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Immunological studies on a modified water-in-oil-in-water (w/o/w) haemmorhagic septicemia vaccine incorporated with *Pasteurella multocida* A: 1 in the external aqueous phase

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Haemmorhagic septicemia (HS) continues to be an important problem for dairy industry in various parts of the world. Though, Asian serotype B: 2 and African serotype E: 2 are the only organisms considered as the causative agents of classical Asian and African HS in cattle and buffaloes (Dawkins *et al*. 1990, Kumar *et al*. 1996) have reported isolation of A: 1, A: 3, F: 3 and F: 3, 4 from HS and HS like diseases in India.

Since association of these serotypes with classical fatal HS still needs to be confirmed, at present it is expected that these commensal serotypes may play a role in creating a complex situation of pasteurellosis where HS is specifically caused by B: 2 which in certain endemic areas may be complicated by commensals serotypes of A and F.

This finding necessitates improvement of conventional whole cell (WC) B: 2 vaccines against HS.

Since parentral immunization with inactivated WC results in immunity that is specific for homologous but not heterologous serotypes of *Pasteurella multocida* (Adler *et al*. 1996), continued efforts are needed to develop methodologies capable of producing vaccines that induce high level, long-lasting and cross-protective immune response against different *P.multocida* serotypes. To achieve the objectives of cross protection against multiple serotypes, it is essential either to identify and use common protective antigen/antigens (Rimler 2001), or induce the organism to synthesize conserved antigens like iron- regulated OMPs (IROMPs) (Adler *et al*. 1996) or to incorporate representatives of serotypes in multivalent formulations.In the present study, possibility of bivalent vaccine incorporating both *P. multocida* A:1 and B:2 serotypes in multiple emulsion formulation were evaluated.

Bacterial strains: Pasteurella multocida vaccine strains (B: 2) P_{52} and bovine A: 1 were procured respectively from Division of Biological Standardization and Bacteriology and Mycology, Indian Veterinary Research Institute, Izatnagar, Bareilly and were revived on brain heart infusion and blood agar. Dense growth of each strain was obtained by growing it in fermenter using the media and parameters recommended (Mishra 1991). The harvest of each strain was tested for purity and identity, formalized (0.5% formalin, v/v) and stored at 4°C until use.

Adjustment of biomass: The biomass of P_{52} and A:1 culture was determined as per Mishra (1991). Initially, the dry mass of each serotype was suitably diluted with 0.5% formal saline so that the bivalent vaccine P_{52} and A: 1 contained 1 mg WC antigen per dose (2 ml) in the internal and external phases, respectively. The antigenic mass of monovalent P_{52} vaccine was similarly adjusted to 2 mg/dose before being emulsified in the internal phase.

Preparation of vaccine: The monovalent P_{52} vaccine was prepared according to Verma and Jaiswal (1996). The product contained 1 mg/ml of whole P_{52} cells in the internal aqueous phase. For preparation of bivalent vaccine, a suitable quantity of w/o emulsion containing 0.5 mg/ml P_{52} cells in the internal aqueous phase was prepared. This was further mixed with equal volume of 2% Tween 80(Hi Media) formal saline containing alum precipitated whole cells of A: 1 as per Verma and Jaiswal (1996). The biomass of A: 1 WC was adjusted in such a way that the final product contained 0.5 mg/ml of

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the antigen in the external aqueous phase. Sterility and safety of both the products was tested according to the method described in the Drugs and Cosmetic Act (2004). Stability of the products was checked for 14 days according to the method of Rawat and Jaiswal (2004).

Immunization of rabbits: Healthy adult New Zealand White rabbits (32) of either sex, weighing not less than 1.5 Kg were divided into 2 groups of 16 animals. Each animal was checked for absence of natural anti *P. multocida* P_{52} and A: 1 antibody by slide agglutination test (Bain *et al*.1982). Each animal of group I was immunized once with 2 ml bivalent vaccine through deep I/M route. The animals of group II received a single I/M injection of monovalent P_{52} vaccine. Eight rabbits were kept as unimmunized controls in each group. Animals from each group were bled on 0,7,14,21,30,60 and 90th DPV. Sera samples were collected and stored at –20°C until used.

Humoral immune response: Single dilution ELISA technique (Briggs and Skeeles 1984) was followed for detecting antibody titers against sonicated antigens of P_{52} and A: 1.

Direct challenge test: Eight rabbits from each vaccinated group were further divided into two groups containing four rabbits in each group. On 21st DPV rabbits of first group were challenged with 100 LD_{50} of *P. multocida* P_{52}, where as second group were challenged with 100 LD_{50} A: 1. Similarly the remaining eight rabbits from each group were challenged on 90th DPV. Two uninoculated control rabbits were also challenged with *P. multocida B: 2* (P_{52}) and A:1.

