

## OUTER MEMBRANE PROTEIN PROFILING OF *PASTEURELLA MULTOCIDA*

R. MUNIR, D. SHAHWAR<sup>1</sup>, U. FAROOQ, I. NAWAZ<sup>1</sup>, I. SHAHZAD<sup>1</sup> AND A. KHANUM<sup>1</sup>

Animal Health Laboratories, Animal Sciences Institute, National Agricultural Research Centre,  
Park Road, Islamabad, <sup>1</sup>University of Arid Agriculture, Muree Road, Rawalpindi, Pakistan

### ABSTRACT

Outer membrane proteins (OMPs) play a significant role in the pathogenesis of Pasteurellosis. OMPs of *Pasteurella multocida* (*P. multocida*) B:2 strain were characterized by using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) technique. Molecular weights of OMPs were determined by plotting graph between  $R_f$  value and log of molecular weight. A total of 6 polypeptides ranging from 15 kDa to 91 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. OMPs, therefore, can be used to identify strain specific markers that would form a useful basis to differentiate them from field isolates of *P. multocida* of same serotype i.e., B:2. However, this was a preliminary type of work and further research using various field isolates as well as vaccinal strains are needed.

**Key words:** *Pasteurella multocida*, SDS-PAGE, outer membrane protein profiling.

### INTRODUCTION

Haemorrhagic septicaemia (HS) is an acute and fatal disease of bovines caused by a bacterium, *P. multocida*. Serotypes B:2 and E:2 of *P. multocida* prevail in Asia and Africa, respectively (Carter, 1955; Heddleston *et al.*, 1972). HS is an economically important disease as only in Punjab province estimated losses due to this disease are 2.17 billion Pakistani rupees (Anonymous, 1996).

Serological identification of *P. multocida* is based on capsular antigens A, B, D, E and F, and somatic antigens 1-16 (Carter, 1955; Heddleston *et al.*, 1972; Rimler and Rhoades, 1987). Somatic serotypes are distinguished by agar gel diffusion precipitation test (Rimler and Rhoades, 1989). Serotyping for both capsular and somatic antigens is very useful for detection and identification but it provides insufficient information for epidemiological studies (Amonsin *et al.*, 2002). Some reports indicate that a significant proportion of *P. multocida* isolates is un-typeable (Namioka and Bruner, 1963; Manning, 1982).

Many biochemical and serological methods have been used to study *P. multocida*. These include catalase, indole, oxidase and sugar fermentation tests. Due to limitations of these methods, more accurate methods to investigate HS are being used. In the present study, *P. multocida* was characterized on the basis of outer membrane proteins (OMPs) by using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

### MATERIALS AND METHODS

#### Bacterial source

*P. multocida* was recovered from stocks kept at  $-20^{\circ}\text{C}$  in lyophilized condition at Animal Health Laboratories, National Agricultural Research Centre, Islamabad, Pakistan. This lyophilized isolate was revived in balb/c mouse by giving subcutaneous injection. Heart was collected aseptically by dissecting dead mouse after 24 hours (De-Alwis, 1999). Mouse heart blood was inoculated on brain heart agar (Oxoid Ltd., England), incubated at  $37^{\circ}\text{C}$  for 24 hours and colonies were observed. The bacteria were confirmed morphologically and bio-chemically by using "API 20<sup>®</sup> NE" kit (bioMérieux, USA), as described earlier (Wilson *et al.*, 1993).

#### Capsular and somatic sero-typing

Rapid slide agglutination test was performed by using capsular type B antiserum obtained from National Veterinary Laboratories, Ames, Iowa, USA (Carter, 1955; Heddleston *et al.*, 1972; Rimler and Rhoades, 1987). For somatic serotyping, core antigen was extracted and isolates were tested against reference antigen by AGPT (Heddleston *et al.*, 1972; Rimler and Rhoades, 1989).

