



Immune response and viscosity of haemorrhagic septicaemia oil adjuvant vaccine at different water-oil proportion

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ABSTRACT

The Office International des epizooties (OIE) and Food and Agriculture Organisation (FAO) recommends the formulation of oil adjuvant vaccine (OAV) against haemorrhagic septicaemia (HS) with water-in-oil ratio of 50: 50. In present study, 3 different OAV were formulated with water-in-oil ratios; 60: 40 (OAV60), 50: 50 (OAV50) and 40: 60 (OAV40). The immune response study was carried out in mouse model for 45 days. Viscosity of the 3 OAV formulations; OAV60, OAV50 and OAV40 were 426.77 centi poise (cP), 222.40 cP and 128.50 cP respectively. Thus, new formulated vaccine, OAV40, having 10 % less aqueous phase recorded 42% low viscosity compared to standard existing vaccine, OAV50. The immune response was quick in higher aqueous phase vaccine OAV60, which showed significant rise in anti *Pasteurella multocida* specific Log₁₀ ELISA titer on 14th days post-immunization (DPI) and peak titer on 21st DPI. But vaccine with higher oil phase, such as OAV50 and OAV40, had late but significantly higher and sustained antibody titer up to 45th DPI. All the 3 OAV formulations was protective as it produced 100% protection at 100 LD₅₀ and 60 to 80% protection at 1000 LD₅₀ and 10000 LD₅₀, after challenge on 45th DPI. These results indicated that higher oil proportion in emulsions was effective in reducing viscosity and inducing higher level of sustained antibody response without compromising protection.

Key words: Haemorrhagic septicaemia, Oil adjuvant vaccine, *Pasteurella multocida*, Viscosity

Haemorrhagic septicaemia (HS), one of the most important diseases of cattle and buffaloes, causes huge economic loss in India and accounts for 46 to 55% of all bovine deaths in past four decades (Dutta *et al.* 1990). At present, several vaccines are used for the control of HS and among these oil adjuvant vaccine (OAV) is considered as the most potent vaccine with 1 year protection (Dhanda *et al.* 1956, Muneer *et al.* 2005). However, high viscosity and poor syringeability of the OAV is considered major constraint for its wide application and sometimes also responsible for the swelling and necrosis at the site of injection (Verma and Jaiswal 1998, Tabatabaei *et al.* 2007). Efforts were made to

reduce the viscosity of OAV by formulation of multiple emulsion (Verma and Jaiswal 1997) and through replacing paraffin oil by Mercol 52 (Shah *et al.* 1997). But, still these vaccines did not find way for field application either due to poor immunogenicity or increased cost. Water to oil proportion in the OAV was the crucial factor in its viscosity, antigen release rate and immune response (Aucouturier *et al.* 2001, Jansen *et al.* 2005). Commercial OAV against HS is formulated by emulsifying 50% aqueous antigenic phase with 50% oil phase by applying lanolin as emulsifier (Bain *et al.* 1982, OIE 2008). Keeping all these in mind, the present study was planned to develop the OAV of low viscosity by formulating 3 different water and oil proportions, including the standard proportion prescribed by OIE. The immune response and challenge study of these vaccines were studied in mice model and compared with standard OAV.

MATERIALS AND METHODS

Mice: Swiss albino mice weighing 18 to 20 g were procured from Laboratory Animal Resource section, IVRI, Bareilly, Uttar Pradesh. They were kept for acclimatisation for 15 days before start of vaccination trial.

Growth of *Pasteurella multocida* and inactivation:

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Pasteurella multocida serotype B: 2, strain P52 was collected from the standardization division, IVRI, Izatnagar and was maintained on blood agar slants at 4°C during study. Bacterial biomass was produced by growing *P. multocida* on nutrient agar with yeast extract and caesamino acid at 37°C for 18 h. The biomass of the harvest was determined as per Mishra (1991). The harvested culture was washed thrice with 0.5% formal saline and resuspended in formal saline to match with Brown's opacity tube no. 10 and kept for inactivation at 37°C for 24 h.

