



Outer Membrane Proteins of *Brucella abortus* Vaccinal and Field Strains and their Immune Response in Buffaloes

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ABSTRACT

Outer membrane proteins (OMPs) of three strains of *B. abortus* i.e. S19, RB51 and a local field isolate of biotype 1 were isolated through disrupting cells to generate membranes by centrifugation and sodium lauryl sarcosinate solubilisation of inner membrane proteins. Distinct OMP profiles of each strain were seen on SDS-PAGE. SDS-PAGE analysis of S19 and field isolate revealed eight protein bands in each strain. The OMPs of S19 had molecular masses 89.0, 73.0, 53.7, 49.0, 38.0, 27.0, 22.3, and 17.7 kDa, while field isolate had OMPs of 151.3, 89.0, 75.8, 67.6, 37.0, 27.0, 24.0 and 19.0 kDa. *B. abortus* RB51 yielded 11 OMP bands ranging from 12.5 to 107.1 kDa, with 34.2, 15.8 and 12.5 kDa as additional OMPs. Western immunoblot analysis using antisera raised against all three strains in buffaloes indicated an almost similar pattern of immuno-reactive OMPs in S19 and field strain. Two OMPs of molecular weight 37-38 and 19 kDa were immuno-reactive in all strains in buffaloes. There is possibility of use of these OMPs in a recombinant vaccine for *B. abortus*. A distinct protein of molecular weight of 151.3 kDa was identified in field strain but not in both vaccine strains of *B. abortus*. Use of this OMP in a diagnostic assay may differentiate between vaccinated and infected animals.

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INTRODUCTION

Brucellosis is an economically important contagious bacterial infection of livestock worldwide. The disease is characterized by reproductive failure, particularly abortion in females and orchitis with sterility in males. Other manifestations of the disease include: still births, reduced milk yield, high frequency of retained placenta, prolonged calving interval and hygroma in knee joints (Radostits *et al.*, 2004). In cattle and buffaloes, biovars of *B. abortus* are mainly responsible for brucellosis (OIE, 2008). It is also wide-spread zoonotic infection in the developing as well as developed countries (Godfroid *et al.*, 2005). Seroprevalence of the disease has also been reported in horses (Wadood *et al.*, 2009)

Outer membrane proteins (OMPs) are important immunogens in most of the gram negative bacteria. Investigations on *Brucella* OMPs were initiated in 1980 by sequential extraction methods using different enzymatic and detergent treatments and the characteriza-

tion of these proteins was carried out on the basis of molecular masses (Tibor *et al.*, 1999; Cloeckaert *et al.*, 2002). The role of various outer-membrane and other cellular proteins in provoking immune response against *Brucella* has been investigated in cattle and murine model by immunoblot analysis and humoral as well as cell mediated immune response studies (Onate and Folch, 1995; Stevens *et al.*, 1995; Vemulapalli *et al.*, 2000). Immunoblotting and enzyme linked immunosorbant assays indicated the usefulness of OMPs as differential diagnostic antigens in cattle (Gupta *et al.*, 2007; Mathur *et al.*, 2008). Moreover, several OMPs were tested as a candidate for subunit and recombinant vaccine in various species of animals (Cassataro *et al.*, 2007).

However, there are no reports on the evaluation of *Brucella* OMPs which are immunogenic in buffaloes, the prime dairy animal of South Asia. The present study was designed to identify the OMPs of two commonly used vaccine strains and a local field strain of *Brucella abortus* that are immunogenic in buffaloes.

MATERIALS AND METHODS

Bacterial strains and cultivation

Three strains of *B. abortus* i.e. avirulent smooth S19 vaccine strain, rough RB51 vaccine strain and a locally isolated biotype 1 strain were used in the present study. Each strain was grown in bulk on trypticase soy agar enriched with 0.1% yeast extract in Roux flasks at 37°C for 48 hours in 5% CO₂ atmosphere. Pure culture of each strain was harvested in normal saline and pelleted by centrifugation (1700×g). The pellet of each strain was washed twice with 0.85% normal saline.

