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Immunogenicity of Lipopolysaccharides from *Pasteurella multocida* Type B against Hemorrhagic Septicemia in Buffaloes

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Hemorrhagic septicemia (HS) is a fatal disease of cattle and buffaloes causing heavy economic losses. The causative organism (*Pasteurella multocida*) was isolated and identified from blood samples of infected animals on the basis of morphological and molecular characteristics. The lipopolysaccharides (LPS) were extracted by treating the cells with proteinase K and quantified following per iodic acid method. This LPS was mixed with *Saccharomyces cerevisiae* and different concentrations were injected subcutaneously in buffaloes. The OD values of ELISA showed that the antibody level against LPS was higher in Group D and it gradually decreased from 10th to 30th day post inoculation. Hence it was found that the LPS was immunogenic and its immunogenic activity can be improved by the addition of carrier proteins from *S. cerevisiae*.

Key words: *Pasteurella multocida*, Hemorrhagic septicemia, LPS, ELISA, Immunity & Buffaloes.

Hemorrhagic septicemia (HS), a bacterial infectious disease caused by gram-negative bacterium *Pasteurella multocida*, is one of the threatening diseases of animals, adversely affecting their health and reducing their production. Buffaloes are more susceptible to this disease as compared to cows or other domesticated animals (Ataei *et al*., 2009). A study conducted by Farooq *et al*., (2011) exposed that the morbidity, mortality and case fatality rates due to HS were greater in young calves than the adults, both in buffaloes and cattle. Poor immune response of vaccine has been reported against this disease due to immunosuppressive effects of other diseases like trypanosomosis (Singla *et. al*., 2010; Singla *et al*, 2012). From 5% to 90% mortality rate has been recorded in India and Philippines in different outbreaks (Molina *et al*., 1994), whereas in Faisalabad, Pakistan, 100% morbidly and 31.48% mortality in buffalo calves due to HS has been reported by Khan *et al*. (2011). Different serotypes (A, B, C, D and E) of *P. multocida* have been detected among the livestock population (Kumar *et al*., 2004). Stereotype B: 2 and E:2 of *P. multocida* cause HS in cattle and buffaloes in Asia and Africa respectively (Carter, 1955, Nawaz *et al*., 2006). These are known to possess a type IV fimbriae (pili) as one of the virulence factors (Shivachandra, 2012).

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Pakistan has a cattle population of 38.3 million and buffalo population of 23.7 million heads (Pakistan economic survey, (2012-13); Afzal, (2010); Khaliq and Rahman, (2010)). In Pakistan, the highest prevalence of HS was recorded in Khanewal district (49 %) while the lowest (0.78%) was recorded in Jamshaid Saddar (Karachi) during 2000 to 2005 (Farooq *et al.*, 2007). In a study conducted by Zahur *et al*. (2007), an average loss of Rs. 0.2 million per farm was calculated. 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).

It has been shown that LPS from *P. multocida* assist in adhesion of neutrophils and transmigration through endothelial cells (Galdiero *et al*., 2000). However, there are conflicting reports about the endotoxic properties of LPS isolated from *P. multocida*. LPS isolated from serotype B: 2 strain was shown to be endotoxic, and intravenously administrated LPS could reproduce clinical signs of HS in buffaloes (Horadagoda *et al*., 2002). The main objective of the study is to extract LPS from *P. multocida*, then quantify it and immunologically evaluate in buffaloes by ELISA.

MATERIALS AND METHODS

The Infected field samples were collected from Landhi, Cattle Colony. The animals showing typical signs and symptoms of the disease were identified. The blood from the jugular vein and nasal swabs was collected from live animals while the heart blood was collected from dead animal during necropsy. The samples were transported on ice. These samples were cultured on the nutrient agar, CSY agar, blood agar and Mc-Conkey's agar for isolation and identification of the causative organism. Finally presence of the organism was confirmed by PCR amplification of 16S ribosomal DNA using forward primers: AGAGTTTGATC CTGGCTCAG and reverse primer: ACGG (ACT) TACCTTGTTACGACTT. The nucleotide sequence of amplified 16S rDNA gene was determined and phylogenetic analysis of sequences was carried out (data not shown) using Basic Local Alignment Search Tool (Ashraf *et al.*, 2011).