Preparation of oil adjuvant vaccine: Three different oil adjuvant vaccines were prepared by taking 3 different ratios of aqueous phase containing antigen and oil phase containing sterile liquid paraffin oil. The OAV60, OAV50 and OAV40 contained the water to oil ratio 60: 40, 50: 50 and 40: 60, respectively (Table 1). The mixture of aqueous antigenic phase and liquid paraffin oil phase was emulsified with 6 part of lanolin as emulsifier in a commercial blender. Total four cycles of 5 min with 5 min interval between each cycle was run at medium speed switch (approximately 10000 rpm) at RT. Emulsion was tested for stability by drop test (Bomford 1997) immediately after preparation and by keeping it at 4°C, RT (25°C) and at 37°C for 14 days as per Bain *et al.* (1982). Vaccine was checked for sterility by inoculation on blood agar plate and tested for safety by injecting 0.2 ml vaccine by intramuscular route in mice (Drugs and Cosmetic act, Government of India 1994).

Viscosity of vaccine: The viscosity of the 3 different oil adjuvant vaccine formulations was measured in duplicate by electronic viscometer at 140 rpm.

Immunization of mice: Each of the 3 vaccinated group (OAV60, OAV50 and OAV40) and unvaccinated control group had forty mice. The each mouse of OAV60 group was vaccinated intramuscularly by 0.2 ml, OAV50 group by 0.25 ml and OAV40 group by 0.3 ml of their respective oil adjuvant vaccine. The different volume of vaccine was administered in different group so that each mouse across the group received 0.12 mg of antigenic mass. The unvaccinated control was injected by 0.2 ml of sterile phosphate buffer saline. Blood samples were collected from the retro-orbital sinus at 0, 7, 14, 21, 30, 45 day's post vaccination and sera were stored at -20°C till analysis.

Evaluation of immune response: Serum antibody level of immunised mice was evaluated by single dilution ELISA with some modifications (Briggs and Skeeles 1984). In brief, ELISA plate was coated by 100µl of sonicated *P. multocida* antigen (30µg/ml) prepared in carbonate-bicarbonate buffer (pH 9.6) and kept at 4°C for overnight. The plate was washed thrice with PBS-T and non specific sites were blocked by 5% skimmed milk powder at 37°C for 1 h. Test sera was added in triplicate at 1: 400 dilution followed by addition of goat anti-mice HRPO conjugate in each well at 1: 5000 dilution and kept for 1 h. OPD was used as substrate and the reaction was stopped by the addition of 0.5 N H₂SO₄ after

10 min. The absorbance was taken in ELISA reader at 492 nm. The absorbance was transformed into ELISA titer by using the formula of single dilution ELISA (Briggs and Skeeles 1984).

Challenge trial: Median lethal dose (LD₅₀) of *P. multocida*, strain P₅₂ was calculated according to Reed and Muench (1938). For challenge, each vaccinated group of mice were further divided in 3 subgroup containing 5 mice each, and were challenged with 3 different doses; 100 LD₅₀, 1000LD₅₀ and 10000 LD₅₀ of *P. multocida* (P₅₂ strain). Unvaccinated control mice were also divided into 3 groups, each containing 5 mice and inoculated with 3 different challenge doses. Challenged mice were observed for 7 days and number of mice died and mortality pattern was carefully recorded.

Statistical analysis: Comparison of means of serum titers was performed by analysis of variance (ANOVA) using SPSS 10. Tukey's test was used as post hoc comparison. Viscosity of different OAV formulations were performed in duplicate and averaged for calculation of mean.

RESULTS AND DISCUSSION

Indian *P. multocida*, P52 strain was used in the present study. This has the ability to cause fatal septicemic infections in susceptible species including buffalo-calves and hill-bulls after experimental challenge. The strain due to its antigenic superiority is widely used for vaccine formulation in India.

Commercial OAV against HS is formulated in the water to oil ratio of 50: 50 (Bain *et al.* 1982, OIE 2008). Therefore, OAV50 in the present study served as vaccinated control for the comparison of viscosity and immune responses. The new OAV formulations have the water to oil ratio of 60: 40 (OAV60) and 40: 60 (OAV40).

All the three OAV formulations were found sterile and safe. These three OAV formulations were tested for stability and remained stable at 4°C, RT and at 37°C, and did not separate into water and oil phase up to 14 days.

