

## Recombinant OMP28 antigen-based indirect ELISA for serodiagnosis of bovine brucellosis

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### ARTICLE INFO

#### Article history:

Received 31 August 2009

Accepted 4 December 2009

Available online 16 December 2009

#### Keywords:

*Brucella*  
Diagnosis  
OMP28  
ELISA  
Recombinant protein

### ABSTRACT

Brucellosis is a zoonosis of both public health and economic importance in many developing countries including India. Early detection and segregation of the infected animals are important in order to control the disease. Serodiagnostic tests for brucellosis is mainly based on detection of antibodies developed against lipopolysaccharide (LPS) component of cell. In this study we evaluated a protein antigen, 28 kDa outer membrane protein (OMP28), of *Brucella melitensis* as an alternative to LPS. Recombinant OMP28 was produced in *Escherichia coli* system. The efficacy of purified OMP28 was studied in an indirect enzyme-linked immunosorbent assay (ELISA) for diagnosis of brucellosis in field sera collected from different regions of country. Using known negative and known positive serum samples it was found that OMP28 is immunoreactive to *Brucella* infected cattle, sheep, goat and dog sera. Three hundred and eighty two cattle sera were screened by OMP28 antigen-based ELISA and the results were compared to rose Bengal plate agglutination Test (RBPT). Recombinant OMP28 antigen-based ELISA has shown sensitivity of 88.7%, specificity of 93.8% and accuracy of 92.9%. It was concluded that recombinant *B. melitensis* OMP28 could be used as a protein antigen for diagnosis of brucellosis in domestic animals.

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### 1. Introduction

Microorganisms belonging to the genus *Brucella* are Gram negative, facultative intracellular bacteria of zoonotic importance. Like other intracellular pathogens, brucellae are virulent mainly due to their ability to avoid the bactericidal phagocyte functions and to proliferate within macrophages, leading to the establishment of a chronic infection in the host. Brucellosis is a major zoonotic disease widely distributed in both animals and humans, especially in developing countries. The prevalence of the disease is very high in India [1]. Because of serious economic and medical consequences of brucellosis, efforts have been made to prevent the disease through the use of vaccines. Similarly, control of brucellosis is dependent upon reliable methods for the identification of *Brucella* in livestock and humans.

The lipopolysaccharide (LPS) of smooth *Brucella* species is by far the strongest antigen when compared to other antigenic molecules. Consequently, *Brucella* LPS has been considered the most important antigen during immune response in brucellosis. LPS elicits long lasting serological response in both vaccinated

and infected animals [2–6]. The serological procedures being used in the diagnosis of animal brucellosis are complement fixation test, rose Bengal plate test, standard tube agglutination test, milk ring test and enzyme-linked immunosorbent assay [7]. These tests are mainly based on the detection of antibodies directed against the lipopolysaccharide (LPS) portion of the cell membrane. Therefore it is difficult to differentiate between vaccinated and infected animals using LPS-based serological tests [3,4]. In addition, tests based on anti-LPS antibodies give false positives because of cross-reactivity with other Gram-negative bacteria like *Yersinia enterocolitica* O:9, *Salmonella* species, *Escherichia coli* [8–10].

Therefore, a major goal in immunological studies of brucellosis has been the identification of non-lipopolysaccharide antigens, which could be useful to circumvent drawbacks of LPS antigen. The 28 kDa OMP (OMP28) of *Brucella melitensis* belongs to group III antigens. OMP28, also been termed as BP26 and CP28, is an immunodominant antigen localized in the periplasm and this protein has been a target molecule for detection of anti-*Brucella* antibodies [11–13]. In the present study we described expression and purification of *B. melitensis* OMP28 protein in *E. coli* system. We further evaluated the diagnostic potential of purified recombinant OMP28 of *B. melitensis* for screening cattle sera collected from different regions of India.

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## 2. Materials and methods

### 2.1. Bacterial strains plasmid

*B. melitensis* 16M strain was used in the present study to clone and express 28 kDa OMP gene. The organism was grown in brain heart infusion broth for extraction of nucleic acid. *E. coli* DH5 $\alpha$  was used as host and plasmid pPROExHTb (LifeTechnology, USA) was used as expression vector.

