Analysis of Outer Membrane Proteins of *Pasteurella Multocida* Strains Isolated from Buffaloes Affected with Hemorrhagic Septicemia.

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Abstract: The outer membrane proteins of 9 field isolates of *Pasteurella multocida* from buffaloes affected with hemorrhagic septicemia along with 4 reference strains, separated electrophoretically at a 12% acrylamide in Sodium Dodecyl Sulphate- Polyacryamide Gel Electrophoresis. The reference strains belonged to serotype B: 2. The separation showed 17 to 30 protein bands with various molecular weights. The densitograms of the examined isolates were compared. The highest difference in the molecular weights and optical density of protein subunits were observed in the range of 35 to 55 kDa. The 4 types of electrophoretic profiles were observed among 9 isolates. A band range of 139 to 150 kDa was observed in all strains, however the most commonly occurring proteins were found to be of 21, 30, 33, 36, 50, 54, 58, 60, 75, 86, 90 and108 kDa. The results showed that all the isolates have almost similar profiles with little differences, which indicated that all isolates were from B:2 strain.

Key words: *Pasteurella multocida* / protein / buffalo / SDS-PAGE / serotype

INTRODUCTION

Pakistan has a cattle population of 24.2 million and buffalo population of 26.3 million heads (Anonymous,2004-05). Haemorrhagic Septicaemia (HS) is one of the most common and major infectious diseases of dairy animals (cattle and buffalo) in Pakistan, having mortality rate of about 70% and responsible for annual losses of several hundred million dollars to animal production (May et al., 2001). The highest prevalence of HS was recorded in Khanewal district (49.0 %) and highest importance (75.6 %) was recorded in Faisalabad district during 2000 to 2005 (Farooq et al., 2007).

Clinical symptoms of HS appear after a very short incubation period following exposure to virulent microorganism. The average incubation period is approximately 30 hours (De Alwis, 1995). The classical clinical symptoms of HS may be characterized by three phases .The first phase is characterized by temperature elevation with anorexia and sometime salivation. The second phase is respiratory distress with profuse salivation and nasal discharge. The terminal phase is recumbency and death (Shah, 1998).

*Pasteurella multocida* is responsible for major animal diseases of economic significance in both developed and developing countries whereas human infections related to this bacterium are infrequent (Dziva et al., 2008). *Pasteurella multocida* is a pathogen in diseases of a variety of domestic and feral mammals and birds. HS in cattle, bison and buffalo is a specific disease produced by certain strains of *Pasteurella multocida* (Rimler, 1997). HS is prevalent in south and south East Asia (serotypes B: 2 & B: 2, 5) and (serotypes E: 2 & E: 2, 5) in tropical Africa (Farooq et al., 2007). Serotype B: 2 has been seen in Asia, Southern Europe and rarely in North America (Lu et al., 1988). Lipopolysaccharides and proteins are the two major somatic antigens exposed on the cell surface of *Pasteurella multocida* (Opacca, 2002). The outer membrane proteins (OMP) can play a key role in the pathogenesis of pasteurellosis (Srivastava, 1998). Several OMPs are immunogens and the antibodies produced against these OMPs demonstrate a strong protective action, such antigens may be used as a component of subunit vaccines (Kedrak et al., 2002; Opacca, 2002). The immunogenicity of selected OMPs of *Pasteurella multocida* was demonstrated in rabbits (Confer et al., 2001), calves (Dabo et al., 1997) and chikens (Zhang et al., 1994). Moreover, the protective action of OMPs of serotypes B: 2 against hemorrhagic septicemia was reported (Srivastava, 1998). Most serological tests for detection of *Pasteurella multocida* infection have depended on the use of whole-cell lysate or component of the bacterial OMPs to detect the antibodies in the sera of infected animals (Kawamoto et al., 1993; Peterson et al., 1997).
The main focus of the study was to identify and compare electrophoretic profiles of the outer membrane proteins of Pasteurella multocida strains isolated from local buffaloes affected with hemorrhagic septicemia. In this way we want to check the seroprevalance of Pasteurella multocida for emergence of new strains in local population, as previously only one strain was reported in this region and same was used for vaccine preparation. Electrophoretic protein pattern of local isolates were compared with B: 2 reference strain for their classification. Further studies are required to check the immunogenicity of different proteins alone and in combinations to design a strategy for formulation of sub-unit vaccine in future.