#### Mass cultivation and bacterial cell extraction

*P. multocida* isolate was cultured in 20 flasks each having 100 ml brain heart infusion broths at  $37^{\circ}\text{C}$  overnight. Purity of each flask was monitored. Culture was centrifuged at 10,000 rpm for 30 minutes at  $4^{\circ}\text{C}$  in 50 ml falcon tubes and pellet was collected.

### Bacterial cells washing and cells disruption

Pellet was suspended in 10 ml normal saline and kept overnight at 4°C. Suspension was centrifuged at 10,000 rpm for 20 minute at 4°C and supernatant was discarded. Procedure was repeated twice and finally the pellet was suspended in 4 ml of 10 mM of N-(2-hydroxyethyl) piperazine- N'-2 ethane sulfonic acid (HEPES) buffer (pH 7.4) and bacterial cells were disrupted in a sonicator (Branson Sonifier 450) for 2 minutes (30 seconds for 4 times), centrifuged at 1700g for 20 minutes at 4°C and approximately 3 ml supernatant was collected (Srivastava, 1998).

### Preparation of OMP enriched fraction and protein estimation

The supernatant obtained was ultra-centrifuged at 100,000g for one hour at 4°C, the pellet was collected and was re-suspended in 2 ml of 2% sodium lauryl sarcosinate detergent in 10 mM HEPES buffer (pH 7.4). It was incubated at 22°C for an hour and was ultra-centrifuged at 100,000g for one hour. Pellet was dissolved in 5 ml distilled water after washing twice with distilled water. OMPs were determined by Lowry's method (Barta, 1993). Dilutions of bovine serum albumin (BSA) from 0.1 to 0.5 mg/ml were made along with the 1:5 and 1:10 dilutions of the OMPs. Optical density of standards (BSA), pure OMP and its dilutions was taken at 750 nm wave length in spectrophotometer. A standard graph was plotted between BSA concentrations and corresponding optical density values. Protein concentrations of OMPs were estimated from this standard graph.

### SDS-PAGE of outer membrane proteins

An amount of 20 µl of sample buffer was mixed with 10 µl of OMPs and boiled for 5 minutes (Pal *et al.*, 2002). OMPs were analyzed using 5% stacking gel and 15% separating gel. The protein concentration in sample was adjusted at 15-20 µg/µl and an amount of 15 µl sample was mixed with 1 µl of bromophenol blue and loaded in gel. Molecular weight markers were also loaded in two wells and electrophoresis was performed by applying 30 mA constant current and 200 V for 45 minutes. Separating gel was placed in staining solution overnight. Next day staining solution was replaced with de-staining solution and left for 5-6 hours at constant shaking. Gel was rinsed with distilled water and photographed in white light.

Both low and high molecular weight markers were used to compare with proteins and assess their molecular weights. A graph was plotted between R<sub>f</sub> value and log of molecular weight to determine approximate molecular weight of proteins.

## RESULTS AND DISCUSSION

Morphologically *P. multocida* isolate was a Gram negative coccobacilli. The API 20<sup>®</sup>NE kit value was 3000 004 and in analytical profile index it corresponded to *P. multocida* with 93% identification. API 20<sup>®</sup>NE identification system is an effective and rapid method of biochemical characterization of isolates. The only disadvantage is lack of consistency among percentage of identification e.g., 100% identification was found for *P. multocida* sub-species *septica* (Boerlin *et al.*, 2000) and 96.9% for *P. pneumotropica* (Frebourg *et al.*, 2002) by using API 20<sup>®</sup>NE system. Basis of variation is difference in serotypes. Metabolic activity in isolates of similar sero-groups may differ and cause variation in identification. In addition, API systems give different identification percentages even for the same organism (Quinn *et al.*, 1994). Isolate was typed for capsular antigen by rapid slide agglutination test and it showed agglutination against serotype B.

### OMP profiling of *P. multocida*

A total of six polypeptides ranging from 15 kDa to 91 kDa were obtained having two intense bands of molecular weight of 39 and 32 kDa, and four less intense bands of molecular weights 91, 72, 44 and 15 kDa (Fig. 1). The approximate molecular weight from log of molecular weight and R<sub>f</sub> value are given in Table 1.