Preparation of outer membrane proteins enriched fractions

Outer membrane proteins (OMPs) enriched fractions were extracted following method described by Munir *et al.* (2007). Briefly, the harvested cells (0.5g) were re-suspended in 4 ml of 10 mM Hepes buffer (pH 7.4) and sonicated at 100% amplitude at 0.5 minutes interval for four minutes. The cell debris was removed by centrifugation at 1700×g. The supernatant was ultra-centrifuged at 100,000×g and pellet which contained total membrane proteins was re-suspended in 2% sodium lauryl sarcosinate (sarkosyl) detergent and kept at 22°C for 60 minutes to dissolve inner membrane proteins. Sarkosyl insoluble fraction was then sedimented by centrifugation at 100,000×g. The pellet was washed twice with distilled water, dialyzed against distilled water and stored at -20°C. The protein contents of outer-membrane enriched extract was determined by Lowry's method (Barta, 1993), using bovine serum albumin as standard.

SDS-PAGE

The OMP fractions prepared from the three strains were subjected to SDS-PAGE (Laemmli, 1970), using 12% separating and 4% stacking gel in a BIO-RAD mini gel system. The protein samples containing 15 µg proteins were treated with sample buffer and loaded in the wells. Protein marker SM 0661 (Fermentas, Canada) was used as molecular marker. The gel was run at 30 mA constant current for 45 minutes in running gel buffer and stained overnight with coomassie brilliant blue. Destaining was done for 2 hours with continuous shaking. The molecular weights of the bands were calculated from the Rf values.

Preparation of antisera

Anti-brucella specific polyclonal sera against each strain were raised in two buffalo heifers following Garin-Bastuji *et al.* (1990). S19, RB51 and field isolate were injected intradermally as 5×10⁹ living cells. The animals were then given booster injections of killed cells in incomplete Freund's adjuvant at weekly intervals for 5 weeks. Serum was collected after one week of the last injection and stored in aliquots for further use.

Western blotting of OMPs

Immunoblotting of the OMPs was performed by the method given on web (www.clark.edu/faculty/robertson/laboratorymethods/westernblotting). Briefly, OMPs fractionated by SDS-PAGE were transferred on to 0.45 µm nitrocellulose membrane using a mini transblot apparatus at 90 volts constant voltage for 0.5 hours in

chilled transfer buffer. After transfer, the membrane filters were placed in blocking buffer containing 3% bovine serum albumin for one hour at room temperature with continuous shaking in separate plastic boxes. Primary antibodies i.e. serum diluted 1:100 against S19, RB51 and field isolate were added and the membranes were incubated at 4°C overnight with continuous agitation. Nitrocellulose membranes were washed four times with washing buffer. Then 1:1000 diluted second antibody i.e. anti-bovine IgG horse reddish peroxidase conjugated (Cappel, USA) was added. After incubation for one hour at room temperature with continuous agitation, the membranes were washed twice with washing buffer and placed in 100 ml of freshly prepared diaminobenzidine for 2 to 3 minutes. The reaction was stopped by rinsing in water and the membranes were scanned for permanent record.

RESULTS

OMPs of *B. abortus* strains

SDS-PAGE profile of OMPs of all three strains of *B. abortus* is given in Plates 1-3. Eight bands of sarkosyl insoluble fractions (OMPs) of *B. abortus* S19 were detected on the stained gel. These bands included proteins with molecular masses of 89.0, 73.0, 53.7, 49.0, 38.0, 27.0, 22.3 and 17.7 kDa (Plate 1). Among these eight bands, two of the protein bands i.e. 53.7 and 27 kDa stained weakly.

B. abortus RB51 exhibited eleven protein bands of high, medium and low molecular weights. Proteins of high molecular mass seen were 107.1 and 74.1 kDa. The medium molecular mass proteins consisted of 53.7, 37.1, 34.2 and 25.7 kDa bands. Low molecular weight bands observed on stained gel of RB51 were of 21.8, 20.4, 18.1, 15.8 and 12.5 kDa (Plate 2). Three protein bands i.e. 107.1, 21.8 and 18.1 kDa seemed to have higher concentrations than the other proteins, as these proteins had relatively densely stained bands in the gel.

Field strain of *B. abortus* yielded eight protein bands of molecular masses 151.3, 89.0, 75.8, 67.6, 37.0, 27.0, 24.0 and 19.0 kDa on the stained gel (Plate 3). Protein bands of 75.8, 67.6 and 19.0 kDa were weakly stained, while the bands having molecular masses 37.0, 27.0 and 24.0 kDa were densely stained.

Western immunoblot analysis of OMPs

Distinct variations were seen among the immunoreactive OMPs of three *Brucella abortus* strains used in the study. Transblots of S19, RB51 and field isolates against different sera revealed multiple immunoreactive bands. However, sharp reactivity with 37-38 kDa bands was exhibited in all the three strains in each serum.