### 2.2. PCR amplification and cloning

*B. melitensis* 16M DNA encoding 28 kDa outer membrane protein gene was amplified by PCR, using a set of primers (5'-CGC GGA TCC ATG AAC ACT CTG GCT AGC AAT-3' and 5'-CGCAAG CTT TTA CTT GAT TTC AAA AAC GAC-3') designed from the available nucleotide sequence [11]. In order to overproduce the 28 kDa outer membrane protein (OMP28) of *B. melitensis*, the amplified DNA was cloned into expression vector, pPROExHTb (Life Technology, USA). Restriction sites for *Bam*H I and for *Hind* III (underlined) were introduced to oligonucleotides to facilitate cloning. PCR was performed for 30 cycles at 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min. The PCR amplified product was eluted from agarose gel and ligated to the pPROExHTb vector with T4 DNA ligase. The newly constructed plasmid was designated as pOM28 and was transformed into *E. coli* DH5 $\alpha$  cells.

### 2.3. Induction of expression and purification of recombinant protein

*E. coli* cells harboring pOM28 plasmid were grown in LB medium till the OD<sub>600nm</sub> reached to 0.5. The cells were then induced with 1 mM IPTG and allowed to grow further for 6 h at 37 °C. Cells were harvested and analyzed on SDS-PAGE. Purification of the recombinant OMP28 was performed by using Ni-NTA agarose according to the instructions of the manufacturer (Qiagen, USA) and analyzed on SDS-PAGE.

### 2.4. SDS-PAGE and immunoblot assays

The proteins were subjected to electrophoresis on SDS-PAGE according to Laemmli [14] and transferred to nitrocellulose membranes in a semi-dry transblot system (Atto, Japan). The membranes were blocked with 5% BSA solution. Transferred proteins were immunostained either with field sera at a dilution of 1:200. Secondary antibodies conjugated to horseradish peroxidase and were used in the assays. The reactions were developed with Diamino Benzidine (Sigma) and H<sub>2</sub>O<sub>2</sub>.

### 2.5. Recombinant OMP28 ELISA

Clinical sera from cattle, goat, sheep and dog were analyzed by indirect ELISA using recombinant OMP28 were test antigen. The immunoassay plates (Maxisorp, Nunc, Denmark) were coated with purified recombinant OMP28 protein at a concentration of 100 ng per well, diluted in 0.1 M bicarbonate buffer (pH 9.0) and incubated at 4 °C, overnight. The wells were emptied and washed five times with phosphate buffer saline-Tween20 (PBST) and than blocked with 5% BSA. Immunoassay plates were charged with sera at a dilution of 1:100 and incubated at 37 °C for 1 h. After washing with PBST for five times the plates were incubated with HRPO conjugates for 1 h at 37 °C. After washing with PBST, the wells of immunoassay plates were fill with substrate solution containing Ortho-phenyl diamine (OPD) and H<sub>2</sub>O<sub>2</sub>. Colour development was stopped by adding 2 M H<sub>2</sub>SO<sub>4</sub>, after 10 min of incubation of the

plates in dark at room temperature. Absorbance was recorded at 490 nm wavelength in an ELISA reader.

### 2.6. Rose Bengal plate agglutination test

The rose Bengal plate agglutination test (RBPT) was performed on glass plate according to standard method. Sera samples and antigen brought to room temperature. 30  $\mu$ l of antigen is placed on a microscopic glass slide with dispenser upright. 30  $\mu$ l of serum is also placed along side the antigen, serum and antigen are mixed thoroughly. Any degree of agglutination within 3 min was taken as positive. RBPT antigen was procured from Division of Biological products, Indian Veterinary Research Institute (IVRI), Izatnagar.

### 2.7. Evaluation of RBPT and ELISA

The relative sensitivity, specificity and accuracy of recombinant OMP28 ELISA for serodiagnosis of brucellosis were evaluated in comparison to rose Bengal plate agglutination test (RBPT) as described below,

Sensitivity =  $a/(a + c) \times 100$ , where 'a' is the number of sera positive by ELISA and RBPT while 'c' is the number of sera positive by RBPT but negative by ELISA.

Specificity:  $d/(b + d) \times 100$  where 'd' is the number of sera negative by ELISA and RBPT while 'b' is the number of sera negative by RBPT but positive by ELISA.

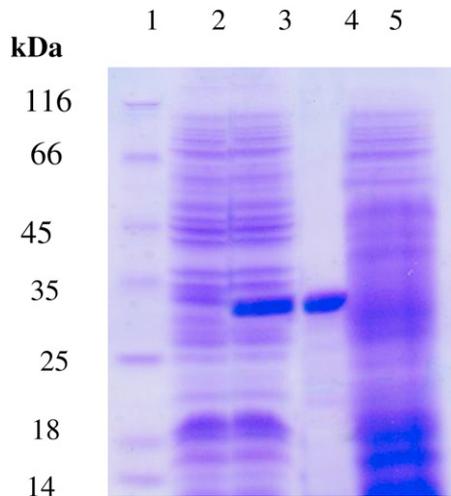
Accuracy =  $(a + d)/(a + b + c + d) \times 100$ .