MATERIALS AND METHODS

Bacterial Strains:
Nine Pasteurella multocida local field isolates of serotype B: 2 (P3652, P3848, P3675, P3856, P3676, P3859, P3847, P3654 and P3677) from buffaloes affected with hemorrhagic septicemia at Veterinary Hospital Landhi Cattle Colony Karachi, Pakistan. Four reference strains-P9012, P2192, P1997 and P1059 (serotype B: 2) obtained from Department of Animal Sciences, Faculty of Agriculture University, Pera Denvya, Sri Lanka.

Preparations of Pasteurella multocida strains for SDS-PAGE:
Thirteen Pasteurella multocida isolates were cultured on BHI (brain heart infusion) medium for 18 hrs at 37°C with shaking at 200 rpm. The cultures were harvested by centrifugation at 10,000 rpm for 20 minutes. The bacterial cell pellets were washed twice with sterile 0.01M phosphate-buffer saline (PBS; pH 7.2). The mean suspension density of each strain (%T=2.0, Abs. 2.0) corresponding to 5x10^5 cfu / ml was evaluated spectrophotometrically using a Jenway 6305 UV / Visible apparatus at 540 nm wavelength. The OMPs were obtained according to the modified method of Morton et al. (1996). The suspension for each strain was mixed with 4x sample buffer with 15% β mercaptoethanol containing 6% SDS, boiled for 5 minutes at 100°C and stored at –20°C until use (11, 15, and 22).

Separation in SDS-PAGE:
The electrophoretic separations were carried out in 12% acrylamide gel according to Laemmli, (1970). A SDS-PAGE Molecular Weight Standard (Fermentas) along with samples were separated on each gel. The separation was started at 100 V, at room temperature for 30 minutes and rest of the gel was run at 30 V until the dye front (bromophenol blue) was 1mm to 2mm from the end of gel. Gels were stained with commassie brilliant blue and the results were analyzed using Dolphin-1D gel Analysis Software (Wealtec). The analysis of the examined strains involved their molecular weight, optical density, the percentage of protein fraction and the rate of the electrophoretic homology.

RESULTS AND DISCUSSION

The electrophoretic separations of whole cell proteins from the field isolates of Pasteurella multocida serotype B: 2 (P3652, P3848, P3675, P3856, P3676, P3859, P3847, P3654 and P3677) from buffaloes affected with hemorrhagic septicemia at Veterinary Hospital Landhi Cattle Colony, Karachi and the reference strains P9012, P2192, P3675, P1059 (serotype B: 2) were explored with 12% acrylamide gel (Fig. 1 and 2). The four types of electrophoretic profiles were observed among 9 strains. Two strains P3847 and P3675 showed similar pattern and revealed protein fractions with molecular weight amounting from 12, 15, 17, 21, 28, 31, 34, 36, 41, 48, 52, 55, 57, 65, 69, 75, 87, 100 and 123 kDa. The highest OD was demonstrated in 48 kDa. Four strains of Pasteurella multocida P3652, P3856, P3676 and P3848 showed the presence of common bands of 12, 16, 21, 25, 29, 31, 34, 37, 41, 50, 53, 59, 75, 90, 104, 117 and 121 kDa while the highest OD value was observed in 53 kDa band. P3859 also demonstrated different protein pattern and homology to the previous strains. The protein bands were observed to be of 14, 18, 21, 30, 33, 39, 43, 47, 51, 54, 58, 64, 75, 85, 108, 119 and 140 kDa.

Examination of 4 reference strains of serotype B: 2 (P9012, P2192, P1997 and P1059) demonstrated the presence of 17 to 26 kDa clearly visible protein subunits. The most frequently observed fractions were with molecular weights of 75, 80 86, 90 and 25 to 55 kDa. The electrophoretic profile of reference strains revealed 80% homology, while electrophoretic profiles of the field isolates demonstrated homology in comparison to reference strains.
Fig. 1: Electrophoretic profiles of the protein of Pasteurella multocida strains of serotype B: 2 isolated from buffaloes affected with hemorrhagic septicemia P3652 (2), P3848 (3), P3675 (4), P3856 (5), P3676 (6), P3859 (7), P3847 (8), P3654 (9) and P3677 (10). Lane 1 comprises the weight standards. Samples were analysed on 12% polyacrylamide gel.