Immuno-reactive proteins of all three strains using antisera raised against S19 strain are shown in Plate 4. S19 antiserum revealed three distinct antigenic proteins of molecular weights 38.0, 17.0 and 14.0 kDa in S19 and field isolate OMPs. However, 17 kDa protein reactive band was weaker in field isolate than S19. RB51 had maximum number of immuno-reactive proteins against S19 antiserum. These included OMPs of molecular weight 107.0, 38.0, 22.0, 19.0, 17.0, 14.0 and 12.0 kDa.

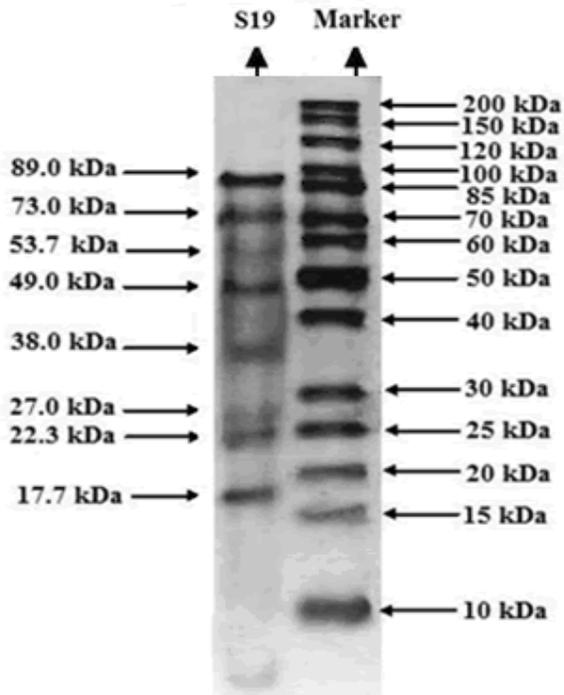


Plate 1: Outer membrane protein profiles of *B. abortus* S19 using SDS-PAGE. Protein marker (0611, Fermentas, Canada) is also shown.

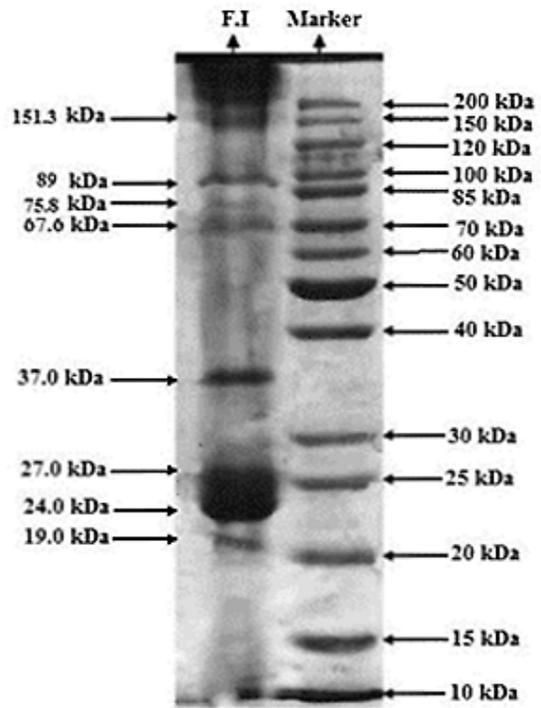


Plate 3: Outer membrane protein profiles of *B. abortus* field isolate (biotype I), using SDS-PAGE. Protein marker (0611, Fermentas, Canada) is also shown.

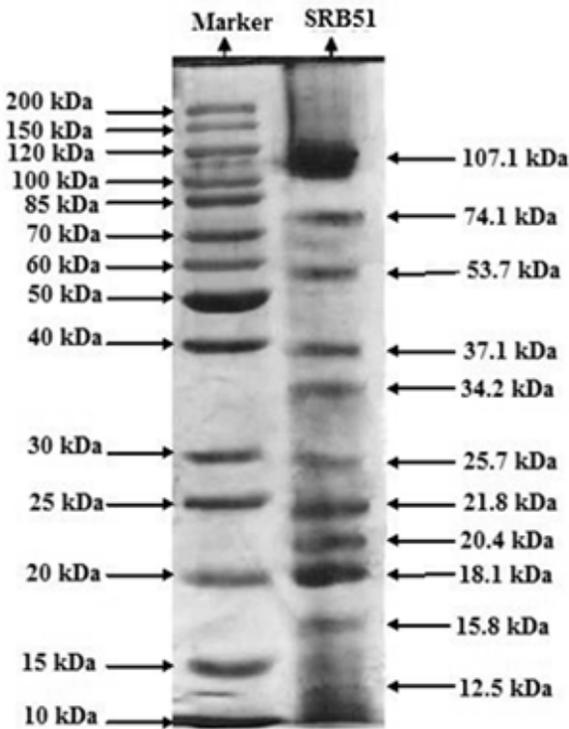


Plate 2: Outer membrane protein profiles of *B. abortus* RB51 using SDS-PAGE. Protein marker (0611, Fermentas, Canada) is also shown.

