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Outer membrane proteins and lipopolysaccharides mediated antibody response against bovine *Pasteurella multocida* type B-2

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ABSTRACT

Aims: The activation of cellular and humoral immunity depends upon nature of antigens. Complex proteins like bacterial outer membrane proteins (OMP) usually successfully activate both humoral and cellular immunity. Whereas antigens like bacterial lipopolysaccharides (LPS) usually elicit T-independent immunity i.e. humoral immunity without the activation of cellular immune wing. The present study was under taken to evaluate the comparative immunologic behavior of both the important molecules (bacterial lipopolysaccharide and outer membrane proteins) of *Pasteurella multocida* alone and in combination in bovine calves in field conditions.
Methodology and results: *Pasteurella multocida* was isolated, purified and identified from an outbreak by mean of culture and biochemical methods. The pathogenicity of the confirmed isolates was done in rabbits (*Oryctolagus cuniculus*) on the principles of Koch's postulates. Alum based vaccine against *P. multocida* was prepared and antibody titer against Outer membrane protein (OMP) and lipopolysaccharides (LPS) were determined by complement fixation test (CFT). The results showed that the antibody titer against OMP and LPS in whole culture vaccine is significantly higher than the respective tested vaccines. These results concluded that OMP no doubt is an active T-dependent immunogenic molecule but its immunogenicity increases many times when combined with LPS in whole culture vaccine.
Conclusion, significance and impact of study: Lipopolysaccharides (LPS) in combination with outer membrane proteins (OMP) synergistically boost the humoral immune response in vaccinated animal.

Keywords: Complement fixation test, lipopolysaccharides, outer membrane proteins, *Pasteurella multocida*

INTRODUCTION

Pasteurella multocida is a gram negative bipolar, non-motile bacteria classified on the basis of capsular composition in five groups (A, B, D, F and E) and 16 somatic groups. It causes diseases in mammals including birds, bovines and human etc. It results in high neutrophil and leukocyte count which leads toward inflammatory responses at the site of infection (Ryan and Ray, 2004). In severe cases, the organism may cross the blood brain barrier to cause meningitis. Buffalo is more susceptible to *P. multocida* infection than cow and show more severe pasteurolosis with profound clinical signs Office of International Epizootic (OIE, 2014).

Pasteurella multocida is classified on the basis of capsular polysaccharide difference. This is called carter classification and designated as A, B, C, D, E and F. This organism is further subdivided into the 16 serotypes (serogroups) (Heddleston *et al.*, 1972). Bacterial lipopolysaccharides are a macromolecule of 10-20 kDA. It contains 1, lipid A (responsible of toxicity); 2, hydrophilic core chain of polysaccharide and 3, O-antigenic oligosaccharide (specific to the bacterial serotype) (Rietschel *et al.*, 1994). Lipopolysaccharides (LPS) is a heat stable endotoxin responsible for the septic shock (septicemia) in human as well as for the induction of immune response in mammalian cells. The endotoxicitic activity of LPS is mainly due to lipid A moiety (Schletter *et al.*, 1995). Capsular LPS is one of the factor that contributed towards the disease development includes adhesion of Pasteurella to the mucosal membrane and avoids phagocytosis. Different serotypes have different composition of capsule. Type A and Type B capsule is composed of hyaluronic acid. Type D and Type F contains heparin and chondroitin respectively. These compounds are same to those that found in the species of the host which was colonized by the particular serotype and this molecular mimicry decreases the chance of development of strongest immune response to the organisms. Another major virulence factor is *P. multocida* toxin that stimulates growth of fibroblasts. In septicemia

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conditions, severe endotoxaemia causes serious illness which can be fatal (Dabo *et al.*, 2007).

This bacterium was found to have consist of several OMPs. These OMPs play an important role as immunogenic antigen. This study was based on OMP 87; the gene responsible for this was taken, cloned and amplified in the vector pET32a and over-expressed in *E. coli* as fusion protein. When mice were immunized with this purified protein along with adjuvant it was found to produce 60% to 80% of immunity. So this study indicated that the use of rOmp87 protein along with suitable adjuvant was responsible for producing immunity against Hemorrhagic Septicemia and pasteurellosis in livestock (Kumar *et al.*, 2013).

This study was designed to investigate how LPS and OMP was implicated in the immune response against *P. multocida* infection.

