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DIFFERENTIATION OF CLOSELY RELATED VACCINAL STRAINS OF *PASTEURELLA MULTOCIDA* USING SODIUM DODECYL SULPHATE-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

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ABSTRACT

The present study was carried out to observe inter-strain variation at molecular level in the two vaccinal types (B:3,4 and B:6) of *Pasteurella multocida*. The micro-organisms were identified and characterized by using conventional microbiological tests. The results of these tests were unable to show any definite variations between the two vaccinal strains studied. Whole cell SDS-PAGE profiles showed similarities in the two vaccinal strains. However, SDS-PAGE profiles of envelope showed differences between the two vaccinal strains. The 85, 27 kDa major and 70, 40 kDa minor proteins were common in both strains, while the 50, 32 and 29 kDa major proteins were found in B:3,4 but absent in B:6. SDS-PAGE profiles of outer membrane protein (OMP) showed that 26 minor bands were common. The 50 and 32 kDa major proteins were found in B: 3,4 and absent in B:6, while 46 kDa minor protein was found in B:6 only. So, the present study concluded that the two vaccinal strains of *Pasteurella multocida* had differences in OMP, lipopolysaccharides and whole cell extracts.

Key word: Vaccinal strains, *Pasteurella multocida*, SDS-PAGE.

INTRODUCTION

Haemorrhagic septicemia (HS) is caused by *P. multocida* and manifested by an acute and highly fatal septicemia mainly in cattle and buffaloes (Carter and De-Alwis, 1989). The disease is prevalent in Pakistan with as high prevalence as 49% in one district (Farooq *et al*., 2007). The disease is caused by two specific serotypes of *P. multocida*. Serotype B:2 is more frequently found in Asia (Munir *et al*., 2007) and E:2 is more frequent in Africa (De-Alwis,1992).

The pathogen consists of five capsular serogroups, A, B, D, E and F, and there is a relationship between the capsular serogroup and disease predilection (Boyce and Adler, 2001; Chung *et al.,* 2001). At present, the most acceptable and widely used serotype designation system is a combination of Carter capsulate typing and Hedleston somatic typing. Using this method, the Asian and African hemorrhagic septicemia serotypes are designated B:2 and E:2, respectively and non-HS type B strain of Australian origin as B:3,4 (De-Alwis, 1990).

Most strains of *Pasteurella* form a polysaccharide capsule or envelope. It is composed of polysaccharides, lipopolysaccharides (LPS) and variety of proteins (Knights *et al.,* 1990). Capsulation did not affect the LPS profile. Proteins and lipids are present among the network of polysaccharides. Both LPS and poly-saccharides play a role in passive haemagglutination. Prince and Smith (1996) described the three-antigen complexes alpha, beta and gamma polysaccharides protein complex, a serogroup-specific polysaccharide and LPS. Muniandy *et al.* (1993) observed that the immunogenicity of certain LPS was due to the presence of contaminating outer membrane protein (OMPs).

The outer membrane proteins (OMPs) of Gram-negative bacteria have a role in disease processes as they act at an interface between the host and pathogen (Lin *et at*., 2002). Outer membrane proteins (OMPs) of *P. multocida* play significant role in the pathogenesis of pasteurellosis and have been identified as potent immunogens. Thus, a variation in OMPs profile among the isolates may help in epidemiological survey. The objective of the present study was to determine differences in OMP, LPS and whole cell extracts from two vaccinal strains of *Pasteurella multocida* using SDS-PAGE.

MATERIALS AND METHODS

Microorganism used in the study

The present work was conducted in the Biotechnology Laboratory of National Veterinary Laboratories, (NVL), Islamabad, Pakistan, in which two vaccinal strains of *Pasteurella multocida* B: 3,4 and B:6 were characterized by biochemical tests and SDS-PAGE. The vaccinal strains were obtained from the seed bank of Vaccine Quality Control (VQC) section of NVL. Staining and biochemical tests (catalase test, oxidase test, urease production test, hydrogen sulphate production test, nitrate reduction test and motility test)

were performed before further characterization. After obtaining pure cultures, analysis was done on SDS-PAGE for whole cells, envelope and OMPs.