## 3. Results

The detection of antibodies by serological methods is very useful in diagnosis of brucellosis. Recombinant forms of purified protein may be an alternative reagent to replace the crude antigenic preparation. This study was performed to characterize the immunoreactivity of the full-length recombinant OMP28 protein of *B. melitensis*. The amplification of 28 kDa outer membrane protein gene from *B. melitensis* was achieved by polymerase chain reaction using a set of primers which were designed based on the sequence with accession no U30815 [11]. Genomic DNA from *B. melitensis* 16M strain was extracted and used as template for amplification of omp28 gene. On amplification, a product of 753 bp DNA fragment was observed which was further confirmed by nested PCR (data not shown). The PCR product contained complete open reading frame (ORF) of omp28 gene with ATG as start codon and TAA as stop codon. The omp28 ORF encodes 250 amino acids.

### 3.1. Production and purification of the recombinant OMP28

*E. coli* harbouring pOM28 plasmid was grown in LB medium and the cells were induced with 1.0 mM IPTG. High level expression of 28 kDa OMP was achieved (Fig. 1). Recombinant OMP28 was mostly accumulated in the cytoplasm of *E. coli* transformant as inclusion bodies. To determine the target protein solubility, the cells were lysed by treating with lysozyme (1 mg/ml) for 30 min on ice followed by sonication. The cell lysate was centrifuged at 10 000 rpm for 30 min. The supernatant contains the soluble protein whereas the pellet contains insoluble proteins. We could detect majority of the expressed protein in the insolubular fraction. The expressed protein was accumulated as insoluble aggregates, termed as inclusion bodies, which could only be extracted and purified under denaturing condition using 8 M urea. Approximately 20 mg protein could be purified from 1 L of induced culture.



**Fig. 1.** SDS-PAGE analysis of total cell lysate of *Escherichia coli* producing recombinant OMP28. Lane 1. Protein molecular mass marker (kDa); Lane 2. Uninduced *E. coli* cell lysate; Lane 3. Induced *E. coli* cell lysate; Lane 4. Purified OMP28; Lane 5. *Brucella melitensis* cell lysate.

### 3.2. Immunoreactivity of recombinant OMP28 protein

To evaluate immunoreactivity of purified protein, Western blot was performed with sera (1:200 dilution) from *Brucella* infected animals of different species. The sera from sheep, goat, cattle and dog, positive by classical serology, reacted to purified recombinant OMP28 (Fig. 2). Immunostaining reaction was weak with dog sera. Three hundred and eighty-two cattle sera were collected from different parts of the country and were tested by indirect ELISA against the recombinant OMP28 antigen. To determine the cut-off value for ELISA, ten known positive and twenty known negative sera for bovine brucellosis were used. The cut-off value of ELISA was determined at a fixed serum dilution of 1:100. Mean OD<sub>490nm</sub> value of twenty negative sera + 3 SD (standard deviation) was considered as cut-off value for declaring a serum as positive or negative for brucellosis. The cut-off value has been determined as 0.192. The sensitivity, specificity and accuracy of recombinant OMP28 based ELISA relative to the reference method, RBPT, are shown in Table 1.

**Table 1**

Evaluation of diagnostic values of recombinant OMP28 antigen-based ELISA in comparison to rose Bengal plate agglutination test (RBPT).

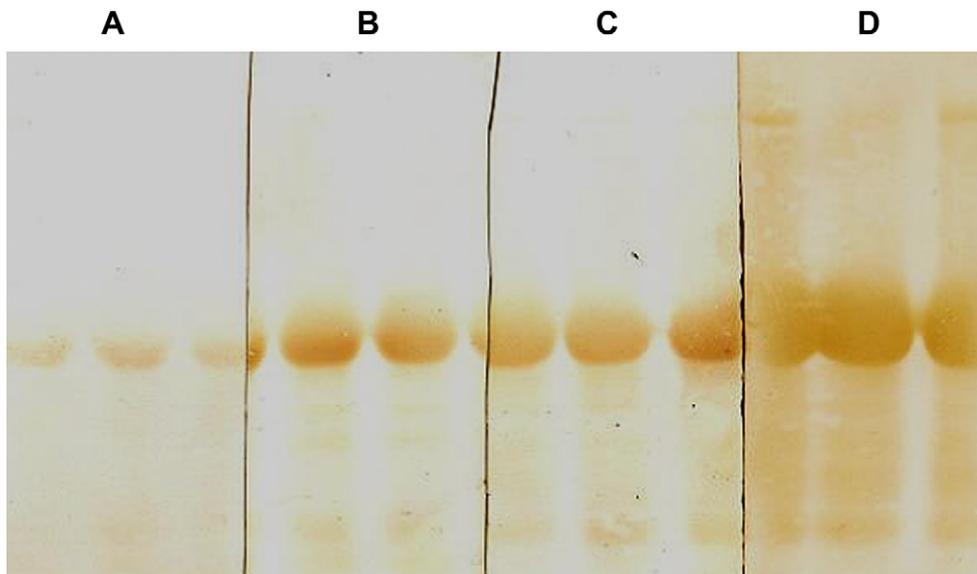
	RBPT +	RBPT –	Total
ELISA +ve	63 (a)	8 (b)	71
ELISA –ve	19 (c)	292 (d)	311
Total	82	300	382