Viscosity of the 3 different OAV formulations was 426.77 cP (OAV60), 222.4 cP (OAV50) and 128.5 cP (OAV40) (Table 1). It was observed that increase in 10% aqueous antigenic phase (OAV60) compared to existing standard vaccine (OAV50) lead to the increase of 92% viscosity while the reduction of aqueous phase by 10% (OAV40) lead to the reduction of viscosity by 42%. Similar trend was reported by Jansen *et al.* (2005) in Infectious bronchitis and New castle disease virus vaccines. However the magnitude of viscosity in present study was in higher side of 128.5 cP to 426.77 cP compared to 50 cP to 243 cP in the study of Jansen *et al.* (2005) at similar water oil proportion. This deviation might be due to use of different emulsifier and mineral oil combination i.e., lanolin and paraffin oil in present study as compared to Arlacel and Marcol-52 by Jansen group. Yang *et al.* (2005) observed that hydrophile-lipophile balance value (HLB) of emulsifier affects the viscosity of the OAV; lower

Table 1. Physical properties of 3 different oil adjuvant vaccine formulations

Particulars	Vaccine groups		
	OAV60	OAV50*	OAV40
Aqueous antigenic phase (%)	60	50	40
Liquid paraffin (%)	40	50	60
Water/oil ratio	60/40	50/50	40/60
Vaccine volume/ dose in mouse (ml)	0.2	0.25	0.3
Antigenic mass/dose in mouse (mg)	0.12	0.12	0.12
Viscosity (cP)**	426.77	222.38	128.54
Viscosity compared to standard existing vaccine	92% increase	Standard vaccine	42% reduction

*The OAV50 is standard existing vaccine and served as vaccinated control. **viscosity was measured in duplicate and data presented here is average of 2 in centi poise (cP).

the HLB value of emulsifier, lower is the viscosity of resultant emulsion. Arlacel has lower HLB value (HLB 4.3) compared to lanolin (HLB 11), so more effective in reducing viscosity. However, lanolin was used for emulsion preparation in present study because it is cheap, easily available and widely used for preparation of HS oil adjuvant vaccine.

Oil adjuvant vaccine (OAV) is the most potent killed vaccine available to control the outbreak of haemorrhagic septicaemia in cattle and buffaloes (Shah *et al.* 1997, Muneer *et al.* 2005) and foot-and-mouth disease in cloven footed ruminants (Bowersock and Martin 1999, Aucouturier *et al.* 2001). The limiting factors in widespread use of this vaccine was high viscosity causing problems of slow inoculation under field conditions, poor stability and adverse reactions at the sites of inoculation (Verma and Jaiswal 1997, Tabatabaei *et al.* 2007). Therefore, attempts were made to reduce viscosity by making double emulsion (Verma and Jaiswal 1997, Kumar *et al.* 2007) or using improved oil such as Mercol 52 (Shah *et al.* 1997). But all these have little success either due to low degree of immunogenicity or cost

increase. To formulate OAV with lower viscosity aqueous phase of w/o emulsion was reduced by 10%. But, this reduced aqueous antigenic phase was compensated by increase in dose of vaccine, 0.3 ml for OAV40 as compared to 0.25 ml for OAV50 to contain the same 0.12 mg of antigenic mass. In present vaccine formulation antigenic mass was taken at brown opacity tube 10. The dose of vaccine can be reduced by taking the brown opacity tube no. 12 or more to compensate the reduced aqueous phase.

