# CHARACTERIZATION OF *PASTEURELLA MULTOCIDA* ISOLATES OF BUFFALO ORIGIN FROM GUJARAT STATE OF INDIA BY OUTER MEMBRANE PROTEIN PROFILE ANALYSIS

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## ABSTRACT

Haemorrhagic septicaemia caused by *Pasteurella multocida* is one of the most fatal diseases affecting Indian buffaloes. The disease is of high economic significance in India due to the high mortality rate in the affected population. The outer membrane proteins (OMPs) are virulence factors of *P. multocida* which play an important role in the pathogenesis of Pasteurellosis. In the present study, *P. multocida* B: 2 field isolates (n=7) and a vaccine strain ($P_{52}$) were grown under normal and iron restricted conditions. OMPs were extracted by sarkosyl method. Characterization of OMP rich extracts from isolates grown under normal conditions by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) revealed a total of 11 to 13 bands of about 16 to 86 kDa. Two bands of molecular weights 68 and 71 kDa were present only in the OMP profile of vaccine strain. Under iron restricted conditions, two additional protein bands of molecular weights 112 and 125 kDa were expressed in all isolates including the vaccine strain. Based on band intensity, 31 and 37 kDa proteins were assumed to be the major proteins expressed under both normal and iron restricted conditions. Further studies are required to study the role of major proteins and iron regulated outer membrane proteins (IROMPs) in the virulence of the bacteria.

**Keywords**: buffaloes, *Bubalus bubalis*, *Pasteurella multocida*, outer membrane protein, SDS-PAGE, major protein, iron regulated outer membrane protein

## INTRODUCTION

*Pasteurella multocida* is a small, nonmotile, gram-negative, indole positive, fastidious and facultatively anaerobic coccobacillus which is found in the nasopharynx and gastrointestinal tract of apparently healthy domestic and wild animals. *P. multocida* can be a primary or secondary pathogen in the disease process of domestic animals and birds (Rimler and Rhoades, 1989) and is the causative agent of numerous, economically important diseases, including bovine haemorrhagic septicaemia, avian fowl cholera, enzootic pneumonia and swine atrophic rhinitis (De Alwis, 1992). In India, *P. multocida* have been recovered from various outbreaks in different agro climatic geographical regions of the country (Kumar *et al.*, 2004) and haemorrhagic septicaemia alone causes 46 to 55% of bovine deaths (Dutta *et al*., 1990). This shows the economic impact of this pathogen on Indian livestock industry.

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Outer membrane proteins (OMPs) and lipoproteins interact with signaling pathways in host cells and play roles in host response and or host evasion (Mclean, 2012). OMPs of *P. multocida* act as an interface between host and pathogen (Lin *et al.*, 2002) and have a significant role in the bacterial pathogenesis (Srivastava, 1998). Many studies conducted on immunogenicity of selected OMPs (Dabo *et al.*, 1997; Confer *et al*., 2001) revealed that some OMPs can serve as potent immunogens (Hatfaludi *et al*., 2010; Borkowska-Opacka and Kedrak, 2002).

Iron being an essential factor for bacterial growth and replication plays a role in the establishment and advancement of infection. Due to the presence of iron-binding glycoproteins such as transferrins and lactoferrins, the host iron is largely unavailable and the free ionic concentration of iron *in vivo* is inadequate to support microbial growth (Ruffolo *et al.*, 1998). In order to survive under these iron limiting conditions, pathogens like *P. multocida* express a number of iron regulated proteins and low MW siderophores which aid in the sequestration of iron binding host glycoproteins (Hu *et al*., 1986).These OMPs involved in acquisition of iron from the host are also regarded as potential virulence factors (Snipes *et al.*, 1988).

The study was aimed to characterize and to compare the outer membrane protein profiles of *P. multocida* isolates of buffalo origin grown under normal and iron limited conditions.

## MATERIALS AND METHODS

### Bacterial strains

Seven *P. multocida* strains isolated from buffalo affected with haemorrhagic septicaemia and a vaccine strain $P_{52}$ was used for the present study. The field isolates had been isolated and typed based on capsular antigens as per the method of Townsend *et al.* (2001) at the Department of Microbiology, College of Veterinary Science and Animal Husbandry, Anand. The details of the isolates used are furnished in Table 1. The reference vaccine strain $P_{52}$ was procured from Animal Vaccine Institute, Gandhinagar.