MATERIALS AND METHODS

Isolation and purification of *Pasteurella multocida*

The sample obtained from the infected animals was cultured on blood agar and MacCkonkey's agar. The plates were incubated at 37 °C for 24 h. The characteristic colonies were obtained and were used for purification purpose in casein sucrose yeast extract agar.

Biochemical characterization

Biochemical profiling was used to identify the bacteria at genus and species level. The suspected *P. multocida* was tested for oxidase, indole production, catalase, carbohydrate (sucrose, lactose, glucose and maltose) fermentation tests (Cappuccino and Sherman, 2008).

Molecular characterization

Molecular characterization of the isolated *P. multocida* was performed by using specific primers as described by OIE 2014.

Mass culturing

Purified and characterized isolate was used as seed for the preparation of mass culture. Briefly; casein sucrose yeast (CSY) extract media was prepared and sterilized by autoclave at 121 °C at 15 pounds for 15 min. The purified inoculum was poured in sterilized CSY media and was incubated. The sample was taken for dry mass calculation. Formalin was added at the rate of 0.5% for bacterial inactivation.

Extraction of LPS

Bacterial LPS was extracted as described in OIE 2014. Briefly, dense culture was centrifuged at 6000 rpm to obtain the bacterial pellet. The pellet was re-suspended in 10 mL normal saline and was kept in water bath at 56 °C for 30 min for the removal of LPS from bacterial cell wall. After incubation, the culture was centrifuged at 6000 rpm for 10-15 min to obtain LPS in supernatant.

Extraction of OMP

Pasteurella multocida OMP antigen was prepared following the method of Thomson *et al.* (1990). In this method, disruption of whole culture was done in a French press at 20,000b/in 1 mM EDTA and 20 mM Tris HCl, adjusting the pH at 7.8. After centrifugation at 10,000 ×*g* for 20 min, 1% W/V solution of Sarkosyl (N-lauroylsarcosine) was added to the supernatant, and the mixture was incubated at 48 °C temperature overnight. After centrifugation, the pellet was re-suspended in distilled water and was stored at −20 °C. Later protein concentration was determined by a modified Lowry method.

Vaccine production

Three types of vaccines were prepared:

i. Whole culture vaccine (Containing both LPS + OMP)
ii. Vaccine with bacterial proteins (containing OMP)
iii. Vaccine contains only Bacterial LPS

Antibody titration

The vaccine was injected in bovine calves subcutaneously (S/C). Serum samples were collected at 0, 14, 28, 35 and 48 days' post vaccination. Antibody titer was estimated by complement fixation test.

RESULTS AND DISCUSSION

On blood agar, characteristic colonies (smooth, translucent and glistening greyish colonies, 1 mm in diameter were observed after an incubation period of 24 h whereas no colonies grew on McConkey agar evidencing as *P. multocida* since bile extract that contains in MacConkey agar inhibits *P. multocida.* The yielded colonies appeared to as gram negative being stained bipolar (OIE 2014) and were biochemically were positive for catalase, indiole, sucrose, glucose, lactose and negative for oxidase and maltose confirming the identity of *P. multocida*.

Animal model test for confirming the LPS-capsule were tested in two healthy rabbits, kept at clean and safe rabbit house of Quality Operation Laboratory, University of Veterinary and Animal Sciences, Lahore Pakistan. Each rabbit was injected with 1 mL of suspension of cultured *P. multocida* and examined for 24-48 h. During post-mortem (when the infected rabbits succumbed after) when blood form its heart was taken out and streaked onto blood agar and MacConkey agar plates, typical colonies of *P. multocida* grew on blood agar in contrast to none of yielded growth on MacConkey agar confirmed colonies of *P. multocida* which were attested on Gram staining showing Gram- negative coccobacilli.

The PCR was carried out by using type B2 specific primers as follows; the amplified product was run on

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Electrophoresis chamber along with DNA ladder. The result showed that there is an amplified product of 620 bp showing the confirmation of *P. multocida* type-B1.

The aerated culture produced a dry mass of 17 mg/mL when calculated by filter method. From this mass following vaccine were prepared;

1. Whole culture vaccine (3 mg/dose, 2.2 mg/dose, 1.5 mg/dose).
2. Purified vaccine (3 mg/dose, 2.2 mg/dose, 1.5 mg/dose).
3. LPS vaccine (LPS extracted from 3 mg/mL, 2.2 mg/mL, 1.5 mg/mL)

Each vaccine was titrated for antibodies against two both LPS and OMP antigen of *P. multocida.*

The antibody titer showed that there is no significant difference (P=0.23) between the individual antibody titer against OMP and LPS (titer checked against both OMP and LPS antigen) in animals vaccinated with whole culture vaccine as shown in Tables 1, 2 and 3.