Sensitivity =  $63/71 \times 100 = 88.7\%$ ; specificity =  $292/311 \times 100 = 93.8\%$ ; accuracy =  $355/382 \times 100 = 92.9\%$ .

### 4. Discussion and conclusion

The live attenuated *Brucella* vaccines that are currently available induce a high-titer antibody response against the O polysaccharide of the LPS, which interferes with the serologic diagnosis of the disease. Anti-protein antibody response is often delayed as compared to anti-smooth LPS response and was limited to animals which develop an active brucellosis infection [20]. In contrast to LPS antigen, a partially purified cytosoluble protein antigen could differentiate between *B. melitensis* infected and *B. melitensis* Rev.1-vaccinated sheep [12]. Therefore, the identification of protein components of *Brucella* bacteria which elicit an antibody response in the majority of infected animals would improve diagnosis of the disease and contribute to the development of new vaccinal strategies.

In this study, antibodies to the OMP28 antigen of *Brucella* were assessed in different species of animals. OMP28 antigen-based indirect ELISA was compared with RBPT, a test based on whole cell LPS antigen. RBPT is a spot agglutination technique and can give both false as well as true positives [15]. The sensitivity and specificity of the test varies with pH of the antigen, ambient temperature and temperature of antigen and test serum [16]. Tests based on anti-LPS antibodies have extensive cross-reaction with other gram negative organisms like *Y. enterocolitica* O:9, *Salmonella urbana*, *E. coli* O:157 etc, which leads to ambiguous diagnosis of animal brucellosis [4]. Therefore, there is always a search of non-LPS candidate antigen for diagnosis of brucellosis. Several *Brucella* proteins have been described as targets of the humoral immune response [2,6,13,17–22]. In the present study we have used 28 kDa outer membrane proteins (OMP28) of *B. melitensis* for identification



**Fig. 2.** Immunoblot analysis of purified OMP28 protein with *Brucella* positive sera. A. Dog sera; B. Sheep sera; C. Goat sera; D. Cattle sera.

of anti-*Brucella* antibodies in the sera collected from different parts of the country. Our result is consistent to previous reports describing usefulness of this protein for detecting anti-*Brucella* antibodies in different species of animals [12,23,24]. OMP28 is a conserved protein found in different species of brucellae. High level expression of *Brucella* OMP28 was achieved in *E. coli* system. Contrary to previous reports [24,25] we could detect majority of expressed as inclusion bodies in the cytosol of the cell. Formation of inclusion bodies is a common feature when protein expresses at a high level. Aggregate formation increased the stability of the expressed protein. Expressed OMP28 protein could be purified after several days of induction without degradation of the protein. OMP28 protein was expressed with N-terminal histidine tag, and thereby could be purified by using Nickel-NTA agarose resin.

In conclusion, our study has shown that indirect ELISA, using recombinant OMP28 protein, yielded high sensitivity and specificity for detection of anti-*Brucella* antibodies in the clinical serum samples of cattle collected from different regions. The same antigen could also serve as potential diagnostic agent for other species of animals like buffalo, sheep, goat and dog. This study has also shown that immunoreactive OMP28 protein of *B. melitensis* could be expressed at a high level in *E. coli* system and further purified to almost homogeneity in a single step. This is suitable for bulk production of antigen in a very short period of time and also avoids biohazard risk of handling of *Brucella* for preparation of LPS antigen.

## Acknowledgements

This work was supported by a grant from the Department of Biotechnology, New Delhi, India. The authors are thankful to the Director, Indian Veterinary Research Institute, Izatnagar for providing necessary facilities to carry out this study.

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