### Preparation of OMPs from *P. multocida* strains

The bacterial strains were cultured under normal (BHI broth) and iron restricted (BHI medium supplemented with 150 µM of 2, 2'-dipyridyl) conditions. The cultures were incubated for 18 h at 37ºC. OMPs were obtained

Table 1. Details of the isolates used for outer membrane protein profile analysis.

| Sr. No. | Isolate ID | Host | Year | Samples used for isolation | Region | Capsular type |
|---|---|---|---|---|---|---|
| 1 | PAB-78-1/13 | Buffalo | 2013 | Bone marrow | Patan | B:2 |
| 2 | PAB-80-3/13 | Buffalo | 2013 | Exudate | Patan | B:2 |
| 3 | PAB-81-4/13 | Buffalo | 2013 | Blood | Patan | B:2 |
| 4 | PAB-83-6/13 | Buffalo | 2013 | Tissue | Amreli | B:2 |
| 5 | PAB-84-7/13 | Buffalo | 2013 | Tissue | Amreli | B:2 |
| 6 | PAB-86-9/13 | Buffalo | 2013 | Tissue | Ahmedabad | B:2 |
| 7 | PAB-89-1/14 | Buffalo | 2014 | Tissue | Gandhinagar | B:2 |

as per the protocol described by Choi-Kim *et al.* (1991) with minor modifications as per Munir *et al.* (2007). The culture in BHI broth was subjected to centrifugation at 10,000 rpm for 30 minutes at 4ºC. The pellets obtained were then washed twice in PBS and resuspended in 10 mM HEPES buffer (pH 7.4). These cells were subjected to sonication for ten cycles of 10 seconds each, with 10 seconds interval between each cycle for cooling. The unbroken cells and debris were removed by centrifugation at 1500 xg for 20 minutes. The supernatant was collected and centrifuged at 100,000 xg for 60 minutes at 4ºC. The pellet was resuspended in HEPES buffer containing 2% sodium lauroyl sarcosinate (sarkosyl) detergent and incubated at 22ºC for 60 minutes. The detergent insoluble outer membrane protein enriched fractions were pelleted out by centrifugation at 100,000 xg for 60 minutes at 4ºC. The pellets were resuspended in distilled water and stored at -20ºC. The protein content of OMPs was estimated by using Nanodrop 1000 spectrophotometer at 280 nm using bovine serum albumin as a standard.

**Separation in SDS-PAGE**

Electrophoretic separation of the outer membrane proteins of *P. multocida* strains was performed in 4% stacking gel and 12% resolving gel as per Laemmli, 1970. The separation was carried out at a constant voltage of 100 V, at room temperature and the gel was stained with 0.5% Coomassie blue. The molecular weight of the OMPs was determined by GelAnalyzer software. A pre-stained protein molecular weight marker was used for comparison of the molecular size of OMPs.

## RESULTS AND DISCUSSION

The electrophoretic protein profiles revealed the presence of 11 to 13 protein fractions of about 16 to 86 kDa (Table 2). The band patterns of all the buffalo field isolates revealed presence of 11 common bands with MWs of ~16, 20, 22, 24, 26, 31, 37, 45, 46, 50 and 86 kDa respectively. Reference strain, $P_{52}$ revealed the presence of 13 bands with protein fractions of ~16, 19, 22, 24, 26, 31, 37, 45, 46, 49, 68, 71 and 86 kDa. Based on the intensity of bands, polypeptides with MWs of 31 and 37 kDa were assumed to be the major OMPs in field isolates and vaccine strain.

Under iron restricted condition, all the isolates along with vaccine strain revealed the expression of two additional proteins of MWs 112 and 125 kDa. The intensity of the bands with MWs 20, 24, 26, 31 and 37 kDa were found to be downregulated in isolates grown in iron restricted conditions whereas 50 and 86 kDa bands were found to be upregulated (Figure 1).

Outer membrane proteins (OMPs) of gram negative bacteria are important virulence factors involved in colonization, invasion and pathogenesis. It is important to have thorough knowledge of the outer membrane proteome of *P. multocida* which will help in the identification of potential virulence factors, diagnostic antigens, drug targets, and vaccine candidates (Prasannavadhana *et al*., 2014).