When the whole culture vaccine (containing OMP and LPS both) was compared with purified vaccine (containing bacterial proteins like OMP only), it was revealed that titer of both anti-OMP (antibodies against OMP) and anti-LPS (antibody against LPS) in whole culture vaccine is significantly high (P=0.04) than anti-OMP (antibodies against OMP) produced by purified vaccine as shown in Tables 1, 6 and 7.

In the same manner, when the whole culture vaccine (containing OMP and LPS both) was compared with LPS vaccine (containing bacterial LPS only), it was revealed that titer of both anti-OMP (antibodies against OMP) and anti-LPS (antibody against LPS) in whole culture vaccine is significantly high (P=0.005) than anti-LPS (antibodies against LPS) produced by LPS vaccine as shown in Tables 1, 4 and 5. These results clearly conclude that both OMP and LPS synergize each other to produce significantly better immune response than the respective purified (without LPS) and LPS vaccine (without OMP).

The capsule (LPS) of the *P. multocida* serogroups A, B, D and F consists of hyaluronic acid, a polymeric form of D-glucoronic acid and N-acetyl-D-glucosamine. Capsular antigen of serogroup B was made up of mannose, fructoseamine and glucose (Wikie *et al.*, 2012). The molecular weight of the LPS was 90 kDA. Outer membrane proteins in all Gram-negative bacteria were extending from one end into the extracellular environment to the other end into the periplasmic space. It has been found that outer membrane protein constitutes 2% to 3% of the genome of Gram-negative bacteria. Outer membrane proteins were as potential targets for vaccines as well as antimicrobial drugs. The structure of the OMP family consists of β-sheet which are anti-parallel and form a β-barrel structure. The OMPs has a number of functions including to act as multimeric porins, enzyme and recognition proteins (Timothy *et al.*, 2007).

In the present study Whole Culture Vaccine containing LPS and OMP and the vaccine containing only LPS injected into the calves and it was observed that WC vaccine produced peak titer at 35 days (64 for 3.0 mg; 53.3 for 2.2 mg and 26.6 for 1.5 mg) and then declined at 48 day (53.3 for 3.0 mg; 32 for 2.2 mg and 16 for 1.5 mg). As far as LPS vaccine was concerned when it was checked for the Ab against LPS by CFT using LPS as an Ag, the result showed that peak titer was produced at 48 days (35 for 3.0 mg; 64 for 2.2 mg and 32 for 1.5 mg).

Our results indicate that LPS remains an effective immunogen being the most important part that confers immunity when used in WC-vaccines along with bacterial protein. However purified LPS whenever used as a vaccine it conferred partial immunity (Adler *et al.*, 1996; Shivachandra *et al.*, 2014). Purified LPS of *P. multocida* was found to be protective at lower doses when induced experimentally in mice (Muniandy *et al.*, 1998).

It was also revealed that in the cell wall of gram negative bacteria LPS is an important component playing its important role in the immunity. Toll like receptor 4 recognized extracellular LPS and cause the stimulation of cytokine transcription in innate immunity. This showed that LPS is responsible for the activation of immunity (Yang *et al.*, 2015). So in the absence of LPS as in purified vaccine if high antibody titer was obtained then it means another component was present in the bacterial body that also has role its role in the production of immunity. Many studies revealed that OMP (outer membrane protein) was important for protection along with LPS. The role of LPS in immune response was investigated by Alder (1996) using monoclonal antibodies (MAbs). He found that MAbs against LPS only opsonized *P. multocida* but did not produce any significant bactericidal effect. Therefore, it is concluded that LPS played a partial role in immune response against PM infection (Adler *et al.*, 1996).

In the present project, whole culture vaccine (LPS and OMP) and the purified vaccine (OMP) injected into the calves. It was observed that WC vaccine produced peak anti-OMP titer at 35 days (170.6 for 3.0 mg/dose,128 for 2.2 mg dose and 106.6 for 1.5 mg dose) and titer was then declined at 48 days (128 for 3.0 mg/dose ,107 for 2.2 mg/dose and 53.3 for 1.5 mg/dose).

Table 1: Showing the statistical analysis of vaccines.