Passive mouse protection test (PMPT): PMPT was carried out as per Rimler (1996). Mice (32) were divided in 2 groups of 16 mice each. First group were used to check ability of 21st DPV sera collected from vaccinated rabbits to passively protect mice against direct challenge with homologous strain of *P. multocida* B:2 and A:1 where as second group were used for 90th DPV sera. 0.5 ml of pooled, filter-sterilized sera of 21st and 90th DPV from each vaccinated group was inoculated in eight mice. Four mice were then challenge infected with *P. multocida* B:2 and another four mice were challenged with *P. multocida* A:1 and were kept under observation for 7 days. Post mortem was conducted on dead mice to confirm the cause of death by isolating the organism from heart blood.

Statistical analysis: Data obtained in the study are expressed as mean± standard deviation (SD) and were analyzed by Duncan's and Turkey's post hoc test using SPSS version 11.0 and was used to detect significant variance between different vaccine groups.

P. multocida serotype B:2 and A:1 were grown on BHI blood agar. Pure gram-negative, non-motile oxidase and indole positive non-hemolytic colonies which failed to grow on Mac conkey agar were used for preparation of vaccine. Dense cultures of both serotypes were produced employing recommended conditions of pH, aerations, growth temperature and agitation (Misrha 1991). The dry mass of the growth was estimated to be 1.8 mg/ml for A: 1 and 1.6 mg/ml for $P_{52.}$ The cultures obtained from fermenter were used for production of vaccine and sonicated antigen for ELISA.

Table 1. Protection % of immunized rabbits in direct challenged test against P_{52} and A: 1

Vaccine	Days of challenge	No. survived/No.challenges	
		P_{52}	A:1
Bivalent P_{52}	21 DPV	4/4 (100%)	4/4 (100%)
	90 DPV	4/4(100%)	2/4(50%)
Monovalent P_{52}	21 DPV	4/4 (100%)	1/4 (25%)
	90 DPV	2/3(66.6%)	0/2(0%)
Control	21 DPV	0/2(0%)	0/2(0%)
	90 DPV	0/2(0%)	0/2(0%)

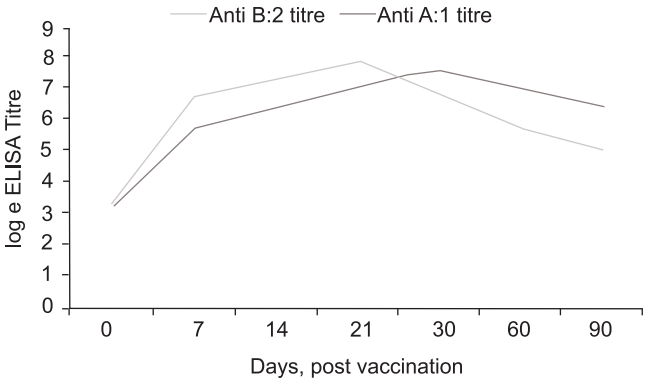

Fig 1. Anti *P.multocida* B:2 and A:1 ELISA titre of bivalent P52 vaccine

In the present study, rabbits were immunized with bivalent P_{52} (B: 2) vaccine containing alum precipitated A: 1 in the external phase showed an early rise of ELISA antibody titers against A: 1 which peaked approximately around 21st DPV (log_e ET 7.79±0.06) and thereafter declined (Fig. 1). On contrary antibody titres against antigen in the internal phase P_{52} showed a slow rise which peaked around 30th DPV (7.46±0.14) and persisted to a significant level up to 90th DPV. On the other hand, monovalent P_{52} vaccine showed a classical immune response in which the ELISA antibody titres against P_{52} peaked around 30th DPV and persisted up to 90th DPV (Fig. 2). Similar pattern were observed in IHA test (Figs 3,4). From the observation the group vaccinated with monovalent vaccine, it may be concluded that whole cell P_{52} (B: 2) bacteria induces a partial cross protective response against bovine isolate of A: 1. Johnson *et al*. (1989) have also reported a cross reactivity among serotypes A, B, D and E of *P. multocida*. They have further reported that antiserum raised against A: 1 protects mice against B: 2.The reason attributed to it may be the common outer membrane protein (OMP) antigens of *P. multocida* including P_{52} (Johnson *et al*. 1991). However, Rawat and Jaiswal (2004) failed to detect

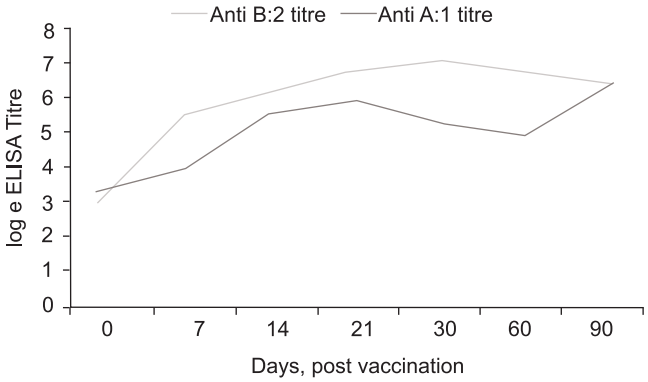

Fig 2. Anti *P.multocida* B:2& A:1 ELISA titers of monovalent P52 vaccine.

