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Improved humoral immune response of oil adjuvant vaccine by saponin co-adjuvantation against haemorrhagic septicaemia in mice and buffalo calves

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ABSTRACT

Oil adjuvant vaccine (OAV) formulations, viz. OAV (OAV without saponin) and S-OAV (OAV with 420 µg saponin/ml) were prepared against haemorrhagic septicaemia using *Pasteurella multocida,* P52 antigen. Three groups of mice, each group with 40 mice, were inoculated with respective OAV, S-OAV and sterile phosphate buffer saline and monitored the ELISA antibody titer for 45 days. In another experiment, 2 groups of buffalo calves, each group with 3 calves, were vaccinated with OAV and S-OAV and monitored the ELISA antibody titer for 4.5 months. Viscosity of OAV was 222.28 and of S-OAV 215.10 centipoise. The anti-*P. multocida* specific serum ELISA antibody titers were significantly higher in S-OAV on 14th days post vaccination compared to OAV and control and remained consistently higher during the experiment. Fluorescent activated cell sorter study revealed that CD4 T cells were significantly higher in S-OAV group as compared to OAV and control, while CD8 T cells was 28.83% higher than control. All vaccinated groups were 100% protected after direct intraperitoneal challenge with 100 LD50 of live *P. multocida,* serotype B, strain P52 on 45th DPV. However, after challenge with 1000 LD50 the S-OAV group had 80% protection as compared to 60% protection by OAV. Trial on buffalo calves further confirmed the finding of mice model as higher ELISA antibody titers were observed by S-OAV group as compared to OAV throughout the experiment period since seventh DPV onwards. Thus, saponin co-adjuvantation of OAV increased the CD4, CD8 T cells population, improved the humoral immune response and protection.

Key words: Buffalo, Co-adjuvantation, Haemorrhagic septicaemia, Oil adjuvant vaccine, *Pasteurella multocida*, Saponin

Haemorrhagic septicaemia (HS), the most important bacterial disease of cattle and buffaloes accounting for 46 to 55% of all bovine deaths in the last 4 decades (Dutta *et al.* 1990), is caused by *Pasteurella multocida* B: 2 in Asia and E: 2 in Africa. Broth bacterin, alum precipitated vaccine, oil adjuvant vaccine and live vaccines are available for the control of HS (Verma and Jaiswal 1998). Oil adjuvant vaccine (OAV) provides minimum of 1 year protection (OIE 2008). It produces strong humoral antibody response (Chang *et al.* 1998). The OAV in comparison to alum precipitated vaccine and broth bacterin produces delayed immune response; a drawback for emergency vaccination against HS (Dawkins *et al.* 1990).High viscosity of resultant emulsion is another concern (Verma and Jaiswal 1998). The immune response of OAV is synergised by application of co-adjuvants (Freund 1951). Saponin and its derivatives are widely used as vaccine adjuvants to stimulate antibody and cell mediated immune response (Sun *et al.* 2009). It forms the part of AS02 combination adjuvant against malaria along with MPL and oil in water emulsion and as an integral part of new vaccine delivery system, ISCOMs (Mutwiri *et al.* 2011). Though work with OAV and saponin as co-adjuvant is limited but experimental trial in foot-and-mouth disease (Smitsaart *et al*. 2004, Xiao *et al*. 2007), *Tenia ovis* (Harrison *et al*. 1999), and *Fasciola hepatica* (Martinez-Fernandez *et al*. 2004) found synergistic immune response. But co-adjuvants in general and saponin in particular have never been tried as co-adjuvants for the OAV against the HS. Thus, in the present study saponin was incorporated into aqueous antigenic phase of OAV and their viscosity, immune response profile, fluorescent activated cell sorter (FACS) based CD4 and CD8 T cell analysis and protection were compared with standard OAV in mice. Subsequently, their immune response study was also conducted in the buffalo calves, the most susceptible natural host.

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MATERIALS AND METHODS

Animals: Swiss albino mice (120) of either sex weighing 18 to 20 g, procured from the institute, were housed in

polypropylene cages with chaffed paddy straw bedding in hygienically controlled environment. Buffalo calves (6), 8-to 12-month- old were procured commercially and kept in hygienically controlled IVRI shed. Feed and water were supplied *ad lib*. The animal experiment was conducted after approval of Institutional Animal Ethics Committee (IAEC).

Growth of Pasteurella multocida*: Pasteurella multocida* serotype B: 2, strain P_{52}, collected from the Standardization Division, IVRI, Izatnagar, was maintained on blood agar slants at 4°C during the study period. 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 Misra (1985). The harvested culture was washed thrice using 0.5% formal saline and re-suspended in formal saline to match with Brown's opacity tube no. 10 and kept for inactivation at 37°C for 24 h.