Sr. #	Vaccines compared	Antibody titer compared (CFT)	Sig. Value
1	Whole culture X Whole culture	Anti-omp (AG-OMP) X anti-LPS (Ag-LPS)	0.23 (NS)
2	Whole culture X Purified Vaccine	Anti-omp (AG-OMP) X anti-OMP (Ag-OMP)	0.04 (S)
3	Whole culture X LPS Vaccine	Anti-LPS (Ag-LPS) X anti-LPS (Ag-LPS)	0.005 (S)

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Table 2: Immune response of WC vaccine for anti-OMP against OMP as antigen

Sr. #	Vaccine	Antibody titer					
	Whole culture vaccine	0 day	7 day	14 day	28 day	35 day	48 day
		GMT	GMT±SD	GMT±SD	GMT±SD	GMT±SD	GMT±SD
1	3 mg/dose	0	16±0	21.3±9.2	170.6±73.9	170.6±73.9	128±0
2	2.2 mg/dose	0	10.6±4.6	16±0	128±0	170.6±73.9	106.6±36.9
3	1.5 mg/dose	0	8±0	10.6±4.6	106.6±36.9	106.6±36.9	53.31±18.4

Table 3: Immune response of WC vaccine for anti-LPS against LPS as antigen

Sr. #	Vaccine	Antibody titer					
	Whole culture vaccine	0 day	7 day	14 day	28 day	35 day	48 day
		GMT	GMT±SD	GMT±SD	GMT±SD	GMT±SD	GMT±SD
1	3 mg/dose	0	12±4.07	16±0	128±0	64±0	85.3±37
2	2.2 mg/dose	0	8±0	8±10.6	85.3±37	128±0	64±0
3	1.5 mg/dose	0	5.3±2.31	8±0	64±0	85.3±37	32±0

Table 4: Immune response of LPS vaccine for anti-OMP against OMP as antigen

Sr. #	Vaccine	Antibody titer					
	LPS vaccine	0 day	7 day	14 day	28 day	35 day	48 day
		GMT	GMT±SD	GMT±SD	GMT±SD	GMT±SD	GMT±SD
1	3 mg/dose	0	4±0	5.3±2.3	16±0	13.3±4.6	8±0
2	2.2 mg/dose	0	2±2	2.6±2.3	8±0	8±0	5.3±2.3
3	1.5 mg/dose	0	2±0	2±0	4±0	5.3±2.3	4±0

Table 5: Immune response of LPS vaccine for anti-LPS against LPS as antigen

Sr. #	Vaccine	Antibody titer					
	LPS vaccine	0 day	7 day	14 day	28 day	35 day	48 day
		GMT	GMT±SD	GMT±SD	GMT±SD	GMT±SD	GMT±SD
1	3 mg/dose	0	5.3±2.31	5.3±2.31	53.3±18.4	64±0	42.6±18.4
2	2.2 mg/dose	0	5.3±2.31	4±0	32±0	32±0	26.6±9.2
3	1.5 mg/dose	0	2.6±1.15	2±2	16±0	21.34±9.2	16±0

Table 6: Immune response of purified vaccine for anti-LPS against LPS as antigen

Sr. #	Vaccine	Antibody titer					
	Purified vaccine	0 day	7 day	14 day	28 day	35 day	48 day
		GMT	GMT±SD	GMT±SD	GMT±SD	GMT±SD	GMT±SD
1	3 mg/dose	0	4±0	3.3±1.15	13.3±4.6	16±0	10.6±4.6
2	2.2 mg/dose	0	2±0	2±2	10.6±4.6	13.3±4.6	8±0
3	1.5 mg/dose	0	1.3±1.15	2±2	6.6±2.3	6.6±2.3	5.3±2.3

Table 7: Immune response of purified vaccine for anti-OMP against OMP as antigen

Sr. #	Vaccine	Antibody titer					
	Purified vaccine	0 day	7 day	14 day	28 day	35 day	48 day
		GMT	GMT±SD	GMT±SD	GMT±SD	GMT±SD	GMT±SD
1	3 mg/dose	0	5.3±2.3	9.3±6.1	64±0	64±0	32±0
2	2.2 mg/dose	0	6.67±2.3	6.67±2.3	64±0	42.6±9.2	26.6±18.4
3	1.5 mg/dose	0	4±0	5.3±2.3	32±0	26.6±18.4	21.3±9.2

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In purified vaccine (OMP only), peak anti-OMP titer was produced at 35 days (85.3 for 3.0 mg/dose; 64 for 2.2 mg/dose and 42.6 for 1.5 mg/dose) and then declined at 48 days (42.6 for 3.0 mg/dose; 53.3 for 2.2 mg/dose and 53.3 for 1.5 mg/dose). The above results concluded that OMP was an effective immunogen as it had produced significantly better antibody titer against bacterial OMP in combination with LPS (whole culture vaccine) as compared to purified vaccine (OMP only).