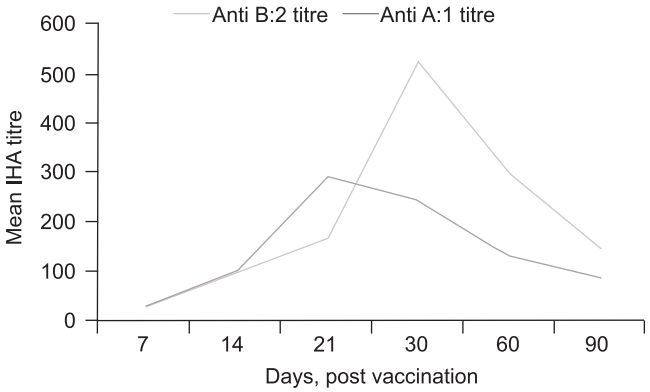

Fig 3. Anti *P. multocida* B:2 and A:1 mean IHA titer of bivalent P52 vaccine.

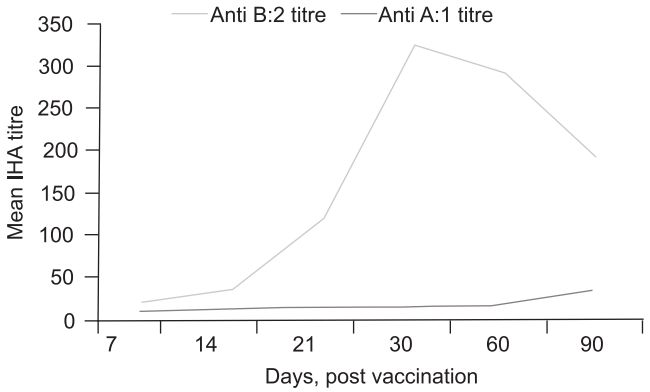

Fig 4. Anti *P. multocida* B:2 and A:1 mean IHA titer of Monovalent P52 vaccine.

cross protection against A: 3 and F: 3 serotypes in PMPT.

Direct challenge infection of rabbits immunized with bivalent vaccine showed 100% protection against both serotypes on 21st DPV. On 90th DPV, though 100% of the rabbits protected against P_{52} challenge, only 50% protection was observed against A: 1 challenge. Monovalent P_{52} vaccine on the other hand, not only showed 100% protection against P_{52} challenge but a partial cross protection (25%) was observed against A: 1 on 21st DPV. On 90th DPV, 66% protection against P_{52} only was observed with monovalent vaccine.

In passive mouse protection test (PMPT), the 21st DPV serum from bivalent vaccine group was found protective against both serotypes. However, on 90th DPV, the passive protection against A: 1 was significantly lower than that of P_{52}. On 21st day, serum from monovalent vaccine group showed a low degree of passive protection against A: 1, which did not persist up to 90th DPV (Table 2).

These observations indicated that antibody response against alum precipitated A: 1 cell in the external aqueous phase was quicker than the P_{52} antigens incorporated in the internal phase. A: 1 titre declined after the 21st DPV whereas the P_{52} titres persisted for 90th DPV. The observation is in conformity with Aucouturier *et al*. (2001) and Salt *et al*. (1998) that the antigen in the external aqueous phase behaves likes aqueous formulation and become immediately available to immune system. Therefore protective immune response against antigen in external aqueous phase is quicker than that of antigen in internal phase. Salt (1998) has demonstrated a protective immune response in bovine and swine against FMD only 4 days after vaccination with such formulations. Therefore, these formulations may prove to be of immense use when emergency vaccination in the areas of HS outbreak involving multiple serotypes of *P.multocida* is required. In the present study, the bivalent vaccine was better than the monovalent since the antibody levels, protection in direct challenge and PMPT were higher in the bivalent group. Excellent possibilities for further investigations on developing improved biological against HS and Pasteurellosis exist through exploiting the external water phase of w/o/w emulsions. Further the methodology may also find a wide application in the field, if alum adsorbed whole cell antigen of the same serotypesB:2(P_{52}) is incorporated in external aqueous phase. Such formulation is expected to elicit a biphasic response, which may persist for more than one year.

Table 2. Assessment of protection % against P_{52} and A: 1 in PMPT

Vaccine	Days of challenge	No. survived/No.challenges	
		P_{52}	A:1
Bivalent P 52	21 DPV	4/4 (100%)	3/4 (75%)
	90 DPV	2/4(50%)	1/4(25%)
Monovalent P 52	21 DPV	3/4 (75%)	2/4(50%)
	90 DPV	3/4(75%)	1/4 (25%)
Control	21 DPV	0/2(0%)	0/2(0%)
	90 DPV	0/2(0%)	0/2(0%)

SUMMARY

A modified double emulsion (w/o/w) vaccine against hemorrhagic septicemia (HS) was prepared by incorporating

a bovine isolate of *Pasteurella multocida* A: 1 in the external aqueous phase and B:2 in internal aqueous phase. Immune studies of the modified vaccine were compared with monovalent P_{52} double emulsion vaccine using rabbit as experimental animal. It was found that the modified double emulsion vaccine provided 100% protection against the challenge of both A: 1 and B: 2 serotypes up to 21st day post vaccination (DPV) but on 90th DPV protection against the antigen in external aqueous phase was only partial.

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