Harvesting of culture

P. multocida strains were harvested by centrifugation in 50 ml falcon tubes at 10,000 rpm and at 8ºC for 15 minutes. The pellet of each tube (total 12 tubes) was first suspended in 5 ml phosphate buffered saline (PBS), vortex (IKA, USA) and then transferred to two tubes and centrifuged again. The supernatant was discarded and the pellet was suspended in 20 ml PBS by vortex, shifted to one tube and again centrifuged. The supernatant was discarded and the pellets were suspended in 3 ml of distilled water by vortex and frozen at -20^{0}C.

The pellet was redissolved in distilled water and sonicated for 30 seconds stroke and 30 seconds cooling upto10 cycles. The amplitude was kept 100 at the cycle 1. The disruptions of the cells were done with the conical probe (3 mm width and 90 mm length).

Protein estimation by Lowry's method

Proteins were estimated by Lowry's method, which was inexpensive and highly reproducible (Lowry *et al*., 1951). Whole-cell and envelope proteins could be calculated by Lowry's method so that the estimated amount was loaded into the wells of SDS-PAGE for better resolution.

Preparation of whole-cell for SDS-PAGE

For the extraction of proteins, 2-3 colonies from thick growth of pure *P. multocida* were taken with the help of sterile loop and dipped in the ependorff tube (1.5 ml) containing 200 µl-distilled water. They were vortexed with the help of mixer thoroughly. After dissolving, the samples were sonicated (dr. Hielscher, type UP 400S).

Preparation of envelope

The culture of *P. multocida* (3 ml) was centrifuged at 20,000 rpm for 30 minutes at 8°C. The pellet was resuspended in 20 mM Tris HCl buffer (pH 7.2) and again centrifuged at 20,000 rpm for 30 minutes at 8°C. The final pellet, rich in envelope, was resuspended in 20 mM Tris HCl buffer pH 7.2 and stored at -20˚C for use in SDS-PAGE.

Preparation of outer membrane protein (OMPs)

The envelop prepared as above was resuspended for selective solubilization in 8 ml of 0.5% (w/v) N-Lauryl Sarcosine sodium salt/ Sarkosyl (Sigma) and incubated at room temperature for 30 minutes. After incubation, the contents were centrifuged at 35700 rpm for one hour at 8°C. The supernatant was discarded, pellet was dissolved in 5 ml of 20 mM Tris HCl buffer (pH 7.2) and centrifuged as above. The supernatant was discarded again and final pellet, rich in outer membrane proteins (OMPs), was resuspended in 0.5 ml Tris HCl buffer pH 7.2 and stored at -20C˚ for further use in protein standardization and SDS-PAGE.

Sodium Dodecylsulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The electrophoresis was carried out using slab type SDS-PAGE Model (V20-CDC, UK) with 12% Poly-Acrylamide gel. A 12% running gel (1.5M Tris-HCl (Scharlau) pH 8.9, 10% SDS (Scharlau), 30% acryl amide + bisacrylamide (Merk) and 5% stacking gel (0.5 M Tris-HCl pH 6.9, 10% SDS, 30% acryl bis) were prepared and polymerized chemically by addition of 16.66 µl N,N', N',N' tertamethylenediamine (Sigma) to the running gel solution and 15 µl to the stacking gel solution. A 250 µl of 10% (w\v) ammonium per sulphate (APS) (Sigma) was also added to the resolving gel and 112.5 µl to the stacking gel solutions (Laemmli, 1970).

A 50 µl of the heated sample was loaded into each well with micro syringe (ILS, Germany). The known molecular weight marker (SDS Marker 661 Ferment's) was also loaded (20 µl) along with the samples. The apparatus was connected with constant electric current (30mA) and the variable voltage till the bromophenol blue (BPB) reached the bottom of the plate.

Staining and destaining of Gel

At the completion of electrophoresis, the gels were put into a container with staining solution containing 0.25% w\v Coomassie brilliant blue (CBR) R-250 dissolved in 50% (w\v) methanol (Scharlau), 10%(v\v) acetic acid (Merck, Germany) in double distilled water. Gels were left in the staining solution for overnight on slow shaking and destained in methanol, acetic acid, and water with shaking (IKA-VIBRAX type VXR, Germany) until the bands became visible above the background. Both staining and destaining steps were carried out while shaking.