Natarajan *et al.* (1985) found that outer membrane protein plays important role in immunity also in other Gram negative bacteria such as *S. typhimurium*. Another study found that iron regulated outer membrane proteins IROMPs of *P. multocida* A:3 232 strain (Pm232, which was a bovine isolate) were vital immunogens in bovines (Prado *et al.*, 2005).

The role of bacterial outer membrane lipoproteins in inducing protective immunity against many infectious diseases could easily be understood by taking in concern the VacJ region is conserved region and widely distributed OMP of *P. multocida* strains. In this study, the gene encoding for VacJ region (OMP) of *P. multocida* serogroup B:2 (strain P52) was expressed and was cloned in vector *E. coli* as a fusion protein. This recombinant purified protein was used to immunize mice without adjuvant (alum) in first experiment and with adjuvant (alum) in the second experiment. No protection was noticed in mice immunized without adjuvant. But more than 50% protection was observed in the mice immunized along with adjuvant. This study indicated that a recombinant VacJ lipoprotein with suitable adjuvants could potentially play a role in producing immunity against pasteurellosis in livestocks (Shivachandra *et al.*, 2014). Kumar also indicated that the use of Omp87 protein along with suitable adjuvant was responsible for producing immunity against HS and pasteurellosis in livestock (Kumar *et al.*, 2013). Beside the role of LPS and OMP in immunity, as found by Ruffolo that *P. multocida* consists of capsule and outer membrane protein and these components have been implicated as immunogens (Ruffolo *et al.*, 1998), they also played important role in pathogenicity. LPS isolated from serotype B:2 strains of *P. multocida* was found to be endotoxic in nature and when given intravenously this LPS reproduced clinical signs of heamorrhagic septiceimia in bovines (Horadagoda *et al.*, 2002). Outer membrane proteins played its role in pathogenicity by acting as a selective barrier that inhibits the entry of many molecules that were toxic to the bacterial cell. This was a very important property for bacterial survival in various conditions. The proteins that are embedded in the outer membrane also fulfilled a number of functions that were necessary for the bacterial survival such as transport of mol. inside and outside of the cell, nutrient uptake and interaction with environmental factors (Tamas *et al.*, 2010). But the LPS which formed capsule over the bacterial cell contributed more towards pathogenicity. The purpose of this capsule was to increases the resistance of a *P. multocida* bacterial cell to phagocytosis caused by and in this way enhanced its virulence as compared to other non-capsulated strains (Borrathybay *et al.*, 2003). Zafar found that *P. multocida* consists of LPS which stimulated the production of Interlukin1, which was associated with change in temperature and weakness of the infected animal (Zafar *et al.*, 2010).

Moreover, it was found that the antibody titer of OMP in WC vaccine was significantly higher than the titer of Ab against OMP in the purified vaccine. These results indicated that OMP in combination with LPS produced better result than alone OMP as clearly shown in this study. Ashraf *et al.* (2014) compare the immunological behavior of LPS and yeast proteins alone and in combination as in the present project. The comparison proved that LPS itself is immunogenic but its immunogenic property increases many times when given along with appropriate dose of yeast used as protein source. In another study Schmerr compared the immunogenicity of LPS taken from *P. multocida* strain with the immunogenicity of whole bacterial cell by inoculated into CF1 mice. The mice which were inoculated with LPS from *P. multocida* (strain P-1234) found to be survived 20% only after giving infection challenge after four weeks. Whereas the mice CH3/HeJ or CF1 that were inoculated with formalized bacterial strain P-1234 from bovine source found to be survived 90% by giving infection challenge after four weeks of inoculation (Schmerr and Rebers, 1979). LPS being non protein in nature do not to stimulate the cellular immunity and hens fails to produce long term immune response. But in combination with bacterial OMP, LPS successfully stimulate both the humoral and cellular immune response required for long term immune response.

CONCLUSION

It was concluded from the above study that lipopolysaccharides (LPS) and outer membrane proteins (OMP) are important immunogenic factors of *P. multocida* which boost up the immunogenic behavior of each other when used in combination than in alone.

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