

Original Research Article

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Isolation and Characterization of the Major Outer Membrane Protein (OMP) of *Pasteurella multocida* Serotype B:2

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ABSTRACT

Outer membrane proteins (OMPs) play a significant role in the pathogenesis of Pasteurellosis. Outer membrane proteins of *P. multocida* are reported to be immunogenic and protective. OMPs of *Pasteurella multocida* (*P. multocida*) B:2 vaccine strain P52 was characterized by using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) technique. The *P. multocida* P52 OMP demonstrated the presence of six OMP bands. The major bands were of 20, 34, 48, 72, 89 and 91 kDa. Potential immunogens of *P. multocida* P52 were identified by electroblot immunoassay. Immunoblotting was performed using the hyperimmune serum raised against the whole cell antigen. Polypeptides separated on 12% SDS-PAGE were transferred to nitrocellulose membrane by semi-dry system. Presence of three polypeptides of 34, 72 and 91 kD size was reported showing reaction with antiserum. A single band of 34kDa in SDS-PAGE was evident and was confirmed by western blot analysis.

Keywords

Pasteurella multocida, SDS-PAGE, Outer membrane protein

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Introduction

Pasteurella multocida is a gram negative coccobacilli and found as a commensal of digestive and respiratory tract of warm blooded animals. It causes diseases in animals (bovines, porcines, rabbits and poultry) immune suppressed by stresses such as viral infections, heat, cold or humidity with aerosol

transmission of infection between animals. Haemorrhagic Septicaemia (HS) is an acute, fatal septicaemic disease of cattle and buffaloes caused by the bacterium *Pasteurella multocida* and occur as a catastrophic epizootic in many Asian and African countries resulting in high mortality and morbidity. Serotypes B:2 and E:2 of *P. multocida* are associated with HS in cattle and buffaloes in

Asia and Central Africa, respectively. In India, it is estimated to cause economic losses of more than 10 million rupees annually (Venkataramanan *et al.*, 2005) thus it is responsible for maximum mortality of livestock in the country (National Animal Diseases Referral Expert System (NADRES) of the Project Directorate on Animal Disease Monitoring and Surveillance, 2012).

The pathogenic potential of *P. multocida* in vertebrate animals was recognized over a century ago and infections are broadly termed as Pasteurelloses. *P. multocida* infects a wide range of animal hosts causing specific infections that manifest differently. Indeed, *P. multocida* has a broad host range, but this peculiar property is poorly understood. Potential virulence factors of *P. multocida* have recently been reviewed (Hunt *et al.*, 2000; Christensen and Bisgaard, 2000; Harper *et al.*, 2006) but no host-specific factors have been identified till yet. The pathogenesis of the disease is a result of complex interactions between host factors (species, age, immune status) and specific bacterial virulence factors which includes LPS, capsule, adhesin, outer membrane etc. (Boyce *et al.*, 2012).

The outer membrane proteins (OMPs) of Gram negative bacteria play essential roles in host-pathogen interactions and in disease processes. Outer membrane proteins of *P. multocida* play a significant role in the pathogenesis of pasteurellosis and have been identified as potent immunogens (Singh *et al.*, 2011). Outer membrane proteins of *P. multocida* have been recognized as immunodominant antigens and are thought to be responsible for cross protective immunity, since lipopolysaccharides (LPS) alone induces only partial protection against pasteurellosis in mice (Ryu and Kim, 2000). The immunogenicity of selected outer membrane proteins of *P. multocida* was demonstrated in rabbits, calves and chickens (Zhang *et al.*,

1994). The outer membrane proteins also have protective role against HS. About 15 to 20 band profiles of outer membrane proteins of molecular weight ranging from 16 to 90 kDa have been documented in all strains of *P. multocida* (Tomer *et al.*, 2002) and OMPs of 39, 46 and 82 to 87 kDa have been identified in all isolates. Outer membrane proteins are important determinants of immunoprotection, hence can serve as vaccine candidates against HS.