Determination of molecular weight of proteins

Molecular weights of whole cell, envelope and outer membrane proteins were analyzed by SDS-PAGE and determined by using a high molecular weight standard marker (Ferment's SDS SM0661). The molecular weight of marker was 10–200 kDa. The marker had 14 proteins and the distance traveled by these 14 proteins of the marker was measured. This marker included 50 kDa reference bands of a greater intensity. By taking the distance traveled by the bands of marker (in cm) along x-axis and molecular weight (in kDa) of the proteins present in the marker along y-axis, a standard curve was obtained. The distance covered by each of the band of the sample was calculated and with the help of standard curve, molecular weights of proteins present in the samples were estimated.

RESULTS

The present study involved microbiological and molecular characterization of two vaccinal strains of *Pasteurella multocida,* namely B:6 and B:3,4. All the cultures showed luxuriant growth on blood agar having translucent grayish or yellowish green colonies. All the microorganisms studied showed whitish gray rough opaque colonies on brain heart infusion agar. The colonial diameter ranged from 1 to 3 mm, details of morphological observations and biochemical tests are given in Table 1.

Whole-cells

Comparative protein profiling was carried out for both vaccinal strains (B:6 and B:3,4), using SDS-PAGE. At least 12 protein bands of 150, 100, 70, 64, 54, 50, 41, 40, 36, 29, 26, and 16 kDa were observed in complex whole cell profile of the both strains (Fig. 1). It was evident from the whole cell profile that large numbers of bands were present.

Envelope

When prepared envelope was analyzed on the SDS-PAGE, the 85, 70, 54 and 27 kDa major and 100, 46, 41, 36 kDa minors proteins were observed in B:6 strain and 100, 85, 50, 46, 36, 32, 29 and 27 kDa major and 70, 54 and 41 kDa minor proteins were observed in B:3,4. Among these, 85 and 27 kDa, major proteins and 70 and 41 kDa minor proteins were common in both strains (Fig. 1). There were quantitative and qualitative differences such as 100, 46, and 36 kDa proteins were quantitatively more in B:3,4 but less in B:6. The 54 kDa major protein was quantitatively more in B:6 but less in B:3,4. The 50, 32 and 29 kDa major proteins were present in B:3,4 but absent in B:6. This shows that quantitatively and qualitatively, differences were present in B: 3,4 and in B:6, indicating differences within the strains of *P. multocida* (Fig. 1).

OMPs

When OMPs were analyzed on SDS-PAGE, the 100, 85, 70, 54, 47, 41 and 36 kDa major and 29, 27 and 26 kDa minor bands could be observed in the B:6 and 50, 32, 29 and 27 kDa major and 85, 70, 54, 41, 36 and 26 kDa minor bands were present in B:3,4 (Fig. 1).

Protein estimation

The result of the protein estimation of *P. multocida* and their standard BSA curve is shown in Table 3. This standard curve was used to calculate the ODs of two strains. The OD of *Pasteurella multocida* strains B:3,4 and B:6 were checked and it was found that these strains had different OD values.

Table 1: Results of morphological, microbiological and biochemical tests

Test performed	Results		Remarks
	B:6	B:3,4	
Colonial morphology	Whitish grey rough opaque	Whitish grey rough opaque	Both the micro-organisms showed whitish grey rough opaque colonies
Colony diameter	2-3 mm	1-2 mm	Colonial diameter ranged from 1-3 mm
Mortality in mice	+ve	+ve	All mice were killed
Growth on MacConkey agar	-ve	-ve	All isolates showed no growth on MacConkey agar
Motility	-ve	-ve	*Pasteurella multocida* is non-motile
Gram stain	-ve	-ve	Pink coccobacilli observed
Catalase production	+ve	+ve	Vigorous bubbling observed with in 30 sec.
Haemolysis	-ve	-ve	No haemolysis was found on blood agar
H_2S production	+ve	+ve	Blackening of the media due to production of H_2S
Urease production	-ve	-ve	No red-pink colony was observed
Oxidase production	+ve	+ve	Immediate change blue purple colour observed
Nitrate production	+ve	+ve	Red colour observed
Indole test	+ve	+ve	Pink ring observed
Citrate utilization test	-ve	-ve	No turbidity observed