In the present study, for OMP extraction, the bacterial cells suspended in 10 mM HEPES buffer were disrupted by sonication, followed by treatment with 2% sarkosyl. Sarkosyl treated suspensions were then subjected to ultracentrifugation for the separation of OMPs.

Extraction by 2% sarkosyl was also performed by Abdullahi *et al.* (1990), Davies *et al.* (1992), Pati *et al.* (1996), Tomer *et al.* (2002)

and Jain *et al.* (2005) with minor modifications. Marandi and Mittal (1995), Marandi *et al.* (1996) and Borkowska-Opacka and Kedrak (2002, 2003) used 1% sarkosyl for OMP extraction, while Ullah *et al.* (2008), Asma *et al.* (2009) and Prasannavadhana *et al.* (2014) used 0.5% sarkosyl extraction method.

In the present study, electrophoretic protein profiles of the buffalo field isolates revealed the presence of 11 protein fractions of about 16 to 86 kDa. The eleven protein fractions with MW of ~16, 20, 22, 24, 26, 31, 37, 45, 46, 50 and 86 kDa respectively were common in all the isolates.

Johnson *et al.* (1991) reported five main protein bands with MW of 27, 32, 37, 45 and 47 kDa. They found that protein with MW 32 kDa was more intensely stained than the rest of the protein fractions in B type bovine isolates. Wasnik (1998) characterized the OMP of *P. multocida* serotype B: 2 and reported 13 protein fractions with MW ranging from 15.92 to 93.92 kDa. OMP characterization of *P. multocida* serotype B: 2 isolates and $P_{52}$ vaccine strain by Pati *et al.* (1996) revealed ten major protein bands ranging from 25 to 88 kDa. Arora and co-workers (2007) studied the OMP profiles of 17 *P. multocida* isolates of bovine origin by SDS-PAGE. All the isolates showed homogenous protein profiles comprising 23 different polypeptide bands ranging in molecular weight from 13 to 94 kDa. On the basis of stain intensity, 32 kDa appeared to be the major protein band followed by 25 and 28 kDa bands. Munir *et al.* (2007) characterized the OMPs of *P. multocida* B: 2 strains and a total of 6 polypeptides ranging from 15 kDa to 91 kDa

Table 2. Details of outer membrane protein profiles of *P. multocida* isolates.

| Sr. No. | Isolate No. | No. of bands | Molecular weights (kDa) | IROMP bands | Molecular weights (kDa) |
|---|---|---|---|---|---|
| 1 | PAB-78-1/13 | 11 | 16, 20, 22, 24, 26, 31, 37, 45, 46, 50 and 86 kDa | 2 | 112 and 125 kDa |
| 2 | PAB-80-3/13 | 11 | 16, 20, 22, 24, 26, 31, 37, 45, 46, 50 and 86 kDa | 2 | 112 and 125 kDa |
| 3 | PAB-81-4/13 | 11 | 16, 20, 22, 24, 26, 31, 37, 45, 46, 50 and 86 kDa | 2 | 112 and 125 kDa |
| 4 | PAB-83-6/13 | 11 | 16, 20, 22, 24, 26, 31, 37, 45, 46, 50 and 86 kDa | 2 | 112 and 125 kDa |
| 5 | PAB-84-7/13 | 11 | 16, 20, 22, 24, 26, 31, 37, 45, 46, 50 and 86 kDa | 2 | 112 and 125 kDa |
| 6 | PAB-86-9/13 | 11 | 16, 20, 22, 24, 26, 31, 37, 45, 46, 50 and 86 kDa | 2 | 112 and 125 kDa |
| 7 | PAB-89-1/14 | 11 | 16, 20, 22, 24, 26, 31, 37, 45, 46, 50 and 86 kDa | 2 | 112 and 125 kDa |
| 8 | $P_{52}$ | 13 | 16, 19, 22, 23, 24, 31, 37, 45, 46, 49, 68, 71 and 86 kDa | 2 | 112 and 125 kDa |

