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Outer Membrane Protein (OMP) Profiles of *Pasteurella multocida* Isolates Associated with Haemorrhagic Septicaemia by SDS-PAGE and Western Blot Analysis

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ABSTRACT

Haemorrhagic Septicaemia (HS) is an acute fatal septicaemic disease of cattle and buffaloes. It is caused by *Pasteurella multocida* serotype B:2. The disease is of great economic importance in India mainly due to the high mortality in susceptible populations. Bacterial Outer Membrane Proteins (OMPs) play an important role in infectious process of many bacteria. In the present study, OMP of *P. multocida* (serogroup B) field isolates (n = 12) and a vaccine strain (P-52) were extracted by a sarkosyl method and characterized using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. A total of 9-14 different polypeptide bands were observed with approximate molecular weight ranging from 16-123 kDa. On the basis of stain intensity, 32 kDa protein appeared to be the major protein band followed by 37, 26, 29, 89 and 72 kDa bands. Two protein bands of 123 and 46 kDa were present only in vaccine strain. Protein band of 69 kDa was present only in four of the field isolates and vaccine strain whereas OMP with 43 kDa was detected in only four of the field isolates and absent in remaining field isolates and vaccine strain. Immunoblotting identified 32, 37, 72 and 89 kDa protein bands as immunogenic OMPs. It can, therefore, be concluded that outer membrane proteins play a significant role in the pathogenesis of pasteurellosis. Several OMPs act as immunogens for which the antibodies produced against them demonstrate a strong protective action and such OMP antigens may be used as component of subunit vaccines.

Key words: *Pasteurella multocida*, haemorrhagic septicaemia, outer membrane protein, OMP profile, SDS-PAGE, Western blotting

INTRODUCTION

Haemorrhagic Septicaemia (HS), an acute fatal septicaemic disease of cattle and buffaloes, is caused by *Pasteurella multocida* serotype B:2. The disease is of great economic importance in India mainly due to the high mortality in susceptible populations. Bacterial Outer Membrane Proteins (OMPs) play an important role in infectious process of many bacteria. Being surface exposed epitope of gram negative pathogens, OMP may induce immune responses useful both in diagnosis and

prophylaxis. Pati *et al.* (1996) and Srivastava (1998) reported that OMPs of *P. multocida* were immunogenic and protective to buffalo calves and rabbits, respectively. Variation in OMP profile among the isolates of *P. multocida* from HS cases may help in epidemiological survey. Analysis of OMPs of *P. multocida* by polyacrylamide gel electrophoresis (PAGE) has been successfully used to identify the protective antigens and to study the overexpression and repression of proteins under different growth phases (Chawak *et al.*, 2000; Tomer *et al.*, 2002; Filia *et al.*, 2009; Tan *et al.*, 2010; Abusalab *et al.*, 2013).

The present study reports the OMP profile of different field isolates of *P. multocida* obtained from HS affected buffaloes in Karnataka in comparison with the standard vaccine strain (P-52) using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Further, the immunogenic proteins were identified by immunoblotting.

MATERIALS AND METHODS

Bacterial strains: A total of 12 isolates of *P. multocida* obtained from buffaloes died of HS in Karnataka state were used in the present study. They were designated as PM 01-12. The vaccine strain (P-52) procured from Institute of Animal Health and Veterinary Biologicals, Hebbal, Bangalore was used as reference strain. The isolates were earlier confirmed as *P. multocida* serogroup B using multiplex Polymerase Chain Reaction (m-PCR).