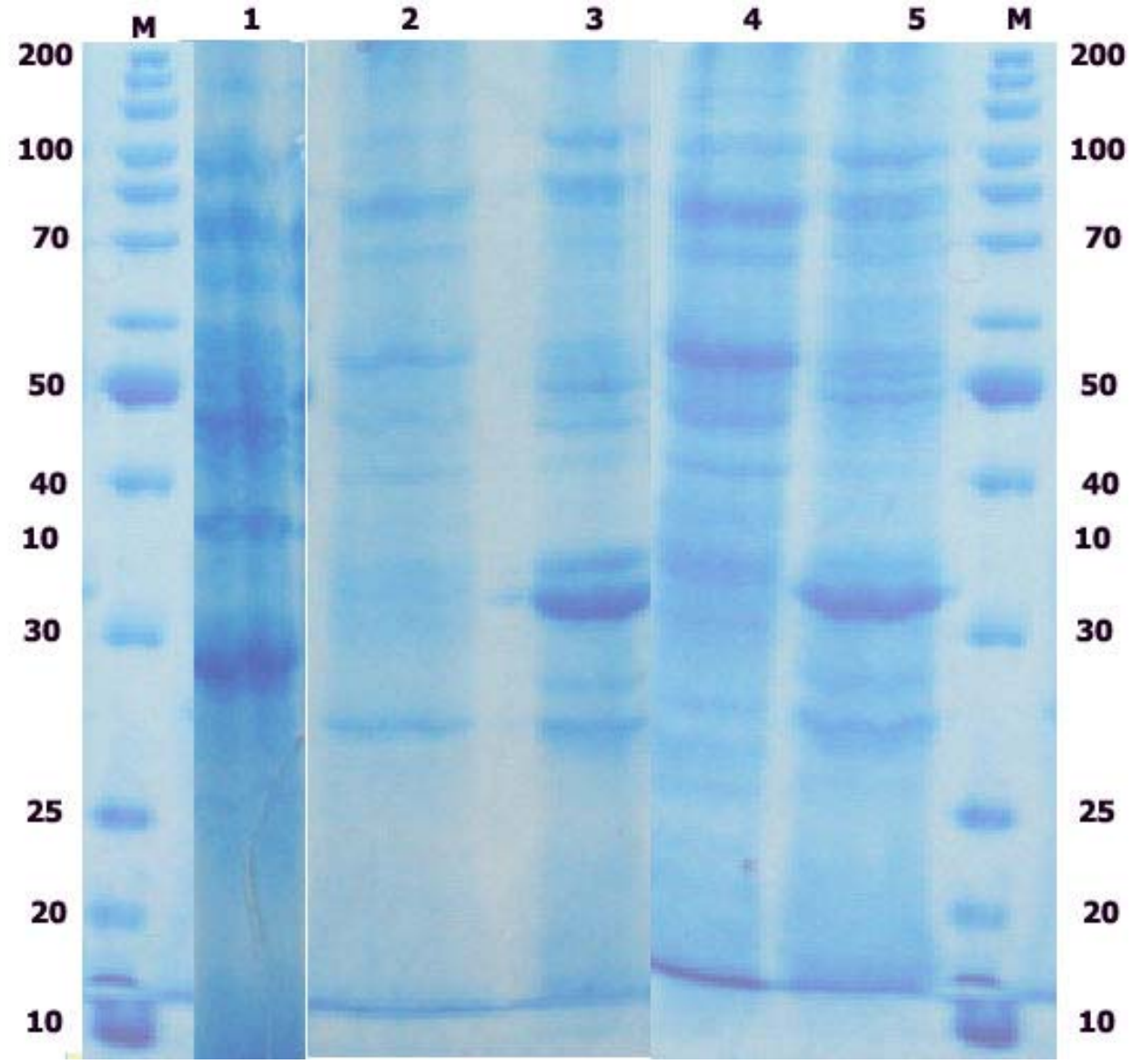

Fig. 1: Protein profile of *Pasteurella multocida* : M = Molecular marker, Lane 1 = whole cell (B :3,4), Lane 2 = Envelope (B :6); Lane 3 = Envelope (B:3,4), Lane 4 = Outer membrane (B:6) and Lane 5 = Outer membrane (B:3,4)

DISCUSSION

Pasteurella multocida is an aerobic, chemo-organotrophic organism. Two *P. multocida* strains used in the study were obtained from the seed bank of Vaccine Quality Control (VQC) section of NVL. The colony morphology was best studied on common laboratory media such as nutrient agar and brain heart infusion agar. Special media such as casein-sucrose-yeast supported abundant growth. The optimum growth temperature was 37°C after 18-24 hours culture. Similar results have been reported by Shigidi and Mustafa (1979), Arawwawela *et al.* (1981) and Wijewardana *et al. (*1986).

