

Comparison of the Common Immunogenic Protein Components of *Pasteurella multocida* Serotypes B:2 and B: 3,4

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Abstract

Serotype B of *Pasteurella multocida* is a major agent of haemorrhagic septicaemia (HS), a form of blood poisoning widespread in cattle and buffalo from Southern Asia. The vaccines currently used against the disease are not potent enough, being effective only for a limited duration only. In an attempt to identify immunogenic components of *P. multocida* for use as live/subunit vaccines, the outer membrane proteins (OMPs) of B:2 and B:3,4 serotypes were isolated and their profiles compared. Three common immunogenic components; polypeptides of molecular weight (MW) 39.0, 33.5, 31.0 kDa, were identified by immunoblotting. The immunogenicity of identified OMPs was also confirmed using antisera generated against them. The antigenic OMPs identified in current research could be explored in the formulation of new, safe and effective vaccines for indigenous buffalo and cattle breeds with a prolonged efficacy.

Keywords: Cattle, OMP, Vaccine, Haemorrhagic septicemia

Pasteurella multocida B:2 ve B:3,4 Serotiplerinin Ortak İmmunojenik Protein Komponentlerinin Karşılaştırılması

Özet

Pasteurella multocida B serotipi Güney Asya'daki sığır ve mandalarda yaygın olarak görülen ve kanda septisemi ile seyreden hemorajik septisemi (HS)'nin en önemli nedenidir. Hastalık için günümüzde kullanılan aşılardan yeteri kadar etkili değildir ve sadece sınırlı bir sürede etkisini göstermektedir. Canlı/subunit aşılarda kullanılmak üzere *Pasteurella multocida*'nın immunojenik komponentlerinin belirlenmesi için yapılan çalışmamızda, B:2 ve B:3,4 serotiplerinin dış membran proteinleri (OMP) izole edilmiş ve bunların profilleri karşılaştırılmıştır. İmmunoblotting sonucunda 39.0, 33.5 ve 31.0 kDa moleküler ağırlıklarında 3 adet ortak immunojenik polipeptit belirlenmiştir. İdentifiye edilen dış membran proteinlerinin immunojenitesi ayrıca bunlardan elde edilen antiserumlar ile doğrulanmıştır. Bu çalışmada identifiye edilen antijenik OMP'ler uzun süre etki gösteren yeni, güvenli ve etkin aşılardan formülasyonlarının geliştirilmesine katkı sağlayacaktır.

Anahtar sözcükler: Sığır, OMP, Aşı, Hemorajik septisemi

INTRODUCTION

Haemorrhagic Septicemia (HS), a widely distributed animal disease of tropical countries, is mainly associated with Asian and African countries. Prevalence of HS is mostly observed with moist conditions with most probably disease spread during winter season resulting in high morbidity and mortality rate. Several serotypes of *Pasteurella multocida* (Gram negative bacilli) are responsible for disease pathogenicity. Clinical signs included high temperature, respiratory distress with nasal discharge and frothing from the mouth, leading to death.

Cattle and buffalo are the most susceptible host for HS, buffalos as compared to other animals^[1]. The major *P. multocida* pathogenicity factors include polysaccharide capsule, endotoxins or lipopolysaccharide (LPS) and outer membrane proteins (OMPs) playing an important role for conferring protective immunity in a wide range of hosts^[1-4]. There is a clear correlation between capsular type and the disease; however, contribution to the disease specificity, of a particular type of lipopolysaccharide or the type and amount of bacterial surface proteins is still unclear^[4-7]. Molecular techniques have been employed to understand pathogenicity and epidemiology of *P. multocida* antigenic



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components^[7-11]. The common serological methods, based on capsular and somatic antigen, are; indirect haem-agglutination (IHA) test for capsular serotypes detection and agglutination/agar gel immunodiffusion (AGID) tests for somatic serotyping. Capsular type B belongs to Asian countries and serotype B:2 is the main disease causing agent. Serotypes B:3, B:4 and B:3,4 cause HS in cattle and wild ruminants producing signs and lesions similar to those by serotypes B:2 in water buffaloes and cattle^[12].