The current focus on developing a newer vaccine has identified membrane antigens as potential immunogens in Gram negative bacteria (Zhou *et al.*, 2009). OMP of *P. multocida* have been implicated as protective immunogen. *P. multocida* expresses both heat modifiable protein (OmpA) and porin (OmpH). OmpH possesses both specific and cross-reacting epitopes which are abundantly expressed on the bacterial surface, OmpH is a surface-exposed conserved immunodominant porin that is detected in 100% of bovine isolates and it has been viewed as a potential vaccine candidate (Dabo *et al.*, 2008). It contains a high proportion of antiparallel β -chains, giving it a barrel shape.

The present work shows the OMP profiling of standard vaccine strain P52 by sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE). Further immunodominant proteins were identified by western blotting.

Materials and Methods

For the present study the ampoule containing culture of *P. multocida* serotype B:2 (strain P52) was streaked directly on streaked on blood agar plate for isolation of a single colony and incubated overnight at 37°C. The colony characters were observed on blood agar plate. Approximately 0.5 ml of 10^{-5} dilution of 18 hrs. old culture of P52 was injected intraperitoneally into three healthy

mice. All of them died within 36-38 hrs of inoculation. The organisms were reisolated in pure culture from heart blood and spleen of the dead animals.

The purity and identity of the culture was tested by morphological, cultural and biochemical examinations. The biochemical tests were carried out as per the standard protocols described by Cowan and Steel (1970).

Slides were prepared by picking single colony and the air dried heat fixed smear of the culture was then stained with Gram's stain. The blood smear from heart blood of experimentally infected mice stained by Leishman stain. The smears were observed to study the morphology of bacteria under oil immersion microscope.

The isolate was streaked onto blood agar and MacConkey agar plate and incubated overnight at 37°C. The biochemical tests such as indole, methyl red, Voges-Proskauer, catalase, oxidase and nitrate reduction test were performed with *P. multocida* isolate.

Then, the isolate was also subjected to sugar fermentation test using glucose, fructose, galactose, maltose, mannitol, sucrose, salicin, raffinose, inositol and rhamnose.

Isolation of whole cell bacterial proteins

Single colony of bacterial culture was inoculated in 1ml of broth and grown overnight. One ml from overnight grown culture was inoculated in 1 litre of broth and again grown for 12 hrs. Culture was centrifuged at 10,000 rpm for 10 min and pellet was washed thrice in PBS. Pellet were then suspended in 10mM HEPES buffer and sonicated for 10 min in ice. Cell suspension was centrifuged for 30 min at 10,000 X g. The clear supernatant was collected and filtered

through 0.45µm filter paper. The filtrate was used as sonicated antigen for raising hyperimmune serum. Protein concentration was estimated by Nano drop spectrophotometer.

Purification of outer membrane proteins

For the purification of outer membrane protein (OMPs) sonicated antigen was centrifuged at 1,00,000 x g for 1 hr. at 4°C in an ultracentrifuge. The pellet obtained were resuspended in 2 ml of 2 % (w/v) sodium lauryl sarcosine in 10 mM HEPES buffer (pH 7.4) and incubated at room temperature for 1 hr. The mixture was centrifuged at 1,00,000 x g for 1 hr. at 4°C and pellet were washed twice with distilled water. The pellet containing purified OMPs were dissolved in PBS. Obtained proteins (OMPs) were used for analysis of polypeptides and immunoblotting (Choi-Kim *et al.*, 1991).

