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IMMUNOGENIC ACTIVITY OF LIPOPOLYSACCHARIDES FROM *PASTEURELLA MULTOCIDA* IN RABBITS

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ABSTRACT

Lipopolysaccharides (LPS) were extracted from the whole cell of the causative organism (*Pasteurella multocida*) and quantified by using the semi-carbazide method. This quantified antigen was then inoculated subcutaneously into rabbits to stimulate antibody production. The maximum antibody titre was determined by applying indirect ELISA, and was found to have been achieved at one week post inoculation in all groups. It was concluded that with an increase in the dose of LPS antigen, the antibody titre increased and the combination of LPS of *P. multocida* with yeast produced more immunity when compared to LPS alone in rabbits.

Key words: Haemorrhagic septicaemia, Immunity, LPS, ELISA *Pasteurella multocida*.

INTRODUCTION

Pasteurella multocida is the pathogenic organism of one of the most common economically significant animal diseases occurring in both developed and developing countries while in the case of human infections associated with this bacterium they are not frequent (Dziva *et al.*, 2008). The pathogenic organism of haemorrhagic septicaemia (HS), a lethal disease of cattle and buffaloes, is *P. multocida* serotype B:2 (Ataei *et al*., 2009). *P. multocida* is a typical gram-negative bacterium belonging to the gamma -3 subdivision of the purple bacteria phylum (Dewhirst *et al.*, 1992). This microorganism is pathogenic for a wide variety of mammals and birds (Marina *et al.*, 2004). It is associated with atrophic rhinitis of Swine, fowl cholera of poultry and HS of cattle and buffaloes (Pedersen and Bradford, 1981). The most common hosts of HS infection and disease are cattle and buffalo but some other animals such as goats, pigs, deer, sheep and camel are also susceptible to it (Blackall *et al*., 2000). The disease is particularly endemic in Asia and Africa but outbreaks have also occurred in Europe and North America (Rimler, 2000). In South East Asia it is the most important economic disease of livestock that has caused huge economic losses in Africa and India (Verma *et al*., 1998). Pakistan has 31.8 million cattle population and a population of 29.0 million buffalo heads (Anonymous, 2008).

P. multocida causes HS, an economically devastating disease in buffalo and cattle (Sarwer *et al*., 2013). It has five types of capsular serotypes *i.e.* type A, B, D, E and F. Most cases are acute or peracute, resulting in death within 8–24 hours of onset. Because the course is so short, clinical signs may easily be overlooked. HS is caused by *P. multocida* type B:2, B:2,5 and B:5 in Asian countries and type E:2 in African countries (Yap *et al*., 2013). The outer membrane of gram-negative bacteria constitute largely of Lipopolysaccharide (LPS). In the case of gram-negative infection, LPS is involved in the activation of the cellular components and humoral immunity of the host defence system. To fight against gram-negative infection, it is vital that host defences are initiated, although serious and life-threatening symptoms of septic shock can also occur due to uncontrolled stimulation (Botto *et al*., 1997). It has been observed that LPS from *P. multocida* assists in adhesion to neutrophils and transmigration through endothelial cells (Galdiero *et al*., 2000).

LPS isolated from serotype B: 2 strains was shown to be endotoxic, and intravenously administrated LPS could reproduce clinical signs of HS in buffaloes (Horadagoda *et al*., 2002). The aim of the study is to identify and characterize the locally isolated *P. multocida* strains; to extract and quantify LPS from these strains and to evaluate its immunogenicity in rabbits by using Enzyme Linked Immunosorbent Assay (ELISA).

MATERIALS AND METHODS

Isolation of LPS: LPS was extracted using a procedure described by Penn and Nagy (1976) and Shah and Shah (1998). Quantification of LPS was done by the semi-carbazide method of Batley *et al*. (1985) against 3-Deoxy – D –Octulosonic Acid (kdo).

Preparation of antigen:

Table 1. LPS antigen inoculation prepared in rabbits

Group	LPS (µl)	*S. cerevisiae* (µl)	Total dose (µl)
A	30	10	40
B	20	20	40
C	10	30	40
D	10	NIL	10
E	Inactivated *P. multocida* having 1 X 10^9 CFU per ml		1000

For the production of hyper-immune sera thirteen rabbits were inoculated by the subcutaneous route with the suspension prepared as above. The inoculation schedule took place at 15 days intervals with a 50% dose being injected into each experimental animal on each occasion. In this way the total dose was given in two attempts. 14 days after the last inoculation, blood samples were collected from the ear vein of each rabbit in syringes and then blood sera was separated from the blood and stored at 4ºC till use (Wijeiwadena, 1992).

Titre of antibodies against LPS present in rabbit sera were measured using the ELISA technique as described by Horadagoda *et al*., (1993) and Maqbool *et al*. (2002).

Analysis of variance (ANOVA) was used with CRD and DMR (Duncan's multiple range) tests to compare the antibody titre in groups and groups in days (Steel *et al.,* 1997).