Mice serves as a good model for the HS infection as it manifests septicaemic form of disease similar to that observed in the natural hosts (Tabatabaei *et al.* 2002). For the evaluation of immune response by each vaccine, single dilution indirect ELISA was used. The log₁₀ ELISA titer in different vaccinated and control group ranged from 2.79±0.04 to 4.90±0.09 (Table 2). In the group of mice vaccinated with low oil phase OAV60 vaccine, significantly high (P<0.05) ELISA titer was observed on 14th days post-immunization (DPI) and achieved peak on 21st DPI and started decreasing thereof. But the peak antibody titer in group of mice vaccinated with high oil phase vaccine, OAV50 and OAV40, was obtained on 30th DPI and remained maintained at high level upto 45th DPI. The result as high peak titer for high oil content preparations is in consonance with the observation of Jansen *et al.* (2005). The OAV work by forming depot of antigen and letting its slow release over long course of time, protect the antigen from rapid degradation by enzymes and recruit the antigen presenting cells (APC) at the injection site and enhances the antigen uptake by these APC (Aucouturier *et al.* 2001, Aguilar and Rodriguez 2007). It seems that higher oil content of OAV50 and OAV40 slowed down the release of antigen in the beginning. But as time passed, more antigens was released under the action of lipase enzyme, which together with its own immunomodulatory effect lead to significantly higher antibody titer as observed on 30th and 45th DPI.

The LD₅₀ for control group of mice was 10^{-6.7}. Mice from all the 3 vaccinated groups sustained the challenge of 100 LD₅₀ (Table 3). But upon challenge with 1000LD₅₀ and 10000LD₅₀, survival was observed between 60 to 80% in 3 vaccinated groups. The mortality pattern observed in control

Table 2. Log₁₀ ELISA titer of mice immunized with OAV having 3 different water oil proportions

Vaccine groups	Days post vaccination					
	0	7	14	21	30	45
OAV60	2.80±0.03	3.07 ^a ±0.06	3.71 ^b ±0.03	4.13 ^b ±0.17	4.08 ^b ±0.08	4.03 ^b ±0.12
OAV50*	2.87 ^a ±0.01	3.03 ^a ±0.03	3.23 ^{ac} ±0.10	3.75 ^{bc} ±0.28	4.90 ^d ±0.09	4.40 ^c ±0.09
OAV40	2.92 ^a ±0.05	3.01 ^a ±0.03	3.55 ^{ab} ±0.03	3.77 ^b ±0.14	4.80 ^d ±0.10	4.38 ^c ±0.06
Unvaccinated control	2.85 ^a ±0.05	2.79 ^a ±0.04	2.82 ^a ±0.06	2.80 ^a ±0.03	2.90 ^a ±0.02	2.95 ^a ±0.07

Values bearing common superscript within row or column do not differ significantly at P<0.05. *The OAV50 is standard existing vaccine and served as vaccinated control.

Table 3. Protection in mice immunized with oil adjuvant vaccine having 3 different water oil proportions.
Challenge was done on 45th days post immunization

Vaccine groups	100 LD ₅₀		1000 LD ₅₀		10000 LD ₅₀	
	No.challenged/ survived	Protection %	No.challenged/ survived	Protection %	No.challenged/ survived	Protection %
OAV60	5/5	100	5/4	80	5/3	60
OAV50*	5/5	100	5/3	60	5/3	60
OAV40	5/5	100	5/3	60	5/4	80
Unvaccinated control	5/0	000	5/0	00	5/0	00

*The OAV50 is standard existing vaccine and served as vaccinated control.

was different from vaccinated mice. The 100% mortality was recorded with in 48 h at all the 3 level of LD₅₀ challenge dose in control group. While in vaccinated group, the mortality level of 20 to 40% at 1000LD₅₀ and 10000LD₅₀ was observed only after 48 h. Bhatti *et al.* (2005) observed 100% protection with 100 LD₅₀ and 70% to 90% protection with 1000 LD₅₀ in OAV vaccinated Swiss albino mice. The 10% better protection by the group at 1000 LD₅₀ might be due to booster vaccination and 3 times higher antigenic mass (0.37 mg compared to only 0.12 mg in present formulation). Yadav and Ahooja (1983) concluded that a vaccine is potent, if it provides log 2 unit of protection. Thus, at all these standards the present vaccine formulations were found potent.

Thus, in the present study, decrease in aqueous antigenic phase and relative increase in oil phase of OAV significantly decreased the viscosity, produced significantly higher and sustained ELISA titre ($P < 0.05$) and have comparable protection with that of existing standard OAV. Further exploration is needed into various aspects of these emulsions like shear force, the droplet size and local inflammatory response to understand the immune response and duration of immunity. The trial with various emulsifiers having different hydrophilic-lipophilic balance (HLB) value will further reduce the viscosity of the oil adjuvant vaccine.

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