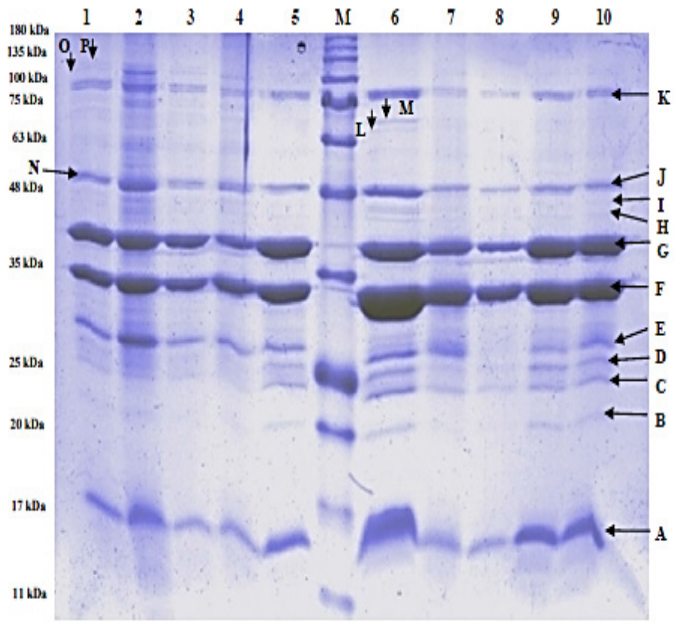


Figure 1. Outer Membrane protein profiles of *P. multocida* isolates on 12% Coomassie Blue stained PA gel.

Lane M- Marker (11 to 245kDa)

Lane 1: OMP profile of $P_{52}$ grown in iron restricted media (BHI broth containing 2, 2' dipyridyl)

Lane 2 to 4: OMP profiles of field isolates grown in iron restricted media (BHI broth containing 2, 2' dipyridyl)

Lane 6: OMP profile of $P_{52}$ grown in normal BHI broth

Lane 7 to 10: OMP profiles of isolates grown in normal BHI broth

A-16 kDa; B-20 kDa; C-22 kDa; D-24 kDa; E-26 kDa; F- 31 kDa; G-37 kDa; H-45 kDa; I-46 kDa; J-49 kDa; K-86 kDa

L-68 kDa, M-71 kDa, N-50 kDa, O-112 kDa, P-125 kDa

were observed which included two intense bands of 39 and 32 kDa, and four less intense bands of 91, 72, 44 and 15 kDa. The characterizations of OMPs of *P. multocida* serotype B: 2 by Dey and Singh (2008) revealed that the MW of isolates and the vaccine strain ranged between 18 and 93 kDa in SDS-PAGE. Asma *et al.* (2009) noticed 4 types of electrophoretic profiles among 9 isolates and the most commonly occurring proteins were found to be of 21, 30, 33, 36, 50, 54, 58, 60, 75, 86, 90 and 108 kDa. Somshekhar *et al.* (2014) extracted OMPs from 12 isolates of *P. multocida* obtained from buffaloes and analyzed them by SDS-PAGE. The OMP profile revealed a total of 9 to 14 bands with approximate molecular weights ranging from 16 to 123 kDa. They observed that protein band of MW 69 kDa was present in only four of the field isolates. They also found that OMPs with MW 89, 72, 48, 37, 32, 29, 26 and 16 kDa were common to all isolates, of which 32 kDa protein was considered to be the major protein.

In the present study, two polypeptides of 31 kDa and 37 kDa could be considered as major OMPs based on the band intensity. This is in agreement with the findings of Tomer *et al.* (2002). They also reported 31 and 37 kDa proteins as the major proteins, though they found an additional major band of MW 33 kDa in the vaccine strain in addition to 31 and 37 kDa protein bands. Similar findings were also reported by Pati and co-workers (1996). The presence of a major protein which is ~32kDa was also reported by Pal *et al.* (2002), Singh and Goel (2002), Arora *et al.* (2007) and Somshekhar *et al.* (2014). Jain *et al.* (2005) found two polypeptides of MW 31.7 kDa and 34.9 kDa as major proteins in serotype B isolates of *P. multocida.*

H.S. vaccine strain, $P_{52}$ revealed the presence of 13 bands with protein fractions of 16, 19, 22, 24, 26, 31, 37, 45, 46, 49, 68, 71 and 86 kDa. Based on the intensity of bands, polypeptides with MW of 31 and 37 kDa were assumed to be the major OMPs.