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Outer membrane proteins of *P. multocida* P52 were analyzed by SDS-PAGE using the method of Laemmli (1970). The vertical slab gel electrophoresis apparatus was used for performing SDS-PAGE by discontinuous buffer system using 12% resolving gel and 4% stacking gel. The vertical slab gel unit was assembled in casting mode with spacers. The solution was mixed well and poured into the sandwich to a level of 4 cm from the top, then a layer isopropanol was gently added to form the uniform gel surface after polymerization. The layer was poured off. Stacking gel was prepared and overlaid on the resolving gel. After putting the comb into the sandwich, the gel was allowed to polymerize. Fifty µl of the sample was mixed with equal volume of 2X sample buffer and boiled for 5 min in a water bath. The comb was slowly removed from the gel after polymerization. The wells were filled with electrode buffer. The samples and protein

marker were loaded in each well. The lower and upper chambers of the tank were also filled with electrode buffer. The electrophoresis unit was connected to the power pack and the gel was run initially at 80V and then at 120V till the tracking dye reached to the bottom of the gel. The gel was stained with Coomassie brilliant blue for two hrs. Later, the gel was destained in destaining solution with intermittent shaking. Finally gel was rinsed in distilled water and scanned. After destaining the molecular weight of polypeptides were determined by the molecular weight analysis tool of the gel documentation system. The molecular weight of protein was determined by comparing with standard protein molecular weight marker.

Identification of immunogenic proteins by western blotting

Polypeptides separated on 12% SDS-PAGE using discontinuous buffer system were transferred on nitrocellulose membrane by semi-dry method of electroblotting as per the method of Towbin *et al.*, (1979) with minor modifications. Five sheets of thick Whatman filter paper were soaked in transfer buffer and placed on centre of graphite anode electrode plate. The distilled water soaked NCM (Nitrocellulose membrane) was then placed on the top of filter papers. The gel was placed on the membrane followed by stacking of five sheets of filter papers soaked in transfer buffer. The assembled transfer stack was covered with cathode plate and current of 0.8 mA/cm² was applied for one hr. The electrophoretic blot was kept in blocking buffer overnight at 4°C. After washing with wash buffer four times for 5 minutes each, the membrane was incubated at 37°C for one hr. with hyperimmune serum against whole cell antigen diluted 1:50 in blocking buffer. After washing 4 times with washing buffer each for 5 min, the blot was again incubated at 37°C for 1 hr. with anti-rabbit horseradish peroxidase conjugate at a dilution of 1:2000 in

blocking buffer. The blot as described above, was transferred to freshly prepared 50 ml of substrate solution containing diaminobenzidine tetrahydrochloride and 6 µl of 30% (v/v) hydrogen peroxide for few min. The reaction was stopped by washing with distilled water. After drying, the membrane was stored in a dark place.

Purification of 34kDa outer membrane protein and its confirmation by western blot

For purification of OmpH, the band corresponding to 34kDa was excised from the preparative gel, of 3 mm thickness, and incubated in a rotary shaker at 30°C overnight in one ml elution buffer (50 mM Tris-HCl, 150 mM NaCl and 0.1 mM EDTA; pH 7.5). Centrifugation was done at 7,000 g for 10 min to obtain the supernatant as OmpH. The protein was tested for the presence of single band in SDS-PAGE.

Results and Discussion

Culture of *P. multocida* P52 was revived on Brain Heart Infusion (BHI) broth and blood agar. Characteristic non-haemolytic colonies were obtained which were gram-negative coccobacilli.

Pathogenicity test of the organism

Mice were inoculated intraperitoneally with 0.5ml of 10⁻⁵ dilution of 18hrs. old broth culture of *P. multocida* P52 and they were found dead within 24-48 hours. The re-isolated colonies showed characteristic of *P. multocida*.

Characterization of culture

P. multocida produced small, smooth, circular, glistening and dew drop like colonies without haemolysis on blood agar (BA) plate on

incubation at 37°C for 24 hrs. The colonies were found to be watery, discrete and translucent with a characteristic odour of the culture (Fig. 1). On microscopic examination of stained smears, the isolate was found to be Gram-negative cocco-bacilli, arranged singly or in pairs. The bipolar reaction exhibited by the isolates following methylene blue staining was an indication of *P. multocida*. No motility was observed in stab culture in semisolid agar at 37°C. There was no growth on MacConkey's lactose agar (MLA). Biochemical characterization revealed that *P. multocida* fermented glucose, fructose, galactose, maltose, mannitol and sucrose but salicin, raffinose, inositol and rhamnose were not fermented. Indole production along with catalase and oxidase production was also shown by the purified organism.