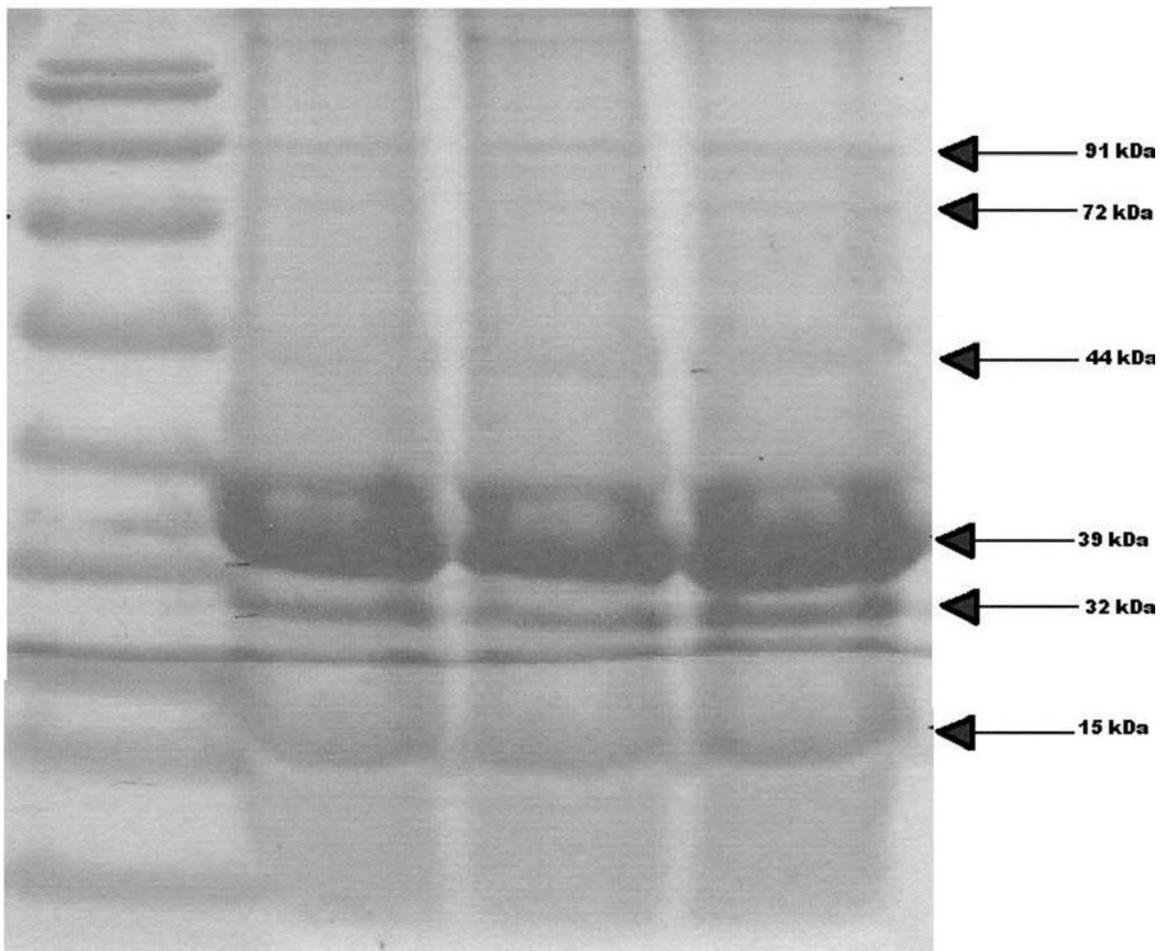
**Table1: Approximate molecular weights of protein**

Band distance (cm)	R <sub>f</sub>	Log of Mol. wt (from graph)	Mol. Wt. (approx.) (Dalton)
1.2	0.150	1.96	91000
1.8	0.225	1.86	72000
3.1	0.380	1.65	44000
4.8	0.600	1.59	39000
5.3	0.660	1.51	32000
6.6	0.825	1.18	15000

Distance covered by solvent front = 8 cm

Pati *et al.* (1996) extracted and characterized OMPs from *P. multocida* B:2 reporting 10 major polypeptide bands of molecular weights ranging from 88 to 25kDa. In our study, six major polypeptide bands of molecular weights 15 to 91 kDa were observed.