Fig. 2: Electrophoretic profiles of the proteins of reference Pasteurella multocida strains of serotype B: 2 P9012 (2), P2192 (3), P1997 (4), P1059 (5). Lane 1 comprises the molecular weight standards

Fig. 3: Comparison of molecular weights
**Discussion:**

In HS, capsular antigens, LPS, protein complex and OMPs are effective immunogens (Carter et al., 1989). In order to improve the immunogenicity of the vaccines, the causative organism has been fractioned and various cell surface components has been studied in past (Borkowsak et al., 1997). Numerous researchers found that *Pasteurella multocida* strains multiplied in *vivo* and in *vivo* on iron- restricted media produced surface antigen revealing protein properties with high molecular weights (Marandi and Mittal, 1995). In the present study proteins were separated in SDS-PAGE and analysed densitometrically electrophoregrams of the *Pasteurella multocida* strains of serotype B: 2 isolated from local buffaloes affected with hemorrhagic septicemia. Johnson et al., (1991) examined the outer membrane protein (OMP)-enriched extracts of avian strains of *Pasteurella multocida* by use of sodium dodecyl sulfate poly-acrylamide gel electrophoresis. Srivastava, (1998) demonstrated an enhanced production of 84 kDa proteins by the strain of serotype B: 2 cultured in iron restricted media. The vaccines prepared using the culture of such strains stimulated the increased production of antibodies in mice, rabbits and cattle as compared to the vaccines with antigens obtained grown under normal conditions. The interest in the potential use of outer membrane proteins (OMPs) for serotyping is rapidly increasing (Buchna and Hildebrandt, 1981; Hamel et al., 1987). The different cell envelope profiles can be distinguished on the basis of electrophoretic mobilities of the heavy and weak proteins. Johnson et al., (1991) determined the proteins profile of capsular serotype B and E strains isolated from animals with hemorrhagic septicemia and placed the isolates in two distinct groups on the basis of the molecular masses (32 to 37 kDa) of the major proteins.

In present experiment the strains of serotype B: 2 isolated from buffaloes and cultured on the BHI medium showed various electrophoretic profiles. Usually additional proteins or an enhanced expression of the OD of proteins (35, 47 to 65 and 75, 87 to 100 kDa) were found. The proteins subunits of 31 kDa to 37 kDa were found in all strains but 4 different patterns of electrophoretic mobility were observed. Similarly Tomer et al., (2002) observed 20 polypeptide OMP bands, out of those three polypeptides of MW 31, 33 and 37 were considered to be major OMP bands and by immunoblotting he found that polypeptide of 37 kDa was the most immunogenic of all the isolates. Jain et al., (2005) revealed two major OMPs of 31.7 and 34.9 kDa in capsular type B isolates. Johnson et al., (1991) reported predominant polypeptide of 32 kDa in capsular type B isolates of *P. multocida*. Pai et al., (1996) reported major OMPs of 30 and 34 kDa in vaccine strain P52. Subunit vaccines comprising OMPs from *Pasteurella multocida* serotype B:2 were also used by him to immunize buffalo calf.

The results of our experiments are similar to the findings of Jain et al., (2005) who observed expression of an additional protein of molecular weight 102 kDa along with other major OMP bands in capsular type B buffalo isolates. Four reference strains of serotype B: 2 were also used to determine their protein profiles and compared them with field isolates. Results revealed that fractions of molecular weight of 25 to 55, 75, 80, 86 and 90 kDa were most frequently observed among all isolates and reference strains.

However, the results showed fluctuations by using different kind of medium, incubation conditions and method of extraction of proteins (Opacka, 2002). For example, Zhao et al., (1995) found iron regulated outer membrane proteins with molecular weight of 74, 87 and 99, Snipes et al., (1988) 80, 84, 96, Choi et al., (1991) 76, 84, and 94, Gilsson et al., (1993) 63, 77, 91 and 99 and Rufflo et al., (1984) 98 or 38, 40, 57, 90, 94, 98 (all values in kDa) with different kind of medium used.

It is concluded that all the isolates and reference strain have almost similar profiles with little differences, which indicated that all isolates were from B: 2 strain of *Pasteurella multocida*. It indicated the prevalence of single strain. It was also found that the protein profile is influenced by incubation conditions, kind of medium used and method of protein extraction, as the growth on BHI medium enhanced the production of high molecular weight proteins. Further studies are required to confirm the immunogenicity of these proteins prior to use them as antigen in subunit vaccine.

**REFERENCES**