Rajkhowa *et al.*, (2012) and El-Jakee *et al.*, (2016) studied cultural, morphological and biochemical characteristics of *P. multocida*. The organism is Gram-negative short ovoid rod with bipolar staining characteristics, non-haemolytic, aerobic to facultative anaerobic and produces indole, oxidase, catalase, ferment carbohydrates with slight gas production and failed to grow on MacConkey agar. Similar type of studies were also conducted by Verma (1991) and De Alwis (1996). Chawak *et al.*, (2000) reported the biochemical characterization of *P. multocida* of avian origin and they were uniform in fermentation of fructose, mannitol, sucrose and glucose. Yadav *et al.*, (2016) isolated *P. multocida* B:2 were from buffaloes and cattle. The isolates were found genetically distinct from standard *Pasteurella multocida* strain P52 (Vaccine strain of India). Two isolates shared same profile while all other isolates shown different profiles. This study provides a clear evidence of presence of more than one isolate in single outbreak of HS and also provide indication of high genetic variation among field isolates of *Pasteurella* sp. and

may be the reason of vaccine failure and outbreaks. Likewise studies conducted by Jogi and Shakya (2013), also revealed that the field strain is different from the vaccine strain which may be a possible reason for outbreaks.

Purification of outer membrane proteins

The *P. multocida* P52 outer membrane proteins were obtained by the method as described by Choi-kim *et al.*, (1991), demonstrated the presence of six OMP bands in *P. multocida* P52. The molecular weights of the major polypeptide bands were in the range of 20 to 91 kDa. The major bands were of 20, 34, 48, 72, 89 and 91 kDa (Fig. 2).

Outer membrane proteins of *P. multocida* are reported to be immunogenic and protective (Srivastava, 1998, Basagoudanavar *et al.*, 2006 and Joshi *et al.*, 2013). Some of the major outer membrane proteins like porins are reported to be highly immunogenic. These are pore forming outer membrane proteins and possess beta barrel structure having extracellular, transmembrane and intracellular domain. They are conserved in gram-negative bacteria showing high homology in primary amino acid sequence and secondary structure (Jeanteur *et al.*, 1991 and Dabo *et al.*, 2008). They form large channels allowing the diffusion of hydrophilic molecules into periplasmic space. They are strong immunogen and have been demonstrated to induce protective immunity in animal models against gram-negative bacterial infections (Luo *et al.*, 1999, Tan *et al.*, 2010 and Joshi *et al.*, 2013).

The outer membrane proteins of *P. multocida* P52 were isolated by the method described by Choi-Kim *et al.*, (1991). The organism was disrupted by sonication and large particles were removed by centrifugation at low speed. The OMPs were separated into detergent insoluble and detergent soluble fractions.

Sonicated antigen was used to induce antibody production. Antiserum raised against whole cell protein was used for western blot analysis. It was possible to identify immunodominant outer membrane protein(s) and recombinant clones containing specific OMP gene.

Omp34 belongs to beta-barrel protein super family. The outer membrane of Gram-negative bacteria acts as a molecular filter for hydrophilic compounds. Proteins known as porins are responsible for the molecular sieve properties of the outer membrane. Porin is a major antigenic outer membrane protein of *P. multocida* and has high immunogenicity in antibody production (Lee *et al.*, 2007).

SDS-PAGE analysis of OMP preparation of *P. multocida* P52 strain revealed the presence of six polypeptide bands. The molecular weight of these polypeptides varied between 20 kDa to 91 kDa.

With little difference, Pati *et al.*, (1996) reported ten polypeptides bands of molecular weight 25 to 88 kDa of the same preparation. Kedrak and Opacka (2002) reported protein bands of 22 to 86 kDa in the OMP profiles of bovine strains (serotype B:2). The polypeptides of 31, 34 and 37 kDa molecular weights gave prominent bands on SDS-PAGE indicating that the same were synthesized in abundance corroborating the observations made by Tomer *et al.*, (2002).