RESULTS AND DISCUSSION

In the first sample, the OD values of group A were at a maximum, indicating a high antibody titre that gradually decreased from group A to D. Average OD values for group A, B, C, D and E on 10th days were recorded as 0.528±0.030, 0.482±0.002, 0.475±0.001, 0.438±0.006, 0.323, respectively (Table 3). The OD values of group A were maximum, indicating a high antibody titer that gradually decreased from group A to D. Group D had minimum OD values, and hence minimum antibody titre was obtained in this group. The mean OD values, SD and CV values are presented in Table 3. The OD value of group E was minimum (0.323) as compared to all above samples. Average OD values for group A, B, C, D and E on 20th days were recorded as 0.442±0.005, 0.399±0.001, 0.388±0.001, 0.354±0.001, 0.310± N.A, 0.054± N.A, respectively (Table 3). The maximum at 20th day (0.442±0.005) and minimum (0.054± N.A) OD values were recorded in group A and E, respectively. The mean OD values for group A, B, C, D and E on 30th days were recorded as 0.294±0.009, 0.239±0.080, 0.213±0.005, 0.205±0.003, 0.190± N.A, 0.066± N.A, respectively (Table 3). The maximum at 30th day (0.294±0.009) and minimum (0.190± N.A) OD values were recorded in-group A and E, respectively. The mean maximum and minimum CV values on 10th, 20th and 30th day were recorded as 5.814 and 0.121; 1.304 and 0.282 and 2.009 and 0.270, respectively (Table 3; Figure 1).

The percentage ß$_o$ of groups at different sampling points were compared, and it was observed that the percentage ß$_o$ (indicating immunity) in the first sample was maximum but that this gradually decreased from the first to the third sampling. The comparison of the percentage of maximal absorbance at the first, second and third sampling points is given in Table 4. The maximum and minimum percentage ßo of groups at 10th, 20th and 30th day was recorded as 212.9 and 110.2 (10th day), 156.7 and 109.9 (20th day) and 149.2 and 104.0 (30th day) in group A and E, respectively. The analysis of variance shows highly significant ($P < 0.01$) differences in various treated groups for antibody titre at different time periods (Table 5). The %ß in the groups at different weeks was compared, and it was observed that the % ß$_o$ (indicating immunity) was at a maximum at the first sampling point but then was gradually decreased from to the third sampling point. The analysis of variance shows that groups and days each had a highly significant ($P < 0.01$) effect and their interaction, that is, groups into days was also highly significant ($P = 0.01$) (Table 5 and 6).

Duncan's Multiple Range Test indicated that all thirty days were significantly different from one another i.e. the value of OD significantly decreased with an increase in time. Similarly, Duncan's Multiple Range Test for groups showed that all the groups had a significant difference from one another ($P < 0.01$) while the control had a significantly lower value of OD from all other groups. The ANOVA test table shows that groups and weeks have highly significant ($P < 0.01$) effect and their interaction, that is, group into week is also highly significant ($P < 0.01$).

The presence of the capsule mainly increases the resistance of a *P. multocida* cell to phagocytosis and enhances its virulence potency when compared with non-capsulated strains (Maheswaran and Thies, 1979; Borrathybay *et al*., 2003). The fact that *P. multocida* is a commonly encapsulated organism is supported by this study, too. On the other hand, the fimbriae which can play important role in colonisation of host tissue (Al Haddawi *et al*., 2000) have not been described so frequently in *P. multocida* (Grund *et al*., 1990). The LPS of *P. multocida* has a profound effect on the mammalian immune system and is of great significance in the pathophysiology of many disease processes, often forming major components of the outer surface of Gram-negative bacteria with immunomodulatory properties (Erridge *et al*., (2002). Accordingly, LPS was extracted using the Proteinase K digestion method (Penn and Nagy,

1976; Shah and Shah, 1998). The quantification tests were performed against KDO, which provided 7.2µg to 19.9µg of LPS in 1 ml of LPS sample in each test tube. The quantified LPS were inoculated as an antigen to check immunity against HS in rabbits, (Carrof and De Alwis, 2003). Lipopolysaccharide-protein complex (endotoxin) is an important constituent of the outer membrane of a *P. multocida* cell. Besides its toxic role in pathogenesis of the disease (Kunkle and Rimler, 1998; Horadagoda *et al*., 2002), endotoxin is also immunogenic at low concentrations (Muniandy *et al*., 1998). LPS molecules are not toxic when they are bound to the bacterial outer membrane but after release from the bacterial wall they are exposed to immune cells and evoke an inflammatory response (Van Amersfoort *et al*., 2003). LPS is released from the bacterial cells when they multiply but also when bacteria are damaged or lysed by various factors present in plasma such as complement and other bactericidal proteins (Smedsrod *et al*., 1994).

The sugar composition of LPS isolated from *P. multocida* was analysed by using KDO as a standard sugar (St. Michael *et al*., 2005). When the immunity developed by pure LPS and LPS along with yeast (with the same quantity of LPS) was compared, it was observed that while LPS is immunogenic, if LPS antigen is given with an appropriate dose of yeast then it can produce more immunity, with immunological results supporting this observation. So, if LPS of *P. multocida* is used in combination with yeast, then this will be more immunogenic as protein-based vaccines which rely on natural sources of antigens are more effective and well-tolerated as discussed by Ryu and Kim (2000).

