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WHOLE CELL PROTEIN PROFILING OF *PASTEURELLA MULTOCIDA* FIELD ISOLATES IN PAKISTAN

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ABSTRACT

Eight haemorrhagic septicaemia (HS) related *Pasteurella multocida* isolates, collected from different localities of Pakistan, were characterized by sodium-dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) technique. After sonification, the bacterial proteins were separated by centrifugation. Proteins from sonicated supernatant were salted out by ammonium sulfate. Sonicated supernatant, as well as ammonium sulfate precipitated proteins, were analyzed. Molecular weights of proteins were determined from graph between R_f value and log of molecular weight. The ammonium sulfate treated samples showed fewer bands with low molecular weights of 54, 45, 42, 40 and 20.5 kDa, while total 31 visible bands were observed in sonicated supernatants ranging from 126 to 11 kDa. The common protein bands in both preparations were of 54 and 23 kDa molecular weight.

Key words: Haemorrhagic septicaemia, *Pasteurella multocida*, SDS-PAGE, whole cell protein profiling.

INTRODUCTION

Haemorrhagic septicaemia (HS) is an acute and fatal disease of cattle and buffaloes in Pakistan and other South-East Asian countries. The disease is caused more frequently by two specific serotypes of *Pasteurella multocida*, serotype B:2 and E:2 in Asia and Africa, respectively (Carter, 1955; Heddleston *et al*., 1972). These serotypes are designated as 6:B and 6:E (Namioka and Bruner, 1963). A few countries, notably Egypt and Sudan, have recorded both serotypes (Shigidi and Mustafa, 1979; Farid *et al*., 1980).

The disease has been much focused by veterinary scientists due to devastating effects on economy of the country. In Pakistan, HS is considered as a disease of economic importance. Only in Punjab province, losses due to HS are reported as 2.17 billion Pakistani Rupees (Anonymous, 1996). Pakistan has a cattle population of approximately 24.2 million and buffalo population of 26.3 million heads (Anonymous, 2004-05). Time trend suggests that course of disease is changing in Pakistan and high rates of morbidity and mortality have been recorded in vaccinated animals, probably due to low number of organisms in vaccines available against HS in market. This necessitates the characterization of the organisms.

Serological identification of *P. multocida* is based on capsular (A, B, D, E and F) and somatic antigens (1-16) (Carter, 1955; Heddleston *et al*., 1972; Rimler and Rhoades, 1987). Sero-groups B and E of *P. multocida* are distinguished by indirect haemagglutination or rapid slide agglutination test and their inability to be affected by certain muco-polysaccharides (Rimler and Rhoades, 1989; Rimler and Wilson, 1994). Agar gel precipitation test (AGPT) can be used for differentiation of somatic serotypes (Rimler and Rhoades, 1989). Although serological and biochemical tests are useful for detection and identification of organisms but these techniques are limited, as they provide insufficient information for epidemiological purposes where discrimination among different strains of the same serotype is important. There is also an inconsistency between typing systems (Brodgen and Packer, 1979). In addition, reports indicate that significant proportion of *P. multocida* isolates is un-typeable (Namioka and Bruner, 1963; Manning, 1982). 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).

Many biochemical and serological methods have been used to study *P. multocida.* These include: catalase, indole, oxidase and sugars fermentation tests. Due to limitations of these methods, more accurate methods are being used to investigate HS like DNA finger printing and protein profiling (Choi *et al*., 1989; Wilson *et al*, 1993).

The objective of the present study was to characterize and differentiate between different isolates of *P. multocida* on the basis of whole cell proteins (WCP) by using sodium dodecyl sulphate polyacryl amide gel electrophoresis (SDS-PAGE).

MATERIALS AND METHODS

Bacterial source

Eight isolates of *P. multocida* isolated from overt cases of HS were recovered from stocks kept at –20°C in lyophilized condition at Bacteriology Laboratory of Animal Health Programme, National Agricultural Research Centre, Islamabad, Pakistan (Table 1).

Table 1: Sero-types of *P. multocida* field isolates

Isolate No.	Locality	Capsular serotype	Somatic Serotype
I	Veterinary clinic, Khushab	B	2
II	Veterinary Research Institute, Lahore	B	2,5
III	Veterinary clinic, Peshawar	B	2
IV	Veterinary clinic, Sheikhupura	B	2,5
V	Veterinary clinic, Islamababad	B	2
VI	Veterinary clinic, Gilgit	B	2,5
VII	Veterinary clinic, Qadirabad	B	2,5
VIII	Veterinary clinic, Karachi	B	2,5

Bacterial revival and culturing

Lyophilized *P. multocida* isolates were suspended in normal saline and revived in balb/c mice by giving subcutaneous injection. Mice died after 24 hours and their hearts were collected after dissecting the mice asceptically (De-Alwis, 1999). Heart blood of each isolate was inoculated on brain heart agar (Oxoid Ltd., Basingstoke, Hampshire, England) and was incubated at 37°C. Colonies were observed after 24 hours and sub-cultured for purity. The bacteria from the purified colonies were confirmed morphologically and bio-chemically using "API 20® NE" kit (bioMérieux, Inc, Durham, USA), as described earlier (Wilson *et al*., 1993).