The results of our study are in close accordance with Munir *et al.*, (2007) they characterized outer membrane protein of *P. multocida* and obtained total of 6 polypeptides ranging from 15 kDa to 91 kDa and Ataei *et al.*, (2009) investigated the immunogenic outer membrane proteins in a wild type *P. multocida* serotype B:2, five proteins viz., 50, 37, 30, 26 and 16 kDa were found in the sonicated cell extract. Present results are somewhat different with the results of other

workers. Wasnik (1998) and Tomer *et al.*, (2002) detected 13 and 20 polypeptide bands respectively, in profiles of outer membrane proteins. Jain *et al.*, (2005) revealed the presence of 11 protein fractions with two major OMPs of 32 and 35 kDa in capsular type B isolates.

Arora *et al.*, (2007) have reported a homogenous outer membrane profile of 17 different *P. multocida* isolates of bovine origin comprising 23 polypeptides ranging in MW from 13 to 94 kDa. On the basis of band thickness and stain intensity 32kDa protein appeared to be the major protein followed by 25 and 28 kDa.

Apart from this, other significant protein bands observed were 13, 34, 44.5, 46, 80 and 84 kDa. Yadav (2008) revealed the presence of eight polypeptide bands. The molecular weight of these polypeptides varied between 16 kDa to 97 kDa comprising of major protein of 34kDa and 87kDa. Joshi *et al.*, (2013) also reported that 34kDa protein was immunodominant.

They also considered that 34kDa protein band represented a type specific marker for the Asian HS isolates so it might be act as a candidate antigen for a subunit HS vaccine and can be exploited in immunodiagnosis of HS.

Identification of immunogenic proteins by western blotting

Potential immunogens of *P. multocida* P52 were identified by electroblot immunoassay. Immunoblotting was performed using the hyperimmune serum raised against the whole cell antigen in order to detect immunogenic proteins in outer membrane proteins. Polypeptides separated on 12% SDS-PAGE were transferred to nitrocellulose membrane by semi-dry system.

Fig.1 Small, smooth, circular, glistening and dew drop like nonhaemolytic colonies on blood agar after incubation at 37°C for 24 hours

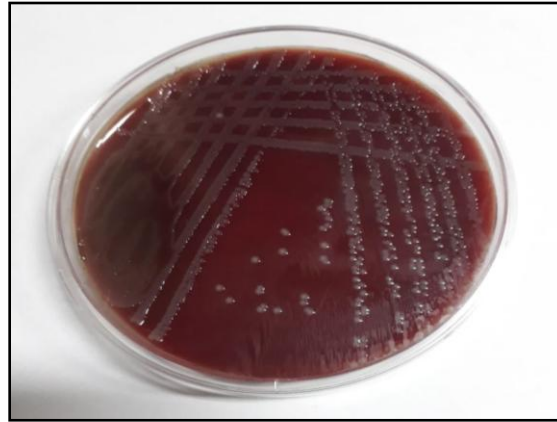


Fig.2 SDS-PAGE of outer membrane protein of *P. multocida* (P52)

Lane M : Protein Marker

Lane 1 & 2 : OMPs of *P. multocida* (P52)