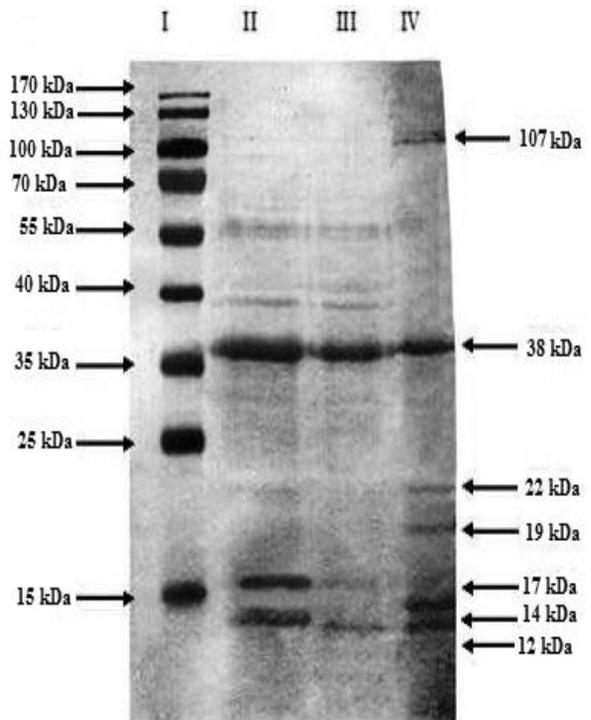


Plate 4: Westernblot analysis of OMPs of *B. abortus* S19 (Lane II), field isolate (Lane III) and RB51 (Lane IV) with high antibody titre serum raised against S19 in buffaloes. Lane I shows protein markers (SM 0671, Fermentas, Canada).

Two immuno-reactive proteins i.e. 37.0 and 19.0 kDa were detected against RB51 antisera in all three strains. Furthermore, similar immunoreactive OMPs profiles of S19 and field isolate using RB51 were detected including 53.7, 49.0, 37.0 and 19.0 kDa proteins. RB51 contained a distinct 107.0 kDa immunoreactive OMP. Immunoreactive proteins of 53.7 and 49.0 kDa were not detected by antiserum raised against RB51 in the Western blotting of RB51 OMPs (Plate 5).

Western immunoblot analysis of OMPs of all three strains using antiserum raised against field isolate showed common reactivity to 37.0 and 19 kDa proteins (Plate 6). Reactive intensity was higher for 37.0 kDa protein than 19.0 kDa. Two additional immuno-reactive proteins were detected in OMPs of RB51. These proteins had molecular weights of 34.0 and 16.5 kDa.

DISCUSSION

OMPs of *Brucella* species were extracted successfully by removing inner membrane proteins through sarkosyl treatment in the present study. Previous studies (Dubray and Bezar, 1980; Santos *et al.*, 1984) have extracted OMPs using different enzymatic and detergent treatments. Sarkosyl treatment is a single step procedure which has given good yield of OMPs from different strains of *B. abortus*. It also avoids enzymatic treatment which may alter the structure of the OMPs.

Distinct profiles of OMPs in three strains of *Brucella* seen in the present study indicate differences in their origin which could be used for differentiation of the strains. Previous studies (Winter *et al.*, 1989) on OMPs of different *Brucella abortus* strains have shown 3 main groups including group-I proteins having molecular masses between 88-94 kDa, group-II between 36 and 38 kDa and group-III between 31 and 34 kDa. Although most of OMPs seen in our study also fall in the same groups, there were two additional distinct protein groups not reported by these workers. These included higher molecular mass proteins (107.1 and 151.3 kDa) and low molecular weight proteins (<20 kDa). Low molecular weight proteins in *Brucella* species have also been reported by Tibor *et al.* (1999). Differences could be attributed to variations in bacterial strains and the method of OMP extraction.