Capsular and somatic serotyping

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

Each *P. multocida* isolate was cultured in 20 flasks having 100 ml brain heart broth at 37°C overnight. Purity of each flask was checked. Culture was centrifuged at 10,000 rpm for 30 minutes at 4°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. This procedure was repeated twice and finally the pellet was suspended in 4 ml of 10 mM N-(2-hydroxyethyl) piprazine- N-2 ethane sulfonic acid (HEPES) buffer (pH 7.4), sonicated in a sonicator (Branson Sonifier 450) for 10 minutes (2 minutes for 5 times) and centrifuged at 1700g for 20 minutes at 4°C. Supernatant having whole cell protein (WCP) was collected (Srivastava, 1998).

Proteins precipitation and dialysis

Ammonium sulfate was added in sonicated supernatant (up to 45% saturation) to precipitate out protein. Two sets of tubes were separated for each isolate. First set was kept overnight at 4°C and next day centrifuged to get precipitate. The ammonium sulfate precipitated fraction (ASPF) was suspended in HEPES buffer.

Second set tubes were vortexed at 5000 rpm for 1-2 minutes and left on ice for 2 hours. After centrifugation at 10,000 rpm for 8 minutes, the pellet obtained was also suspended in HEPES buffer (Srivastava, 1998). Both sets were dialyzed against distilled water for 48 hours.

Protein estimation

Whole cell protein contents of sonicated supernatant (SS) and dialyzed sample (DS) 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 SS and DS. Optical density of standards (BSA), pure SS, DS and their dilutions were taken at 750 nm in a spectrophotometer. A standard graph was plotted between BSA concentration and corresponding optical density. Protein concentrations of SS and DS were estimated from this standard graph.

SDS-PAGE analysis

Each sample was taken in an eppendorf tube and mixed with stock sample buffer, 2-mercaptoethanol and distilled water and heated for 4-5 minutes in boiling water. Then loading dye bromophenol blue was mixed with samples.

Samples from two different stages were used for SDS-PAGE. First sample was taken from SS and second sample was taken from DS after precipitation. The protein concentration of SS and DS was adjusted at 15-20 ug/ul for each isolate before loading it into the wells of the gel. Samples (50 µl) were loaded in each well of the gel. Molecular weight markers were loaded

in two wells and electrophoresis was done by applying 30 mA constant current and 200 V. 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_f value and log of molecular weight to determine approximate molecular weight of proteins.

RESULTS AND DISCUSSION

Morphologically, all isolates were Gram's negative coccobacilli and they reduced nitrate, decomposed tryptophan and were oxidase positive. All isolates were negative for glucose acidification, hydrolysis of gelatin and asculin. These were also negative for presence of β-glactosidase in AUX medium (ammonium sulfate 2g, agar 1.5g, mineral base 82.8 mg, amino acids 250 mg, vitamins/nutritional substances 35.9 mg, phosphate buffer 0.04M (pH 7.1) 1000 ml, final pH 7.1± 0.1).

API 20®NE kit value was 3000 004 and in analytical profile index it corresponded to *Pasteurella multocida* with 93% identification. API 20®NE identification system is an effective and rapid method of biochemical characterization of isolates. The only disadvantage is lack of consistency among percentages 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*., 2000) by using this 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).

Isolates were typed for their capsular antigen by rapid slide agglutination test. All isolates showed agglutination against serotype B. Haemorrhagic septicaemia in cattle, buffaloes and similar animals like deer and elk is associated with *P. multocida* Carter's sero group B (Carter, 1955; Aalbaek *et al*., 1999). Somatic typing was done by AGPT as described by Heddleston *et al*. (1972). Five among eight isolates gave positive result with reference to strain 2 and 5, while remaining three isolates did not react with anti-sera 5 (Table 1).

Pasteurella multocida is identified by somatic and capsular antigens. Isolates identified as 2,5 can belong to any of 5 capsular groups. Somatic antigens were detected by using anti-sera, prepared and evaluated from reference somatic strains. Isolates expressing multiple somatic antigens mislead diagnosticians and research scientists.