Pal *et al.* (2002) reported that OMPs are generally porins, functioning as molecular sieves assisting in the trans-membrane transportation. Heat modifiable characteristics of OMPs from *P. multocida* B:2 were explored to know their basic characteristics under temperature rise. A major band of 32 kDa and two minor bands of approximately 39 and 28 kDa were



**Figure 1: OMPs of the *P. multocida* serotype B:2**

found heat modifiable. This suggests that boiling at 100°C in the presence of  $\beta$ -mercaptoethanol for 5 minutes is sufficient for characterization of OMPs by SDS-PAGE.

Tomer *et al.* (2002) extracted OMPs from six field isolates of *P. multocida* B:2 and a vaccinal strain (P-52) by sarcosyl method and characterized by SDS-PAGE. They recorded about 20 polypeptide bands ranging from 16 to 90kDa in field isolates. The profile of field isolates showed minor differences when compared with the vaccinal strain. The OMP of 33kDa was only expressed in vaccinal strain. Four field isolates expressed an OMP of 39kDa which did not appear in the profile of remaining two field isolates and vaccinal strain P-52. Similarly, an OMP of 25kDa was exclusively seen in the profile of single isolate.

The protective potential of purified lipopolysaccharides is due to its associated proteins which are outer membrane in origin (Kennett *et al.*, 1992). Previous studies have focused on the capsular and lipo-

polysaccharides of *P. multocida*, however, little is known about OMPs of organism (Maheswaran *et al.*, 1973; Ganfield *et al.*, 1976). Analysis of OMPs of several other Gram negative pathogens has resulted in the identification of proteins that have been useful epidemiological and virulence markers (Granoff *et al.*, 1982). OMPs, therefore, can be used to identify strain specific markers that would form a useful basis to differentiate them from field isolates of *P. multocida* of same serotype i.e., B:2 . However, this was a preliminary type of work and further research using various field isolates as well as vaccinal strains are needed.

#### REFERENCES

- Amonsin, A., J. F. Wellehan, L. L. Li, J. Laber and V. Kapur, 2002. DNA finger printing of *P. multocida* recovered from avian source. *J. Clin. Microbiol.*, 40(8): 3025-3031.