Western immunoblot analysis using antisera raised against all three strains in buffaloes indicated an almost similar pattern of immunoreactive OMPs in S19 and field strain, which is supported by Munir (2009), who indicated that S19 was better protective than RB51 against experimental challenge in buffaloes. Different workers (Cloeckaert *et al.*, 1990; Teresita and Cespedes, 2000) identified various OMPs from *Brucella* in Western blotting using both polyclonal and monoclonal antisera. Most of these OMPs were also found immuno-reactive in both vaccinal strains and field isolate using antisera raised in buffaloes.

The use of OMPs of *Brucella* in providing protection against experimental challenge in laboratory animals and cattle heifers has been reported earlier (Oliveira and Splitter, 1996; Montana *et al.*, 1998; Pasquevich *et al.*, 2009). Our study indicated two OMPs of molecular weight 37-38 and 19.0 kDa which were immuno-reactive

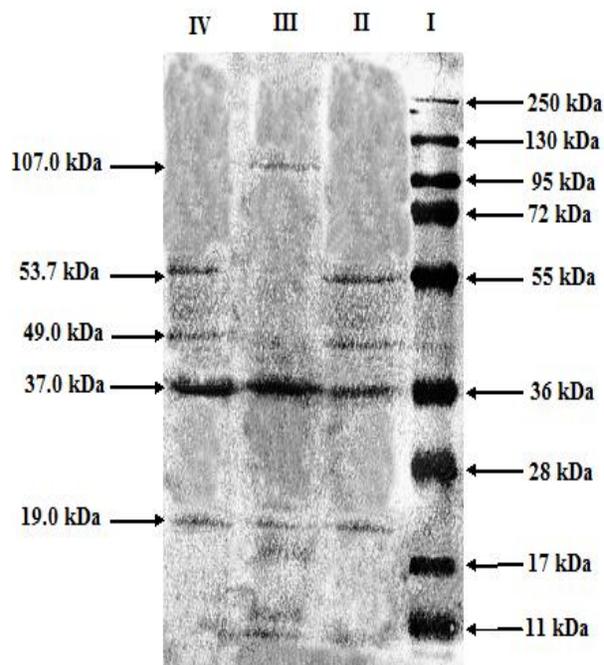


Plate 5: Westernblot analysis of OMPs of *B. abortus* S19 (Lane II), SRB51 (Lane III) and field isolate (Lane IV) with high antibody titre serum raised against RB51 in buffaloes. Lane I shows protein markers (SM 1811, Fermentas, Canada).

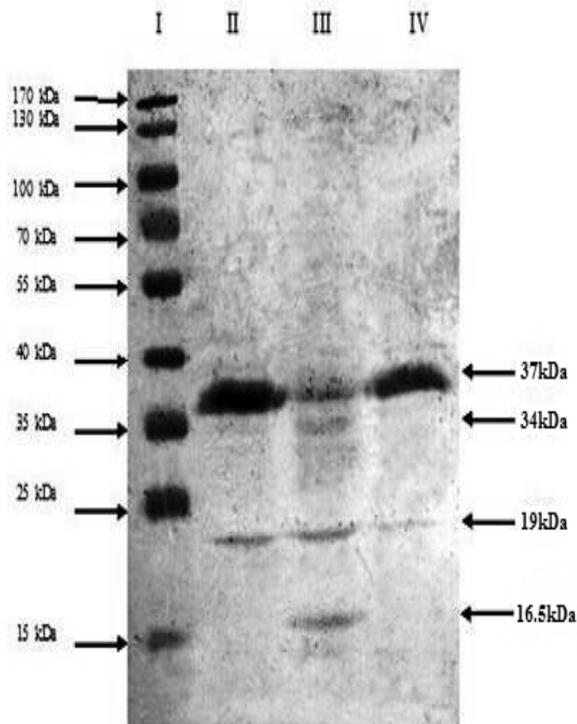


Plate 6: Westernblot analysis of OMPs of *B. abortus* S19 (Lane II), RB51 (Lane III) and field isolate (Lane IV) with high antibody titre serum raised against field isolates in buffaloes. Lane I shows protein markers (SM 0671, Fermentas, Canada).

in all strains in buffaloes. The possibility of use of these proteins in developing a recombinant vaccine for *Brucella abortus* infection in buffaloes needs to be probed further.

A distinct protein of molecular weight of 151.3 kDa was identified in field strain but not in both vaccine strains of *B. abortus*. Use of this protein in diagnostic assays could possibly differentiate between vaccinated and field infected animals in brucellosis. ELISA or other similar sensitive diagnostic assays using this OMP as antigen should be able to clarify this point.

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