An effective treatment of sulphur drugs with antibiotics was reported, but some strains of *P. multocida* show resistance against antibiotic^[13,14]. Immune serum therapy was considered to be ineffective. Commonly, immunity against HS are provided by using three common vaccines namely, (i) bacterins (ii) alum-precipitated vaccine (APV) and (iii) oil-adjuvanted vaccine (OAV). *P. multocida* serotype B:3,4 isolated from a fallow deer in the United Kingdom was used in manufacturing live heterotypic vaccine. The vaccine protected cattle vaccinated subcutaneously against a serotype B:2 challenge and conferred immunity against HS for a year^[15]. Cross-protection by *P. multocida* serotype B:3,4 in a live intranasal vaccine against a subcutaneous challenge with serotype B:2 has been confirmed in Myanmar^[16]. This study revealed outer membrane proteins (OMPs) as common immunogenic components (MW 39.0, 33.5, 31.0 kDa) of *P. multocida* serotypes B:3,4 and B:2, rendering B:3,4 a successful candidate for use as a live vaccine against haemorrhagic septicaemia. Moreover the polypeptides identified could be used in future for the development of sub unit vaccine

In Pakistan, cattle and buffalo populations are at high risk of HS infection owing to high animal losses. In an attempt to provide animals protection against disease, a comparative study was conducted to identify local pathogenic strain specific immunogenic components use as vaccine. The identified immunogenic polypeptides could be explored for subunit vaccine production in future.

MATERIAL and METHODS

For culturing purposes, *P. multocida* strains; B:2 and B:3,4, were obtained from Bacteriology Laboratory, Animal Sciences Institute (ASI), National Agricultural Research Centre (NARC) Islamabad, Pakistan. After reconstitution in normal saline, 0.5 mL of each strain was injected into mice subcutaneously (2 mice for each isolate) maintained in animal house for 24 h. Blood samples were collected from treated animals through cardiac puncture under anaesthesia. Blood was streaked on Trypton Soya Agar medium plates (Oxoid Ltd., England) and incubated at 37°C for 24 h. Positive colonies were initially selected by Gram staining and, for further confirmation, API 20NE identification kit (Biomerix 20NE System) was used. Mass cultivation of pathogenic organisms was performed in 250 mL flasks containing 100 mL brain heart infusion (BHI)

broth kept in shaker incubator at 37°C for 24 h. Liquid cultures were centrifuged at 10,000 rpm for 30 min at 4°C; pellet was re-suspended in normal saline overnight at 4°C followed by centrifugation at 10,000 rpm for 20 min at 4°C. The resulting pellet was again suspended in normal saline, centrifuged and suspended in 10 mM N-(2-hydroxyethyl) piperazine-N-2 ethane sulfonic acid (HEPES) buffer, pH 7.4 (a ratio of 0.1 g pellet wet weight/mL of extraction buffer). The suspended cellular pellet was sonicated (Sonicator G-055 UP 400S) at 100% amplitude for 2 min with 30 sec intervals for cell disruption. The sonicated supernatant was then centrifuged at 1,700×g for 20 min at 4°C and supernatant containing whole cell proteins was divided into aliquots for storage. The study was ethically approved.

Preparation of OMP Enriched Fraction

The OMPs were extracted by the method of Choi *et al.*^[17]. The supernatant was ultra-centrifuged at 100,000×g /2,8500 rpm at Beckman Coulter L-100XP ultracentrifuge using SW41 Ti-Rotor for 1 h at 4°C. The pellet was collected and re-suspended in 2% sodium lauryl sarcosinate detergent and then incubated at 22°C for 1 h. The solution was ultracentrifuged at 100,000×g for 1 h at 4°C. The pellet was kept in distilled water overnight and ultra-centrifuged followed by another washing step and dissolved in Nano-pure water. Next the pellet was dialyzed against distilled water for 8 h using dialysis membrane of 6,000-8,000 Dalton. The final protein concentrations of 3.05 mg/mL for B:2 strain and 1.1 mg/mL of B:3,4 strain were obtained. The protein rich extract was then separated by SDS-PAGE^[18] using 4% stacking and 12% separating gels visualised using Coomassie brilliant blue stain. Molecular weights of proteins were determined through Rf values (Rf = band distance/distance covered by dye front). Molecular weights obtained through plots were then log transformed.