The techniques in molecular biology have significantly increased understanding of the epidemiology of *Pasteurella* diseases such as SDS-PAGE have shown to establish the unique properties of the bacterial proteins (Johnson *et al.,* 1991). When SDS-PAGE (Sodium dodecyl polyacrylamide gel electrophresis) was used to observe the inter-strain variation of the two strains studied in the whole cells preparations, complex profiles were obtained. There were some bands present in B:3,4 and in B:6 which could not differentiate the B:3,4 from B:6. Both the strains had a major proteins band of 36 kDa. The presence of this major protein of similar molecular weight or approximately calculated weights with the little variation in our study (36 kDa) is

Table 2: SDS-PAGE results of the microorganisms

Sr. No.	Major protein (kDa)	Minor protein (kDa)	Whole cell		Envelop		OMPs	
			B:6	B:3,4	B:6	B:3,4	B:6	B:3,4
1.	150	-	✓	✓	×	×	×	×
2.	100	-	✓	✓	×	✓	✓	✓
3.	-	100	×	×	✓	×	×	×
4.	85	-	×	×	✓	✓	✓	×
5.	-	85	×	×	×	×	×	✓
6.	70	-	✓	✓	✓	×	✓	×
7.	-	70	×	×	×	✓	×	✓
8.	64	-	✓	✓	×	×	×	×
9.	54	-	✓	✓	✓	×	✓	×
10.	-	54	×	×	×	✓	×	✓
11.	50	-	✓	✓	×	✓	×	✓
12.	46	-	×	×	×	✓	✓	×
13.	-	46	×	×	✓	×	×	×
14.	41	-	✓	✓	×	×	✓	×
15.	-	41	×	×	✓	✓	×	✓
16.	40	-	✓	✓	×	×	×	×
17.	36	-	✓	✓	×	✓	✓	×
18.	-	36	×	×	✓	×	×	✓
19.	32	-	×	×	×	✓	×	✓
20.	29	-	✓	✓	×	✓	×	✓
21.	-	29	×	×	×	×	✓	×
22.	27	-	✓	✓	✓	✓	×	✓
23.	-	27	×	×	×	×	✓	×
24.	-	26	×	×	✓	✓	✓	✓
25.	16	-	✓	✓	×	×	×	×

Note: Major and minor proteins refer to density of bands in the gel.

consistence with the earlier studies by Johnson *et al.* (1991) and Rimler (1996). In general, the eletrophoretic profiles of whole cells preparations appeared to be homogeneous, regardless of animal species from which they were originally obtained. The 85, 46, 32 and 26 kDa proteins were present in envelope and OMPs but were not found in whole cell indicating that it might be masked in whole cell complex profile.

The envelope profile of vaccinal strains showed differences in proteins of which 85 and 27 kDa major proteins were common in both strains while the 50, 32 and 29 kDa major proteins were present in B:3,4 but absent in B:6. Similar differences have been shown by Knights *et al.* (1990) in *P. multocida* serotypes on the basis of polypeptide profiles.

Differences were also seen between B:3,4 and B:6 when OMPs of strains were analyzed by SDS - PAGE. Quantitative and qualitative differences were present in both strains. This showed that quantitatively and qualitatively differences were present in B:3,4 and in B:6, indicating differences in strains of *P. multocida*. Truscott and Hirsh (1988) demonstrated an outer membrane protein with antiphagocytic activity from *Pasteurella multocida* of avian origin, and concluded that 50 KDa protein band was common in whole cell and OMPs of *P. multocida*.

The selected surface molecule for the study in this case was OMPs whose analysis was extremely useful with major differences between strains as analysed by SDS-PAGE. Therefore, it is proposed that this study may be repeated with larger number of *P. multocida* strains, isolates or serotypes to gain more insight into the structural and functional relationship and epidemiology of the diseases, ultimately leading to the better control of the this most important economic disease of cattle in this country. So, the present study concluded that the two vaccinal strains of *Pasteurella*

Table 3: Optical density at 750nm of the standards used for protein estimation

Standard dilution (%)	Optical density (OD)
1.00	1.35
0.75	1.182
0.50	0.935
0.25	0.524
0.125	0.264
0.0625	0.111
0.03	0.006
***Pasteurella multocida* strains**	
B: 3,4	1.624
B: 6	1.594

multocida were having differences in OMP, LPS and whole cell extracts.

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