Phenotypic limitations have been overcome by development of DNA (Owen, 1989) and protein based techniques (Knights *et al*., 1990). These techniques can precisely differentiate individual strains of closely related bacteria. Ideal typing system can state whether two or more isolates are related or not (Oslen *et al*., 1993).

Serological identification depends on factors like production of anti-sera, problem of cross reactivity and variation in immunological responses between animals. These factors pose problem for standardization. Still serotyping procedures are important from diagnostic point of view. Conventionally, both somatic and capsular serotyping is done before protein profiling. To differentiate strains of *P. multocida*, expression of iron regulated outer membrane proteins and analysis of protein patterns are used (Ruffolo *et al*., 1997; Kedrak and Opacka, 2002).

Protein profiling of *P. multocida*

Ammonium sulfate treated sample showed fewer bands and in the low molecular weight region only, while sonicated supernatant showed more bands all along length of the gel. The approximate molecular weight from log of molecular weight and R_f value are given in Table 2 and Figure 1.

Table 2: Approximate molecular weights of protein

Band distance (cm)	R_f value	Log of Mol. Wt (From graph)	Mol. Wt. (approx.) Dalton
1.9	0.14	5.10	125000
2.1	0.15	5.08	120000
3.1	0.22	4.98	96000
3.4	0.25	4.94	87000
3.6	0.26	4.92	83000
3.9	0.28	4.90	79000
4.2	0.30	4.88	76000
4.4	0.32	4.85	71000
4.7	0.34	4.82	66000
5.0	0.36	4.81	65000
5.3	0.38	4.79	61500
5.7	0.41	4.78	60000
6.2	0.45	4.76	57500
6.4	0.46	4.75	56000
6.8	0.49	4.73	54000
7.1	0.51	4.72	52000
7.6	0.55	4.70	50000
8.1	0.59	4.67	47000
8.5	0.62	4.66	46000
9.0	0.65	4.64	44000
9.9	0.72	4.57	37000
10.3	0.75	4.53	34000
10.7	0.78	4.50	32000
11.3	0.82	4.45	28000
11.7	0.85	4.40	25000
11.9	0.86	4.39	24500
12.1	0.88	4.36	23000
12.4	0.90	4.34	22000
12.6	0.91	4.33	21000
12.8	0.93	4.20	16000
13.3	0.96	4.06	11000