Hyperimmune Sera Production

Antisera were raised in two groups of rabbits (3 animals per group, two for each strain and one as a control) while two groups of animals were used for raising antisera in calves^[19]. The blood of infected animals was collected and serum separated by centrifugation at 3,000 rpm for 10 min.

Immunoblotting of Anti-Sera Proteins

Effectiveness of raised antisera was checked using dot blot method with standard rabbit antisera (Chinchilla breed). Three experiments were performed: one with B:2 and B:3,4 antigens (OMPs and LPSs) and B:2 antisera raised in rabbits; other with B:2 and B:3,4 antigens with B:3,4 antiserum raised in rabbits; third with B:2 and B:3,4 antigens (OMPs and LPSs) and B:2 antisera raised in calves. The primary antisera (raised in rabbits) and secondary antibody (goat anti-rabbit IgG Alkaline Phosphatase conjugated) dilutions were prepared in TEN buffer. The substrate contained nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in alkaline phosphate buffer (APS).

Immunoblotting was performed using standard protocol and transblot apparatus by the protocol given on the manufacturer's website [20] followed by SDS-PAGE using pre-stained markers (SM 0671, Fermentas). Blotting was performed using E844 CONSORT Electrophoretic power supply at 4°C. Two dilutions (1:50 and 1:100) of B:3,4 antiserum were used as the primary antibody following the same conditions as mentioned above. The 1:100 dilution of primary antibody (B:2 antiserum in calf) was prepared using 3% bovine serum albumin (BSA), incubated for 2 h at room temperature with constant shaking. The BSA was removed and antiserum washed thrice with 15 mL of 0.1% phosphate buffered saline with tween (PBST). The horse-radish peroxidase conjugated secondary antiserum IgG antibody (1:1000 dilution) and primary antibody (1:50 dilution) were added followed by three washings with 0.1% PBST. The filter was placed in Diaminobenzidine (DAB) substrate solution and reaction was stopped by rinsing with water. The images were scanned and stored for further analysis. In another experiment, the 1:50 and 1:100 dilutions of B:3,4 antiserum were used as the primary antibody following the same conditions as mentioned above.

LPS Profiling

For LPS extraction from B: 3,4 and B:2 strains was performed using O.I. E Manual (2004) and enzymatic method Bastuji *et al.* [21] respectively. Bacterial strains were mass cultivation using BHI broth and cells were harvested. The extracted LPS were analysed on 4% stacking gel and 12.5% separating gel, along with L4130 as standard LPS marker and then silver stained [21]. The gels were then transferred to nitrocellulose membranes following the method described by Towbin *et al.* [22].

RESULTS

The B:2 and B:3,4 strains of *P. multocida* were found to be Gram negative coccobacilli as they gave pink colour after Gram staining under microscope. The data gathered

from kit-based tests was transformed with a numerical value of 3.000,004 and was confirmed in the Analytical Profile Index.

The OMPs concentration was found to be 3.05 mg/mL for B:2 strain and 1.1 mg/mL of B:3,4 strain. The SDS-PAGE results showed five polypeptides of 94.8, 56.5, 33.5, 31.0 and 17.06 kDa for strain B:3,4 whereas five polypeptides of 71.61, 33.5, 31.0, 23.44 and 17.06 kDa (Table 1) for strain B:2. Among these, three polypeptides (33.5, 31.0, and 17.0 kDa) were common in both of the strains (Fig. 1). As far as the intensity of bands was concerned, the polypeptide having MW of 33.5 kDa showed the most intense band among bacterial strains.

The dot blot results were positive for all the dilutions (1:100, 1:200 and 1:400) of primary antisera (raised against

Table 1. Calculation of OMPs molecular weights using Rf values and log of molecular weight plot

Tablo 1. OMP'lerin molekül ağırlıklarının Rf değerleri ve moleküler ağırlık plot logaritması kullanılarak hesaplanması