Extraction of lipo-polysaccharide (LPS)

LPS was extracted by the method as described by Penn and Nagy (1976). Briefly the bacterial growth was removed from the CSY-plates with a 2.5% solution of sodium chloride followed by shaking of the bacterial suspension for 5 minutes at room temperature. Whole cell extracts were collected by centrifugation for 15min at 10,000 rpm. Then cell surface protein was removed from this extract by the addition of 100 µg of proteinase K per ml and incubation at room temperature for one hour. Subsequently proteinase K was inactivated by heating the mixture for 10 min at 100°C. The proteinase K treated supernatant was referred as whole polysaccharide.

Quantification and SDS-PAGE analysis of LPS

The quantification of LPS was carried out by per Iodic acid method described by Skoza and Mohos (1976). Briefly mix the sample (0.5 ml) of the LPS hydrolysate with 0.25ml of periodic acid reagent and incubate at 37°C for 30 minutes. Cooled and added 0.25 ml of Sodium arsenite solution, mixing until the brown color of iodine disappears. Thiobarbituric acid solution (0.25ml) was added and heated at 100ºC for 7.5minutes.While the reaction mixture still hot, added 1ml of dimehylsulphoxide. Cooled and measure the absorption at 548nm wavelength. The same procedure was repeated with KDO, which was used as a standard solution.

Solubilization buffer (3X) was mixed with purified LPS samples in 1: 3 ratio. The supernatants were mixed separately with chloroform (1:1) and centrifuged at 5000 rpm for 20 minutes. Three distant layers, top layer containing LPS, middle layer had the debris and a bottom layer was constituted of chloroform, were obtained. The purified LPS was mixed with desirable concentration (100 (l /33 (l), and boiled for 5 minutes. A 20 µl /well from each sample was separated on 12.5% SDS gel with a 4% stacking gel under reducing conditions at 100 mA for 2 hr using mini-PROTEAN electrophoresis instrument (Bio-Rad Laboratories, California, USA). The silver staining method for LPS by Hitchcock and Brown (1983) and Fomsgaard et al. (1990) was used to stain the gel.

Preparation of antigen

LPS was extracted from the fresh cultures of *P. multocida* and mixed with yeast

(*Saccharomyces cerevisiae*). LPS alone has poor immunogenic properties so it was mixed with yeast to increase its immunogenicity. A total twenty four female buffaloes of the same age were divided into six groups. Different doses of the antigen were injected subcutaneously into animals of different groups. While one group was injected only yeast (*Saccharomyces cerevisiae*) and treated as negative control. Details of different doses and treatments of each group are shown in table 1.

Antibody titer determination

The serum antibody titers against LPS were determined using ELISA technique. Purified LPS (2µg/well) was coated on ELISA plates to detect antibodies against LPS. Serum antibody titers were determined using IgG alkaline phosphatase conjugate (Sigma) and p-nitrophenyl phosphate as substrate (Maqbool *et al*., 2002).

Statistical analysis

Analysis of variance (ANOVA) was used under Duncan's multiple range test to compare the antibody titer between different groups at different days (Steel and Torrie, 1984).

RESULTS AND DISCUSSIONS

Colonies of freshly isolated *P. multocida* are smooth, non-hemolytic, grayish, translucent, shiny, glistening and approximately having 1 mm diameter after incubation at 37ºC on blood agar for 24 hours. Large colonies are seen on CSY agar, and smaller colonies may develop from older cultures. The suspected colonies were isolated and sub-cultured. Confirmation of the *P. multocida* was done by PCR (Ashraf *et al.*, 2011).

The isolated LPS were quantified against standard Kdo (3-Deoxy-D-Manno-octulosonic acid) by using the per iodic acid method and the results are shown in Table 2. The electrophoretic separation of LPS from *P. multocida* serotype B was shown on 12.5% polyacrylamide gel after silver staining (isolates No. 1, 2 and 3). The extracted LPS from different isolates showed same electrophoretic profiles (Figure 1).

Antibody titer at 10th day post inoculation

The conjugate dilution (1:400) and serum dilution (1:16) were optimized for the antibody titer determination. The OD was determined at 405 nm wavelength using a microplate reader. At 10th day post immunization, the antibody titers of group A were minimum that gradually increased from group A to D. The group D had maximum antibody titers. The mean OD values, S.D and C.V are shown in table 3.

Antibody titer at 20th day post inoculation

The results revealed that group A has a least antibody titer while group D had a maximum antibody titer. The mean O.D values are given in Table 3.