Extraction of OMPs: The Outer Membrane Proteins (OMPs) of the isolates were extracted as per the method described by Davies (1991). Briefly, the bacteria were grown in 200 mL of Brain Heart Infusion (BHI) broth by incubating at 37°C for 18 h in a shaker incubator. The bacteria were sedimented at 6000 g for 20 min, pooled and suspended in 35 mL of 20 mM Tris (pH 7.2). They were then pelleted at 6000 g for 20 min. Bacteria were then re-suspended in 35 mL of 20 mM Tris (pH 7.2) and placed in a 50 mL beaker and sonicated with 0.75 probe at 9020 Hz amplitude on ice for 6 min continuously. Sonicated preparation was centrifuged at 6000 g for 20 min. The supernatant containing the cell wall and OMPs was removed with a pipette and placed into a clear ultracentrifuge tube and pelleted at 60,000 g for 1 h. The supernatant was discarded and the pellet was re-suspended in 1 mL of 20 mM Tris (pH 7.2). Cytoplasmic membrane was then solubilized by adding 4 mL of 0.5% N-lauroyl sarkosine and incubated at room temperature for 30 min. Clumps were broken up by pipetting up and down several times. The OMPs were then transferred to a 5 mL ultracentrifuge tube and pelleted at 60,000 g for 1 h and washed once in 20 mM Tris (pH 7.2). The pellet was finally re-suspended in 1 mL of 20 mM Tris (pH 7.2). The protein concentration was determined using Bradford dye binding method.

Analysis of OMPs by SDS-PAGE: Sarkosine extracted OMPs (15 µg) of each isolate was mixed with an equal volume of 2X sample buffer and heated at 100°C for 3 min. The samples were analyzed in 12% resolving gel and 5% stacking gel as per the protocol described by Laemmli (1970) along with the pre stained molecular weight markers. Electrophoresis was carried out at a constant volt of 100 V until the dye front reached the lower end of the gel. The gel was carefully separated and immersed in 5 volumes of staining solution and placed on a slowly rotating platform for 6 h at room temperature. The gel was destained by soaking in the destainer solution on a slowly rotating platform by changing several times until polypeptide bands were clearly visible. The electrophoretic mobility of proteins was compared with standard pre-stained protein marker and molecular weights of different proteins were determined by calculating the relative front value. A permanent record of the stained gel was made by scanning in a computer scanner.

Western blotting: The proteins were transferred on to nitrocellulose membrane (NCM) as per Sambrook and Russell (2001) using semi dry electroblot apparatus (Bio-Rad, USA) at 15 V for 45 min. Immunoblotting of the NCM having proteins transferred from gel was carried out using buffalo immune serum as primary antibody and rabbit anti bovine IgG-HRPO (horse radish peroxidase) conjugate. Then, the strips were dipped in 0.05% 3'-3' diaminobenzidine tetrahydrochloride. When the desired colour densities were obtained, the reaction was stopped by washing NCM in distilled water.

RESULTS AND DISCUSSION

The OMP profiles revealed a total of 9-14 polypeptide bands with approximate Molecular Weights (MW) ranging from 16-123 kDa (Fig. 1 and 2). Two protein bands with MW of 123 and 46 kDa were present only in vaccine strain whereas, MW 69 kDa was present only in four of the field isolates (PM04, PM05, PM10 and PM11) and vaccine strain. OMP with MW of 43 kDa was present in only four of the field isolates (PM01, PM02, PM04 and PM05) and other OMPs with MW 89, 72, 48, 37, 32, 29, 26 and 16 kDa were common to all the field isolates as well as the vaccine strain. On the basis of stain intensity, 32 kDa protein appeared to be the major protein band followed by 37, 26, 29, 89 and 72 kDa bands.

Marandi and Mittal (1996) demonstrated two major bands with molecular masses of 32 and 37 kDa in sarcosyl-insoluble OMP profiles of *P. multocida* on SDS-PAGE. The results of the present study are in close agreement with that of Pati *et al.* (1996) who observed 10 major and 4 minor protein bands of OMPs of *P. multocida* serotype B:2 on SDS-PAGE analysis ranging from 25-88 kDa with two polypeptides of 30 and 37 kDa as predominant ones. Tomer *et al.* (2002) revealed 20 polypeptide bands in the profiles of the *P. multocida* (serotype B:2) field isolates and vaccine strain and identified three polypeptides of MW 31, 33 and 37 kDa as major OMPs based on the band thickness and intensity of staining. OMP profiles for HS causing *P. multocida* capsular group B were revealed by many other workers. Kedrak-Jablonska and Borkowska-Opacka (2006)