Specimen ID	Distance Covered by Bands (cm)	Rf Values	MW (kDa)	Log MW
SM0431	0.6	0.6/8 = 0.075	116.0	2.064
	1.2	1.2/8 = 0.15	66.2	1.820
	2.2	2.2/8 = 0.275	45.0	1.653
	2.9	2.9/8 = 0.362	35.0	1.544
	4.1	4.1/8 = 0.512	25.0	1.397
	4.9	4.9/8 = 0.61	18.4	1.264
	5.3	5.3/8 = 0.662	14.4	1.158
B:3,4	0.6	0.6/8 = 0.075	94.83	1.976
	1.9	1.9/8 = 0.237	56.5	1.752
	3.2	3.2/8 = 0.4	33.5	1.52
	3.4	3.4/8 = 0.425	31.0	1.491
	4.9	4.9/8 = 0.612	17.06	1.232
B:2	1.3	1.3/8 = 0.1625	71.61	1.855
	3.2	3.2/8 = 0.4	33.5	1.52
	3.4	3.4/8 = 0.425	31.0	1.491
	4.1	4.1/8 = 0.512	23.44	1.370
	4.9	4.9/8 = 0.612	17.06	1.232

Distance covered by dye front = 8.0 cm

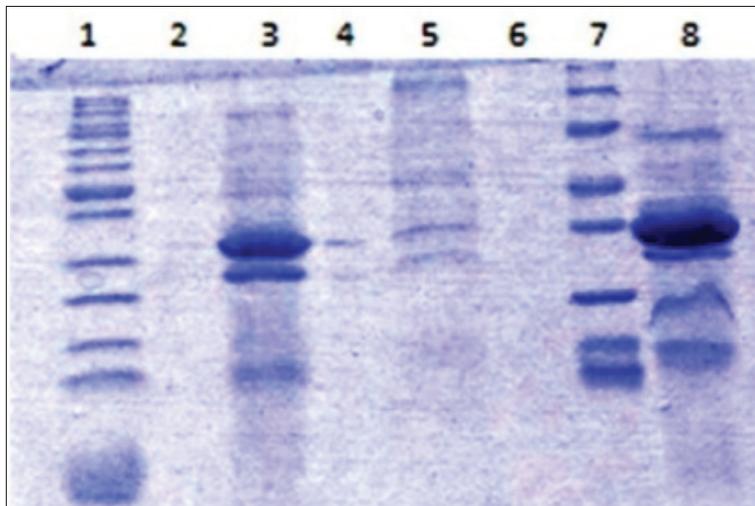


Fig 1. Outer membrane protein profiles of *Pasteurella multocida* strains B:2 and B:3,4

Lane 1: Protein marker (SM 0661), Lane 3 and 5: B:3,4 (1:2 dilution), Lane 7: Protein marker (SM 0431), Lane 8: B:2(1:2 dilution)

Şekil 1. *Pasteurella multocida* B:2 ve B:3, 4 suşlarının dış membran protein profilleri

Kolon 1: Protein markeri (SM 0661), Kolon 3 ve 5: B:3,4 (1:2 sulandırma), Kolon 7: Protein markeri (SM 0431), Kolon 8: B:2(1:2 sulandırma)

Antigens/ Antiserum IDs	Dilution Factors		
	1:100	1:200	1:400
OMPs B:2/ B:2 antiserum			
LPS B:2/ B:2 antiserum			
Positive Control (B:2 Culture)/ B:2 antiserum			
OMPs (B:3,4)/ B:3,4 antiserum			
LPS B:3,4/ B:3,4 antiserum			

Fig 2. Dot blot results of B:2 and B:3,4 antigens against B:2 antiserum raised in rabbits

Şekil 2. Tavşanlardan elde edilen B:2 antiserumuna karşı B:2 ve B:3,4 antijenlerinin Dot-Blot sonuçları

Fig 3. Cross reactivity of B:2 antigens against B:3,4 antiserum raised in rabbits