- Anonymous, 1996. Economic analysis and survey planning. In: Epidemiology Survey, Punjab. Pak. German Bilateral Technical Cooperation, Directorate of Planning and Evaluation, Department of Livestock and Dairy Development., Punjab, Lahore. PP: 4-5.
- Barta, O., 1993. Protein estimation. In: Monographs in Animal Immunology, Veterinary Clinical Immunology Laboratory, Vol. 2. BAR-LAB, Inc, Blacksburg, USA.
- Boerlin, P., H. H. Siegrist, A. P. Burnens, P. Kuhnert, P. Mendez, G. Pr  tat, R. Lienhard and J. Nicolet, 2000. Molecular identification and epidemiological tracing of *Pasteurella multocida* meningitis in baby. J. Clin. Microbiol., 38(3): 1235-1237.
- Carter, G. R., 1955. Studies on *P. multocida*. A haemagglutination test for identification of serological types. Amer. J. Vet. Res., 16: 481-484.
- De-Alwis, M. C. L., 1999. Haemorrhagic septicaemia. Australian Centre for International Agricultural Research (ACIAR) Monograph No 57, Canberra, Australia.
- Frebourg, N. B., G. Berthelot, R. Hocq, A. Chibani and J. F. Lemeland, 2002. Septicemia due to *Pasteurella pneumotropica*: 16s rRNA sequencing for diagnosis confirmation. J. Clin. Microbiol., 40(2): 687-689.
- Ganfield, D. J., P. A. Robert and K. L. Heddleston, 1976. Immunogenic and toxic properties of a purified lipo-polysaccharide protein complex from *Pasteurella multocida*. Infect. Immunol., 14: 996-999.
- Granoff, D. M., S. J. Barenkamp and R. S. Munson, 1982. Outer membrane protein subtypes for epidemiologic investigations of *Haemophilus influenzae* type B disease. In: *Haemophilus influenzae*: Epidemiology, Immunology and Prevention of Disease. Sell, S. H. and P. F. Wright (Eds.). Elsevier Sci. Publi. Co., New York, USA. PP: 43-54.
- Heddleston, K. L., J. F. Gallagher and P. A. Rebers, 1972. Fowl cholera: Gel diffusion precipitation test for serotyping *P. multocida* from avian species. Avian Dis., 16: 925-936.
- Kennett, L., N. Muniandy and T. K. S. Mukkur, 1992. Comparative protective potential of non-living intact cells and purified outer membrane and associated proteins of *Pasteurella multocida* type B:6 grown under iron-regulated conditions. In: *Pasteurellosis in Production Animals*. Pattern, B. B., T. S. Spencer, R. B. Johnson, D. Hoffmann and L. Lehane (Eds.). An International Workshop Sponsored by Australian Centre for International Agriculture Research (ACIAR), held at Bali, Indonesia. PP: 10-13.
- Maheswaran, S. K., G. H. Johnson and B. S. Pomeroy, 1973. Studies on *Pasteurella multocida* A. The capsular polysaccharides from turkey isolates. Avian Dis., 17: 705-715.
- Manning, P. J., 1982. Serology of *P. multocida* in laboratory rabbits: A Review. Lab. Anim. Sci., 32: 666-671.
- Namioka, S. and D. W. Bruner, 1963. Serological studies on *P. multocida* type IV: Distribution of organisms on the basis of capsule and O groups. Cornell Vet., 53: 41-53.
- Pal, A., S. K. Srivasatava and V. P. Singh, 2002. Heat modifiability of outer membrane proteins of *P. multocida* serotype B:2. Indian J. Exp. Biol., 40(1): 106-108.
- Pati, U. S., S. K. Srivastava, S. C. Roy and T. More, 1996. Immunogenicity of outer membrane proteins of *Pasteurella multocida* in buffalo calves. Vet. Microbiol., 52(3-4): 301-311.
- Quinn, P. J., M. E Carter, B. K. Markey and G. R. Carter, 1994. Bacterial pathogens: Microscopy, culture and identification. In: *Clinical Veterinary Microbiology*. Wolf Publishing, London, UK. PP: 59-61.
- Rimler, R. B. and K. R. Rhoades, 1987. Serogroup F, a new capsular sero-group of *Pasteurella multocida* and pasteurellosis. J. Clin. Microbiol., 25: 615-618.
- Rimler, R. B. and K. R. Rhoades, 1989. *Pasteurella multocida*: In: Adlam, C. and J. M. Rutter (eds), *Pasteurella and Pasteurellosis*. Academic Press, London, UK.
- Srivastava, S. K., 1998. Characteristics of whole cell proteins of *P. multocida* serotype B:6. Indian Vet. J., 75(5): 395-398.
- Tomer, P., G. C. Chaturvedi, Minakshi, P. Malik and D. P. Monga, 2002. Comparative analysis of the outer membrane protein profiles of isolates of the *Pasteurella multocida* (B:2) associated with haemorrhagic septicaemia. Vet. Res. Commun., 26(7): 513-522.
- Wilson, M. A., M. J. Morgan and G. E. Burger, 1993. Comparison of DNA fingerprinting and serotyping for identification of avian *Pasteurella multocida* isolates. J. Clin. Microbiol., 31: 255-259.