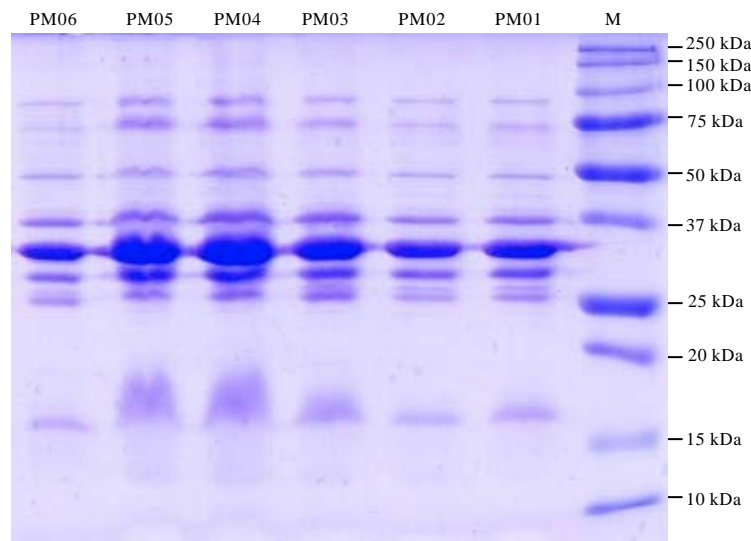


Fig. 1: SDS-PAGE profile of *P. multocida* field isolates, PM 01-06: Field isolates of *P. multocida* serogroup B and M: Pre stained protein molecular weight marker

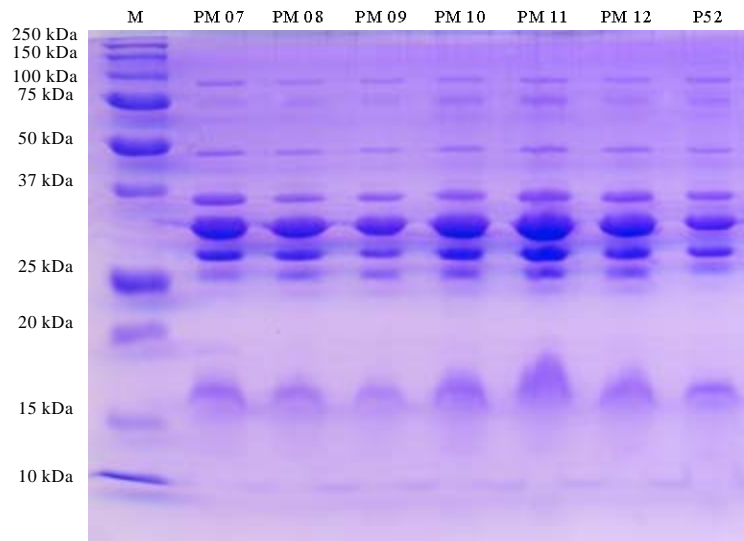


Fig. 2: SDS-PAGE profile of *P. multocida* field isolates and vaccine strain (P52), PM 07-12: Field isolates of *P. multocida* serogroup B, P52: Vaccine strain (*P. multocida* serogroup B) and M: Pre stained molecular weight marker

also reported the presence of proteins with MW ranging from 18-104 kDa in the electrophoretic profiles of OMPs of *P. multocida* serotype B:2, 5 and demonstrated 31-32 and 36-37 kDa as major OMPs based on stain intensity. Similar observations were also made by Arora *et al.* (2007) who reported that a homogenous pattern of 23 distinct protein bands ranging in MW from 13-94 kDa was seen with OMP profiles of *P. multocida* capsular B field isolates and identified 32 kDa protein followed by 25 and 28 kDa as major OMPs. Tan *et al.* (2010) revealed 26, 32 and 37 kDa protein fragments as major OMPs of four local Malaysian strains of *P. multocida* serotype B:2.