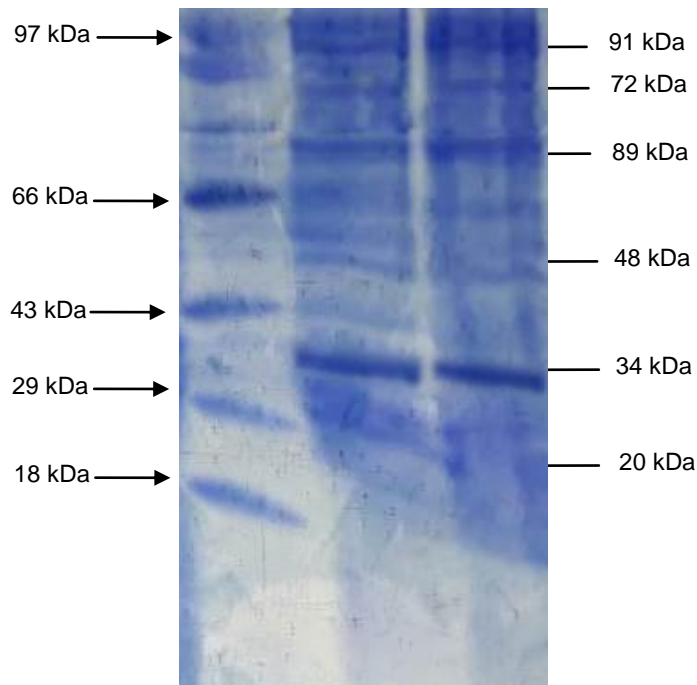
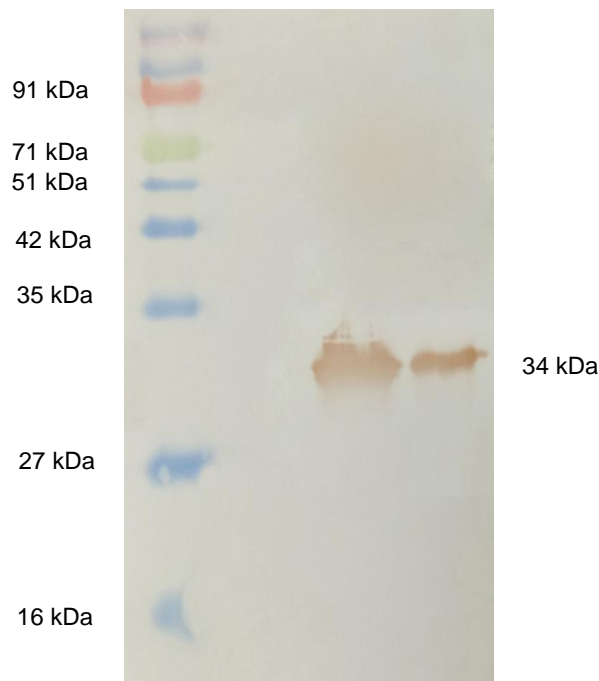


Fig.3 Western blot of Purified OMP of *P. multocida* (P52)

Lane M : Protein Marker
Lane 1 & 2 : 34kDa purified OMP of *P. multocida* (P52)



Presence of three polypeptides of 34, 72 and 91 kDa size was reported showing reaction with antiserum. The results of western blot profiles of major immunogens indicated that all the major protein bands appeared immunogenic; however 34 kDa protein was found to be most immunodominant among them. In the present study, out of six immunodominant outer membrane proteins, three polypeptides of 34, 72 and 91 kDa molecular weights were identified. The results differed little from those reported by Pati *et al.*, (1996) who encountered faint signals of only three outer membrane proteins of 44, 37, and 33 kDa reacting to hyperimmune sera. According to Arora *et al.*, (2007), 32 kDa protein band was found to be immunodominant along with 25 kDa in all the *P. multocida* isolates of bovine origin.

The original objective of this study was to isolate and characterize the *ompH* gene encoding immunopotent outer membrane

protein of *P. multocida* B:2 P52 strain. Although, about six (polypeptide) bands of outer membrane proteins were resolved on SDS-PAGE, all these peptides were not equally immunopotent and reacted differently on immunoblotting. With previous observations, the 34 kDa outer membrane protein was found to be potential immunogen. It was found to react strongly on western blot with antisera raised against sonicated antigen. The results strengthened the studies performed by Yadav (2008) and Joshi *et al.*, (2013) that an immunodominant antigen and might serve as potent immunogen in vaccine preparation. Protein of 34 kDa was found to be immunodominant antigen in our study.

Purification of 34kDa outer membrane protein and its confirmation by western blot

The band corresponding to 34kDa was excised from the gel and incubated in a rotary shaker

at 30°C overnight in one ml elution buffer. A single band of 34kDa in SDS-PAGE was evident and was confirmed by western blot analysis (Fig. 3).

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