It is concluded that the antibody titre against LPS of *P. multocida* proved that LPS is an important immunogenic part of bacterial cell. Similar findings have been reported by Reatz and Whitefield, (2002) who described that the LPS stimulates the innate immune response, whereby the inflammatory response plays a critical role in helping to clear bacteria and prevent infection. So, the LPS of *P. multocida* can be considered for use as an antigen in the development of a vaccine against HS in cattle and buffaloes, subject to further evaluation of its immunogenicity in these ruminants.

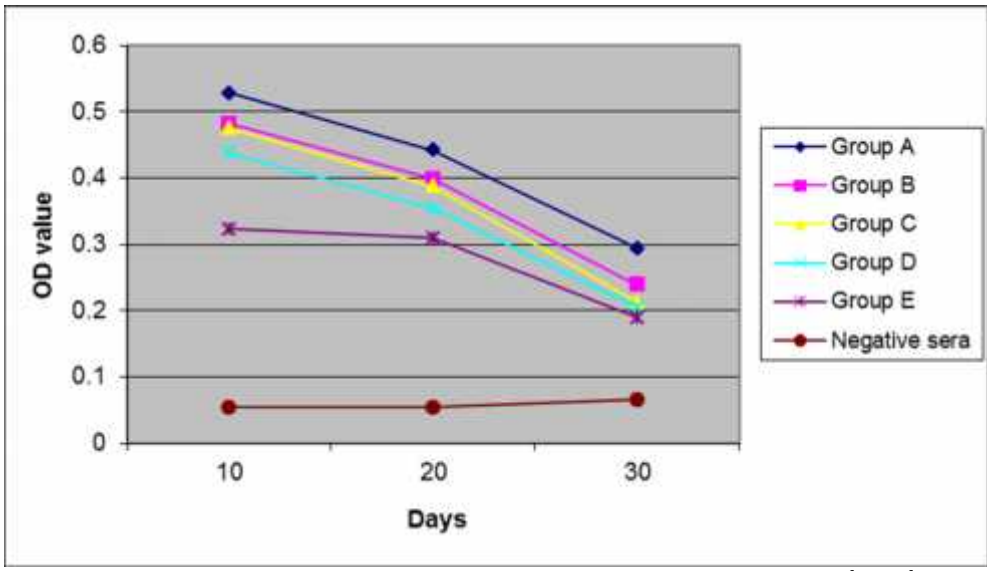

Fig. 1. Comparison of ELISA antibody titers in different groups of rabbits at the 10th, 20th and 30th days post inoculation.

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

No. of Sample	Absorbance at 548 (nm)	Amount of LPS (µg/ml)
1	0.270	7.2
5	0.367	9.78
9	0.380	10.12
17	0.335	8.92
31	0.451	12.02
37	0.269	7.16

Table 3. Average OD values, SD and CV of 10th, 20th and 30th day post inoculation in rabbits

Group	OD ± SD			CV		
	10th day	20th day	30th day	10th day	20th day	30th day
A	0.528±0.030	0.442±0.005	0.294±0.009	5.814	1.304	2.009
B	0.482±0.002	0.399±0.001	0.239±0.080	0.519	0.382	1.467
C	0.475±0.001	0.388±0.001	0.213±0.005	0.121	0.446	0.270
D	0.438±0.006	0.354±0.001	0.205±0.003	1.388	0.282	1.840
E	0.323± N.A	0.310± N.A	0.190± N.A	N.A	N.A	N.A.
-ve serum	0.054± N.A	0.054± N.A	0.066± N.A	N.A	N.A	N.A

Table 4. Comparison of antibody titre of different groups at different time periods.

Group	% β_0 of 10th day	% β_0 of 20th day	% β_0 of 30th day
A	212.9	156.7	149.2
B	194.3	141.4	141.3
C	191.5	137.5	108.1
D	176.6	125.5	105.2
E	110.2	109.9	104.0

Table 5. Analysis of variance for comparison among the groups and days

Source	Degree of Freedom (df.)	Sum of Squares (S.S)	Mean Square (M.S)	F-Value	Prob.
Days	2	0.381	0.191	2384.9443**	0.00
Groups	4	0.106	0.026	330.4310**	0.00
Groups × Days	8	0.016	0.002	25.2284**	0.01
Error	30	0.002	0.000		
Total	44	0.506			

** = Highly significant

Table 6. Analysis of variance for comparison among groups and days with their rank

Source	Control	2	3	4	5	Mean
Groups	0.323 f	0.528 a	0.484 b	0.475 b	0.438 c	6.743 A
Days	0.310 f	0.443 c	0.400 d	0.388 d	0.354 e	5.684 B
Groups × days	0.191 j	0.294g	0.239 h	0.214 i	0.206 i	3.431 C
Total	2.472 E	3.794 A	3.370 B	3.229 C	2.993 D	

Conclusion: It was concluded that with an increase in the dose of LPS antigen, the antibody titre increased and the combination of LPS of *P. multocida* with yeast produced more immunity when compared to LPS alone in rabbits.

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