-ELISA to screen the positive and negative samples of sheep and goat and results of rOmp28-ELISA were compared with ELISA performed with native antigens (CE and SA). All these three ELISA results were also compared with the results of conventional assay (STAT). When compared with total number of positive and negative samples tested in indirect plate-ELISA as well as with conventional methods, rOmp28 was found to react with maximum positive samples of ovine and caprine, compared to native antigens. The seroprevalence was also determined by indirect ELISA and STAT and variation was observed due to the numbers of false positives and false negatives detected by various tests [24,25]. The sensitivity and specificity of the rOmp28-ELISA was compared for ovine and caprine brucellosis considering STAT as gold standard test, it was found more sensitive and specific than ELISA using native antigens. Results of specificity of all three ELISA were not consistent with respect to antigen used and found higher than sensitivity when compared with STAT for both ovine and caprine, similar results were obtained by other workers[26,27]. For diagnosis of brucellosis in cattle, ELISA can be a better option in comparison to CFT, RBPT and STAT[28] because of its better sensitivity over the other tests[29] These findings endorse the diagnostic potential of recombinant outer membrane protein (rOmp28 antigen) of *B. melitensis* in an indirect plate-ELISA over the native antigens for serodiagnosis of ovine and caprine brucellosis.

## 5. CONCLUSION

The result of indirect plate-ELISA using rOmp28 antigen was compared with native antigens for diagnosis of brucellosis in ovine and caprine clinical samples. In comparison with conventional STAT assay high sensitivity and specificity was observed with rOmp28 ELISA. The results conclude the reliability of the rOmp28 antigen indirect ELISA in the serodiagnosis of caprine and ovine brucellosis. Since a single serological test may not be enough in the brucellosis disease diagnosis, rOmp28-ELISA system in conjunction with other serological tests like STAT would be used for screening of large numbers of samples.

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