Antibody titer at 30th day post inoculation

The comparison of O.D values of all groups at different days post inoculation is shown in Figure 2. O.D values gradually increased from Group A to D while gradually decreased from 10th days post immunization to 30th day post immunization. Results showed that group A had a lowest antibody titer while group D had maximum antibody titers.

The % $^{2}_{o}$ representing the antibody titers of different groups at different days were compared, it was found that antibody titers at 10th days post immunization was maximum then it gradually decreased from 10th to 30th days post immunization. Comparison of percent maximal absorbance values at 10th, 20th and 30th days post immunization are given in Table 3. Analysis of variance analysis tests

Table 1. Doses and inoculation schedule for buffaloes

Groups	Buffaloes	Treatments	DoseLPS+ *S. cerevisiae*
A	4	LPS+ *S. cerevisiae*	100 µl+100 mg
B	4	LPS+ *S. cerevisiae*	80µl+120 mg
C	4	LPS+ *S. cerevisiae*	60 µl+140 mg
D	4	LPS	40 µl + 0
E	4	Inactivated *P. multocida* having 1 X 10^9 CFU / ml	1000 µl
-ve control	4	*S. cerevisiae*	0 + 150 mg

Table 2. Estimation of 3-Deoxy-D-Manno-octulosonic acid in LPS by periodic acid method

No. of Sample	Absorbance	µg/ml
1	1.382	18.4
2	1.461	19.4
3	1.376	18.3
4	1.495	19.9
5	1.283	17.1
6	1.410	18.8

showed that different groups have a significant difference in antibody titers at different days post immunization ($P < 0.05$). Duncan's multiple range test indicated that the antibody titer of all three samples were significantly different from one another, i.e. value of OD significantly decreased with increase in time. Similarly Duncan's multiple range test for groups showed that difference between group 3 and 4 were non-significant ($P > 0.05$) while their difference was significant from

Table 3. The Average of optical density (OD), standard deviation (SD) and coefficient of variance (CV) at the 10th, 20th and the 30th days post inoculation in buffaloes

Group	OD ± SD			CV		
	10th day	20th day	30th day	10th day	20th day	3th day
A	0.344±0.0078	0.256±0.0049	0.192±0.007	2.258	1.937	3.682
B	0.356±0.0120	0.269±0.000	0.202±0.001	3.371	0.000	0.700
C	0.372±0.0070	0.281±0.0098	0.213±0.002	1.900	3.522	0.993
D	0.380±0.0127	0.288±0.0035	0.215±0.007	3.349	1.225	3.288
E	0.287±0.0077	0.281±0.0120	0.169±0.002	2.705	5.501	1.251
-ve control	0.033± N.A	0.020± N.A	0.035± N.A	N.A	N.A	N.A

Table 4. ANOVA to compare the groups and days

Source	Degree of freedom	Sum of square	Mean square	F value
Groups	2	0.113	0.056	974.8341**
Days	4	0.018	0.005	78.6578**
Groups and days	8	0.001	0.000	2.9359*
Error	15	0.001	0.000	
Total	29	0.133		

** Highly significant, * Significant

other groups. The group 5 *i.e.* control had a significant least value of OD from other groups.

The sugar composition of LPS isolated from *P. multocida* was analyzed by using Kdo as standard sugar (St. Michael *et al*., 2005). The LPS contained glucose, 2-keto-3-deoxyoctonate, and heptose which was similar to the results described by Rimler *et al.* (1984). The method adopted in this study for extraction of LPS was not similar to the method explained by Rimler *et al.* (1984) who used the phenol extraction method. The LPS of *P. multocida* was quantified by using the semi - carbazide method in order to prepare the appropriate dose (Collins, 1977). All LPS of gram-negative bacteria contained, glucose, 2-keto-3-Deoxyctulosonic acid and heptose. The trihaptose unit is linked to 2-keto-3-deoxyoctulosonic acid (Kdo). So, identification and quantification of LPS were done by using Kdo as a standard (Rimler *et al.*,1984). The quantification of LPS was also performed by using the periodic acid method. Skoza and Mohos, (1976) reported similar findings by using the same methods. The quantity of LPS obtained by per iodic acid methods ranging from 17 to 20µg/ml while Hakimi *et al*., 2006 reported that the amount of LPS was 50µg/ml. The quantification of LPS was carried out to prepare the dose for inoculation in buffaloes. The LPS of isolates of *P. multocida* were characterized by SDS-PAGE. Silver-stained SDS-PAGE purified LPS profiles were similar to those of