The minor differences observed in the molecular weights of different polypeptides by different workers may possibly be due to slight variation in the calculation of Rf values (Arora *et al.*, 2007). The difference could also be attributed to the fact that organisms used at different passage levels *in vitro* express different proteins (Knights *et al.*, 1990). The low degree of diversity in OMP profiles may be attributed to the less genetic diversity in HS causing *P. multocida* isolates described previously by several workers (Dutta *et al.*, 2004; Saxena *et al.*, 2005).

Immunoblotting studies carried out by immune serum from buffaloes vaccinated with formalin killed whole cell vaccine revealed 32, 37, 72 and 89 kDa OMPs as major immunogens (Fig. 3). The results of the present study are in close agreement with Pati *et al.* (1996) who revealed that the 30 and 37 kDa polypeptides present in the OMP preparation of *P. multocida* strain B:2 along with 44 kDa were the chief immunogens. They also detected an immunogenic 68 kDa polypeptide in the sonicated preparation. The reports of Tomer *et al.* (2002) also supports present findings, who demonstrated that 37 kDa polypeptide as the most antigenic OMP in the profiles of all the field isolates, including P₅₂ strain of *P. multocida* associated with HS. They opined that this highly antigenic OMP has potential for further protective and immunodiagnostic studies. Chaudhuri and Goswami (2001) identified 87 kDa OMP as one of the major immunodominant proteins in the OMP preparation of *P. multocida* P₅₂ strain and it had a strong homology with the

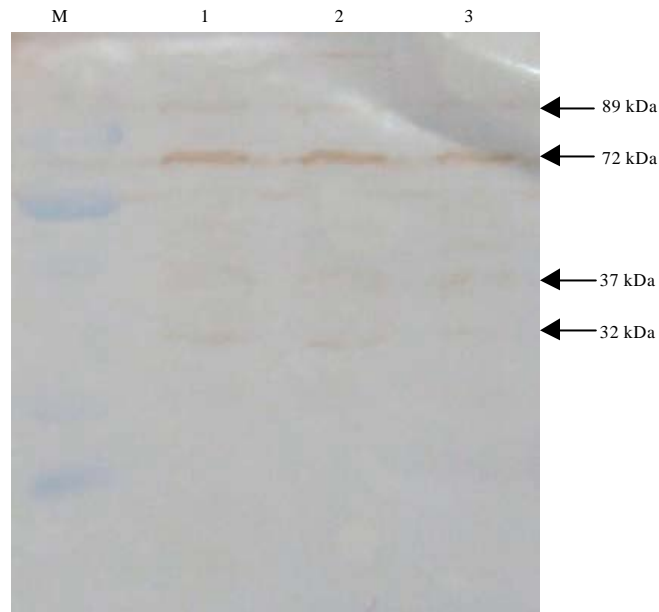


Fig. 3: Immunoblotting analysis of *P. multocida* OMPs, Lanes 1 and 2: Field isolates of *P. multocida*, Lane 3: Vaccine strain (P52) and Lane M: Pre stained molecular weight marker

87 kDa outer membrane antigen (*oma87*) of the *P. multocida* of avian strain. The results of the present study collaborated with Arora *et al.* (2007) who also found 32 kDa to be major immunodominant protein band in the OMP profiles of *P. multocida* field isolates associated with HS including vaccine strain (P₅₂). They along with another study concluded that this band represents a specific marker for the Asian HS isolates. Immunoblotting studies on OMP profiles of *P. multocida* serotype B:2 isolates carried out by Filia *et al.* (2009) and Tan *et al.* (2010) also revealed 32 kDa protein band as immunodominant followed by 37, 45 and 100 kDa protein bands.

Outer membrane proteins play a significant role in the pathogenesis of pasteurellosis. Srivastava (1999) provided evidence that whole cell purified protein vaccine could be attempted to immunize animals against HS and observed that purified protein for vaccine preparation has advantages over conventional whole cell vaccine. Several OMPs act as immunogens and the antibodies produced against them demonstrate a strong protective action. Such OMP antigens may be used as components of subunit vaccines.

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