Distance covered by solvent front=13.8 cm

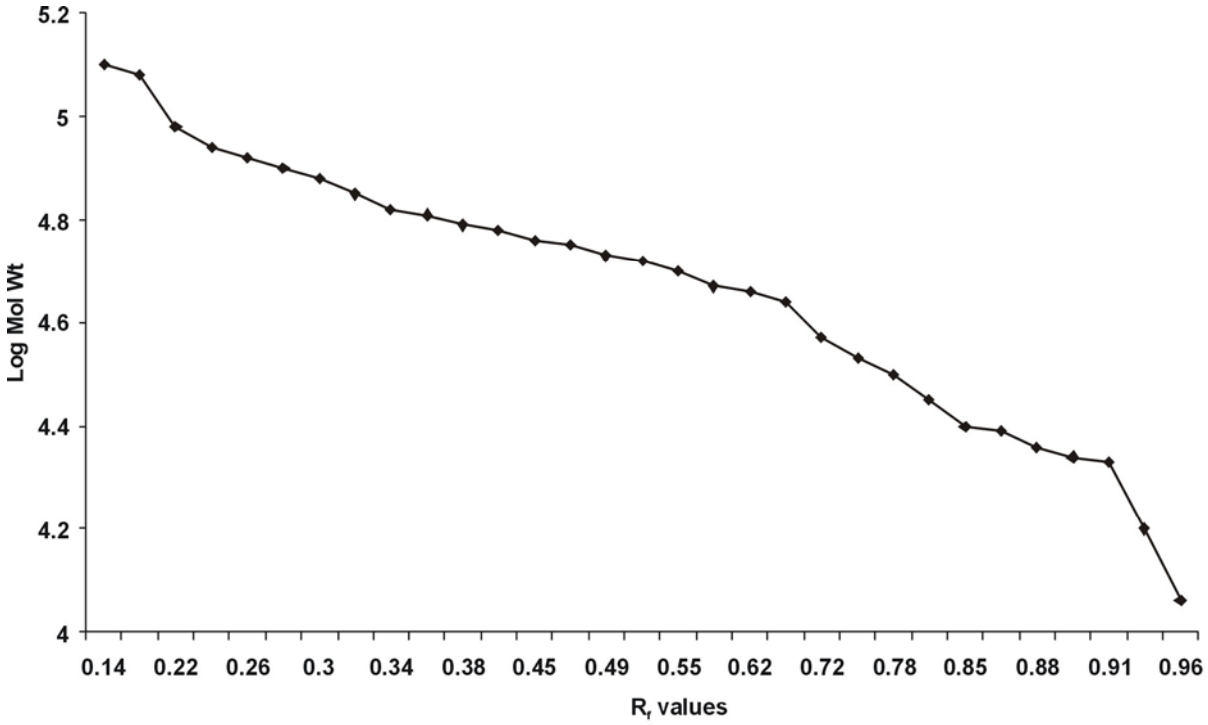

Figure 1: Approximate molecular weight of protein bands

Polyacralamide gel (10%) was used for electrophoresis. Electrophoresis profile of serotype B:2 and B:2,5 showed identical protein profile and no significant difference was observed among SS and DS. In sonicated preparation, a total of 31 polypeptides were observed, having approximate molecular weights shown in Table 2. Most of the low molecular weight proteins were precipitated at 45% concentration of ammonium sulfate including 54, 45, 44, 42, 40, 30, 26.5, 23 and 21.5 kDa (Figure 2).

Molecular weights of highly stained protein fractions were 28, 34, 37, 47, 79 and 83 kDa. From 28 to 47 kDa, all isolates showed most intense protein bands of 34, 37 and 47 kDa. The common protein bands observed in both sonicated and ammonium sulfate precipitated fraction were 54 and 23 kDa (Figure 2).

Ireland *et al*. (1991) analyzed proteins profiles by using sonicated *P. multocida* strains of serotype 1 isolated from fowl cholera cases. They found complex protein profiles with a large number of bands. Patterns obtained by staining with coomassie blue were similar. Srivastava (1998) reported 12 polypeptides ranging from 120 to 30 kDa in sonicated cells, whereas 14 polypeptides in ASPF. He observed some protein bands only in sonicated cells like 120, 105, 81, 60 and 62 kDa, while some only in ASPF like 54, 52, 50, 45, 40 and 30 kDa.

In conclusion, no quantitative difference was observed among different isolates. However, additional studies using different percentage of gels and gradient gels are needed to analyze before recommending this technique to differentiate vaccinal strains from the field isolates.

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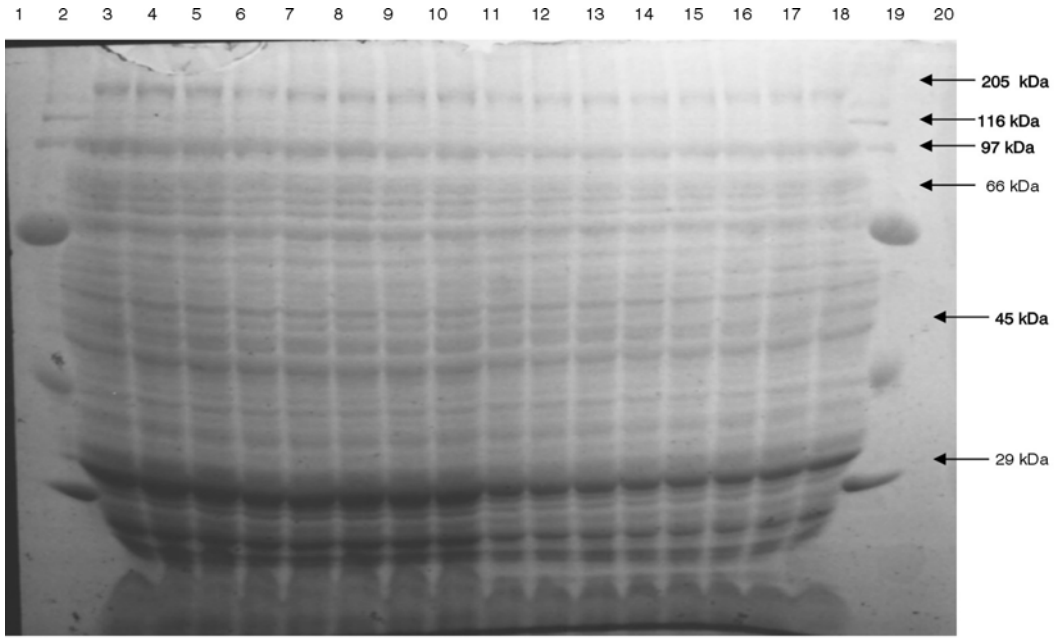

Figure-2: WCP of the PM strains of serotype B:2 and B:2,5

Mol. Wt. Marker (2, 19), PM I (3, 11), PM II (4, 12), PM III (5, 13), PM IV (6, 14), PM V (7, 15), PM VI (8, 16), PM VII (9, 17), PM VIII (10, 18)

PM=*Pasteurella multocida*
WCP=Whole Cell Protein

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