semi rough strains of gram-negative and contained one band that confirmed the purity of extracted LPS that was utilized as antigen. LPS has a vital role in pathogenicity, as mutants with truncated LPS were found attenuated (Harper *et al.*, 2004). The measured quantity of LPS was injected as an antigen to determine its immunogenicity in buffaloes. The LPS represents the outer surface of gram-negative bacteria with immunomodulatory properties (Carrof and De Alwis, 2003). The LPS was injected with *S. cerevisiae* as Berner *et al.* (2008) hypothesized that microparticles of *S. cerevisiae* might serve as an adjuvant to enhance specific immune response. Tsuji and Matsumoto (1988) extracted two different antigens having two antigenic determinants. Heat stable protein and periodate sensitive carbohydrate, these were the major antigenic determinant on the LPS. Confer (1993) stated that somatic antigens, particularly LPS, appear to be a major source of immunity if injected.

The LPS antigen (100µl) was given to experimental buffaloes. The same experiment was performed by Paul (1967), who injected 1.8mg / kg body weight of LPS to calves, and found that the dose in milligram was more toxic rather than immunogenic. In this experimental study, serum antibody titers were detected in buffaloes against a LPS antigen by ELISA, which is a reliable diagnostic tool (Zaoutis *et al.*, 1991).

The group D had maximum % β_o and had a maximum antibody titer value while the group A had a minimum %β_o and hence minimum antibody titer. The group D was given higher doses of LPS, therefore, the more immunity was produced as compared to other groups, which were given LPS with yeast. The LPS was more immunogenic. The results were shown by Marina *et al.* (2004) revealed that the purified LPS produced immunity in buffaloes and there was direct proportionality of LPS dose and immunity. The dose of LPS combined with yeast was less immunogenic in buffaloes, but LPS was more immunogenic as compared to LPS-Yeast.

In 2nd and 3rd sampling, there was also a gradual increase in %β_o from group A to D. As the % ^2_o increased gradually the antibody titers also increased. In case of the days comparison, the 10th day's serum sample had maximum % β_o while 30th day's sample had the minimum % β_o. The %β_o of groups into different days were compared, and then it was observed that the values of % β_o in the first sampling was maximum but it gradually decreased from 10th to 30th days, but the immunity or antibody titer gradually decreased and maximum antibody titer was obtained in 1st sampling or after one month of inoculation.

It was observed that the LPS is more immunogenic in buffaloes as compared to LPS-protein complex. Previously Ryu and Kim (2000) described that results of active protection with LPS-protein complex was more immunogenic as compared to alone LPS in mice and cattle. Because the extraction of LPS by formalin and hot water, phenol method decreased the immunogenicity of LPS antigen while the proteinase K digestion method was used in this experimental study, that is safe and easy as compared to formalin and hot

Fig. 1. Electrophoretic profile of LPS on 12.5% polyacrylamide gel after silver staining (Lane, 1 sample No. 1; lane 2, sample No. 2 and Lane 3 sample No. 3)

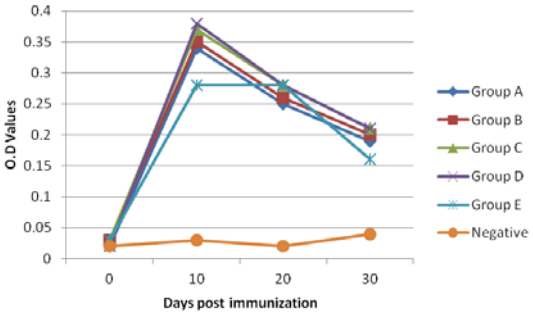

Fig. 2. Comparison of ELISA antibody titers in different groups at the 10th, 20th & 30th days post immunization

phenol water. It was concluded that the sufficient amount of specific antibodies against LPS were produced after immunization. The LPS of *P. multocida* was found immunogenic. The similar results were described by Raetz and Whitfield (2002) they found that the LPS has a vital role in innate immunity, where of the inflammatory response has a significant role to clear bacterial infection.

Further research work on immune-protection studies of the antibodies against LPS is required. Protection against pathogenic *P. multocida* can be assessed by challenge protection tests in susceptible animals. Moreover, expression and purification of the recombinant antigen in suitable system (*E. coli* or yeast) can also lead towards its immunological studies.

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