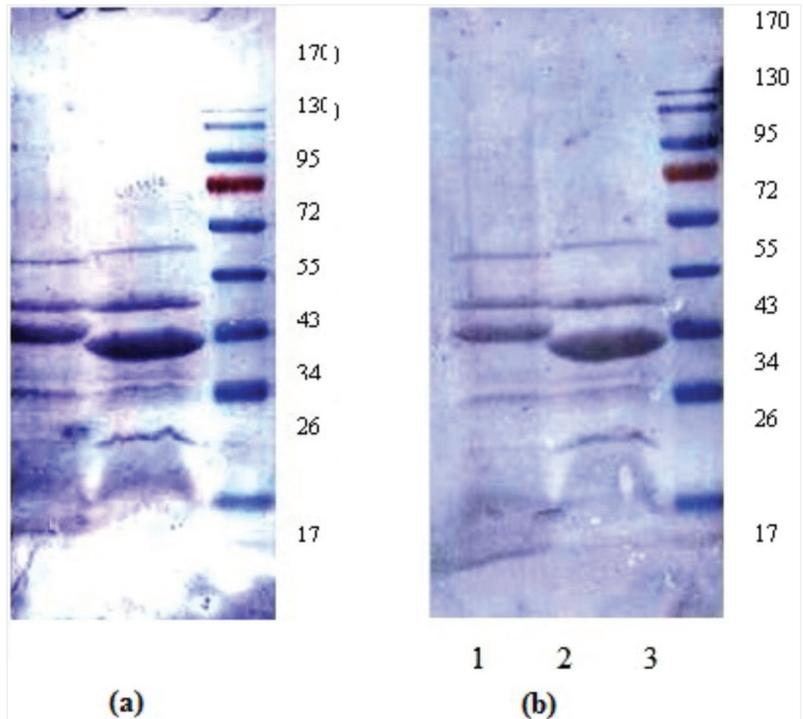
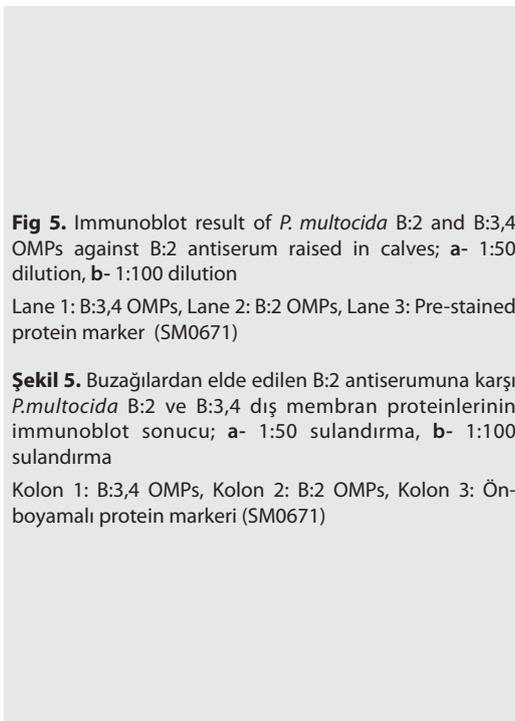
Şekil 3. Tavşanlardan elde edilen B:3,4 antiserumuna karşı B:2 antijenlerinin çapraz reaktivitesi

Antigens/ antiserum	1/100 dil	1/200	1/300	1/400
OMPs B:2/ B:3,4 antiserum				
LPS B:2/ B:3,4 antiserum				
+ive control (B:2 supernatant/ B:3,4 antiserum				
+ive control (B:2 culture /B:3,4 antiserum				
-ive control (B:2 culture/ control antiserum				

Antigens/ antiserum	1/100 dil	1/300	1/100	1/300
	Antiserum calf 308		Antiserum calf 405	
OMP_s B:2				
LPS B:2				
OMP_s B:3,4				
LPS B:3,4				
+ive control (B:2 culture)				

Fig 4. Dot blot results of B:2 and B:3,4 antigens against B:2 antiserum raised in calves

Şekil 4. Buzağılardan elde edilen B:2 antiserumuna karşı B:2 ve B:3,4 antijenlerinin Dot-Blot sonuçları



P. multocida B:2 and B:3,4 in rabbits). The OMPs of B:2 and B:3,4 strains reacted with all three dilutions of their respective antiserum (self reactivity) whereas 1/400 dilution was positive for B:2 LPS only (Fig. 2, Fig. 3). The antisera raised in buffalo calves and B:2 antiserum dilutions (1/100 and 1/300) from two groups of animals

was also checked for its efficacy against its own antigens (B:2 OMPs and LPS i.e., self reactivity) and antigens of the other strain, B3,4. (cross reactivity). The results were positive for OMPs with both the dilutions whereas B:2 LPS gave positive result only with 1/300 dilution only (Fig. 4).