Somshekhar *et al.* (2014) reported the OMP profile of $P_{52}$ vaccine strain having polypeptide bands ranging from 16 to 123 kDa, of which 32 kDa protein being the most intense band. Other bands were of MW 123, 89, 72, 69, 48, 46, 37, 29, 26 and 16 kDa respectively. OMP characterization of *P. multocida* $P_{52}$ vaccine strain by Pati *et al.* (1996) revealed ten major protein bands ranging from 25 to 88 kDa while, Wasnik *et al.* (1998) detected 13 OMP bands ranging from 15.92 to 93.92 kDa. Tomer and co-workers (2002) found 20 polypeptide bands ranging from 16 to 90 kDa in the OMP profile of $P_{52}$ vaccine strain. Even though the range of MW of protein bands obtained in our study was similar (16 to 86 kDa), only 11 protein bands were obtained in our study. Twenty three polypeptide bands were detected in the $P_{52}$ vaccine strain by Arora *et al.* (2007) ranging from 13 to 94 kDa in MW and they assumed 32, 25 and 28 kDa bands to be the major proteins based on stain intensity. The studies conducted by Prasannavadhana *et al.* (2014) revealed 15 polypeptide bands in $P_{52}$ vaccine strain, of which 33 and 38 kDa proteins were found to be major proteins.

The comparison of OMP profiles of the buffalo isolates and the vaccine strain used in the present study revealed that the overall profiles were similar in terms of amount, number and position of protein bands, with only minor differences. The total number of bands in the buffalo isolates was 11 while H.S. vaccine strain expressed a total of 13 protein bands. The two extra bands expressed in the vaccine strain were having MWs of 68 and 71 kDa. These bands were not expressed in the buffalo isolates and this represent some minor variations

between the buffalo isolates and H.S vaccine strain.

The minor differences were observed in the molecular weights of OMP profiles by different workers. The determination of molecular weights solely on the basis of molecular marker is erroneous and could have led to such incompatibilities (Prasannavadhana *et al*., 2014); the same organism used at different passage level *in vitro* may express different proteins (Knights *et al.*, 1990). The pressure of the host environment *in vivo* on the expression of the protein can also cause difference in the molecular weights of the proteins recognized in SDS-PAGE (Prasannavadhana *et al*., 2014)

In the present study, the buffalo isolates and $P_{52}$ vaccine strain were grown in the medium with iron chelating compound (2, 2' bipyridyl), to study the difference in protein profile under iron restricted conditions. The OMP profiles revealed 13 to 15 bands ranging from 16 to 125 kDa including two additional proteins of MW 112 and 125 kDa. These two proteins were expressed only in iron-limited conditions; all the other bands were of the same molecular weight as in the OMP profile obtained in normal conditions. So the 112 and 125 kDa proteins can be considered as the iron-restricted outer membrane proteins (IROMPs). An upregulation of 50 and 86 kDa proteins was also observed upon iron-restriction. Prasannavadhana (2014) reported three additional proteins (110, 125 and 127 kDa) when $P_{52}$ vaccine strain of *P. multocida* was grown in iron restricted conditions. Borkowska-Opacka and Kedrak (2002) reported IROMPs of MW 102 and 110 kDa from *P. multocida* of serotype B: 2, 5 when grown in iron restricted conditions. Jain *et al.* (2005) also reported the presence of an IROMP of 102 kDa in serotype B isolates of *P. multocida*. In contrast to our study, some studies (Jain *et al.*, 2005 and Wasnik, 1998) revealed no additional bands when $P_{52}$ cells were grown in iron restricted conditions. These variations in iron restricted protein expression might be due to the difference in the amount of 2, 2' dipyridyl used for iron restriction and also owing to the difference in the passage levels of the organisms employed in different studies (Knights *et al*., 1990).

However, there are reports that the immunogenicity of IROMPs is higher than OMPs prepared in iron sufficient medium (Srivastava, 1998 and Confer *et al.*, 2001). So a vaccine prepared from IROMPs can come up with better protection than normal vaccines prepared from *P. multocida* grown under iron sufficient conditions.

In summary, outer membrane protein profiling of *P. multocida* buffalo isolates revealed two major proteins of 31.7 kDa and 34.9 kDa. Two iron regulated outer membrane proteins of MW 112 and 125 kDa were expressed by *P. multocida* isolates but further their role in virulence needs to be studied.

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