The immunoblot of OMPs confirmed common bands 33.5, 31.0 and 17.0 kDa among B:2 and B:3,4 strains of *P. multocida* as revealed by SDS-PAGE. The polypeptides having molecular weight of 39.0, 33.5 and 31.0 kDa were found immunogenic in B:2 and B:3,4 strains as revealed by western blotting. The band of molecular weight of 39.0 kDa was not obvious in the gel but was visualised in Western blotting thus proving that this method is more sensitive in detecting immunogenic proteins than Coomassie blue staining (Fig. 5).

As in case of LPS, dot blot results were found positive for 1/100 and 1/300 dilution of primary antisera with 1/1000 dilution of secondary antibody though immunoblots with LPS were unsuccessful.

DISCUSSION

Asian and African cattle and buffalo are highly susceptible to HS infection resulting from *P. multocida*. The disease prevalence, morbidity and mortality rate is high in tropical environments including certain regions of Pakistan. Several serotypes of *P. multocida* exist ranging in disease pathogenicity. Serotype B:2 is the common disease causing agent in Asian water buffaloes while B:3, B:4 and B:3,4 serotypes cause HS in cattle and wild ruminants^[4]. Disease pathogenicity arises from polysaccharide capsule, endotoxins or LPS whereas *P. multocida* OMPs especially B:2 serotype are reported for conferring immunity against disease^[2,4,23]. Therefore, detection of immunogenic proteins common to B:2 and B:3,4 serotypes of *P. multocida* are being explored as future targets for subunit vaccine production against HS infection.

The OMPs of B:2 and B:3,4 serotypes of *P. multocida* were studied for their immunogenicity. The SDS-PAGE profiling of OMPs from B:2 and B:3,4 serotypes identified five proteins with three common bands; 33.5, 31.0, and 17.06 kDa. The polypeptide with 33.5 kDa showed the most intense band between both bacterial strains. No research has been reported regarding the comparative analysis of B:2 and B:3,4 strains. As far as the band intensity is concerned, Jain *et al.*^[24] reported 31.7 and 34.9 kDa as the major OMPs among nine protein bands with MW ranging from 21.1-89.2 kDa. Kedrak and Opacka^[25] found protein bands of 22 to 86 kDa in the OMP profile of serotype B:2 while Pati *et al.*^[23] reported ten protein bands ranges between MW of 25 to 88 kDa. In another study, Johnson *et al.*^[26] demonstrated polypeptides of 32 kDa as the major OMP. With a little difference from our findings, Tomer *et al.*^[27] concluded 31, 33 and 37 kDa as the major OMPs while comparing the outer membrane protein profiles of *P. multocida* B:2 isolates.

The immunogenic and protective role of OMPs against HS infection has been elucidated previously^[22,25,28]. With regards to the immunogenicity of OMPs of *P. multocida* B:2

and B:3,4, both strains reacted with respective antiserum raised in rabbits, thus confirming cross-reactivity. Western blotting analyses revealed three polypeptides from B:2 and B:3,4 strains; 39.0, 33.5 and 31.0 kDa possessing immunogenicity against infection (Fig. 5). Based on quantitative analysis of gel bands, Tomer *et al.*^[16] also reported three polypeptides MW of 31, 33 and 37 kDa, with 37 kDa fraction being highly antigenic. Whereas other study reported polypeptides of 44, 37 and 30 kDa being major immunogens among ten polypeptide bands of 25 to 88 kDa in a study on outer membrane protein from *P. multocida* serotype B:2^[24].

The efficacy of antisera raised in both groups of animals (rabbits and calves) is indicative of the immunogenic nature of both the antigens (OMPs and LPS). Experiments showing cross reactivity of antisera with antigens reveal the common nature of the mentioned immunogenic components also evident from SDS-PAGE analysis of OMPs where three common bands (MW of 33.5, 31.0 and 17.0 kDa) were found among B:2 and B:3,4 strains. Therefore, present study clearly revealed outer membrane proteins (OMPs), as common immunogenic components of *P. multocida* serotypes B:2 and B:3,4 for use as a live vaccine against haemorrhagic septicaemia. Moreover, the polypeptides identified could be employed for the development of subunit vaccine(s).

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