Vaccines: Oil adjuvant vaccines, viz. OAV-oil adjuvant vaccine without saponin, and S-OAV-oil adjuvant vaccine with 420 µg saponin/ml vaccine, were prepared. The OAV was prepared by emulsifying equal proportion of aqueous antigenic phase and sterile liquid paraffin oil with 6 part of lanolin as emulsifier in a commercial blender. Total 4 cycles of 5 min with 5 min interval between each cycle was run at medium speed switch (approximate 10000 rpm) at room temperature. The S-OAV was prepared in similar way as of OAV except that its aqueous antigenic phase contained 420 µg saponin/ml vaccine. The emulsion was tested for stability by keeping it at 4°C, room temperature (25°C) and at 37°C for 14 days (Bain *et al.* 1982). Vaccine was checked for sterility by inoculation on blood agar plate and safety by injecting 0.25 ml of vaccine by intramuscular route in mice (Anonymous 1994).

Viscosity of vaccine: The viscosities of different OAV were measured by viscometer at the CARI, Izatnagar. Before the measurement of vaccine viscosity, formulations were stirred manually to form a uniform suspension and kept at 25°C. The 25 ml of sample was transferred into a steel cylinder provided with the viscometer. Using an appropriate spindle with constant speed (RPM), viscosity of vaccine samples was measured and expressed as centipoises (cP). The accuracy of the instrument was calibrated with standard viscosity fluids.

Immunization of mice: Mice were randomly divided into 3 groups and each group with 40 mice. The first 2 groups were inoculated intramuscularly with 0.25 ml of OAV and S-OAV. The unvaccinated control group was injected with 0.25 ml of sterile phosphate buffer saline. Blood samples were collected from the retro-orbital sinus on 0, 7, 14, 21, 30, 45 days post vaccination (DPV) and sera were separated and stored at –20°C until use.

Immunisation of buffalo calves: Buffalo calves (6) were randomly distributed into 2 groups, each group with 3 animals. These groups were vaccinated by 3 ml of OAV and S-OAV vaccine, by intramuscular route and monitored for ELISA titer at regular interval for 4.5 months.

Evaluation of immune response: Serum antibody levels of immunized mice were evaluated by single dilution ELISA (Briggs and Skeeles 1984). Briefly, ELISA plates were coated with 100µl of sonicated *P. multocida* antigen (30µg/ml) prepared in carbonate-bicarbonate buffer (pH 9.6) and kept at 4°C overnight. The plates were washed thrice with PBS containing 0.05% Tween 20 (PBS-T) and free sites were blocked by 5% skim 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 1h. The OPD was used as substrate and the reaction was stopped by the addition of 3N H_2SO_4 after 10 min. The absorbance was taken in ELISA reader at 492 nm. The absorbance was transformed into ELISA titer as per Briggs and Skeeles (1984). In buffalo calves similar procedure was adopted except sera were diluted 1: 100 and the rabbit anti bovine HRPO conjugate was used at 1:4000 dilution.

Estimation of CD4 and CD8 T cells population in mice: Estimation of CD4 and CD8 T cell lymphocyte population was conducted on blood samples of all the 3 groups of mice in triplicate on 21st DPV. Blood samples were collected in EDTA and immediately transported to laboratory for fluorescence activated cell sorter (FACS) analysis. In brief, 50 µl of blood was treated with a fluorescent dye tagged with monoclonal antibody specific for CD4 T cells (Alexa fluor–647 R Phycoerythrin and monoclonal antibody Alexa fluor 488 mouse IgG) and CD8 T cells by another sets of fluorescent dye and monoclonal antibody. The mixture was incubated at room temperature for 15 min and subsequently centrifuged at 250×g and washed twice with PBS. Ammonium chloride was added to the blood pellet for lysis of RBC and incubated at room temperature in dark for 10 min. The mixture was centrifuged at 200×g for 5 min and after completely decanting the ammonium chloride solution, 0.5 ml of PBS was added. The sample was transferred to the fluorescence activated cell sorter (FACS) and fluorescence activity was plotted on the X-axis and the cell no. on Y-axis.

Direct challenge test: Median lethal dose (LD_{50}) of *P. multocida*, strain P_{52} was calculated according to Reed and Muench (1938). For challenge, each vaccinated and unvaccinated control groups of mice were further divided in 2 subgroup containing 5 mice each and were challenged with 2 different doses; 100 LD_{50}, and 1000LD_{50} of *P. multocida* (P_{52} strain). Challenged mice were observed up to 7 days for mortality, if any.

Statistical analysis: Comparison of means of ELISA titers, CD4 and CD8 T lymphocyte cell counts were 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 mean values were calculated.

RESULTS AND DISCUSSION

The OAV is considered as the most potent vaccine for the control of HS, and provides minimum 1 year protection (OIE 2008). The water to oil ratio in the OAV affects viscosity, immune response and protection (Kumar *et al*. 2011). At present commercial OAV against HS is formulated in the water to oil ratio of 50:50 (Bain *et al.* 1982, OIE 2008). Therefore, the OAV in the present study served as vaccinated control.

Saponin based adjuvants have the ability to modulate the cell mediated immune response and enhances antibody production at very low dose. It has been widely used as adjuvants in many veterinary vaccines. But its toxicity and haemolytic activity are the most limiting factors (Waite *et al*. 2001). Depley vaccine was developed against the HS using saponin and became popular in Iran but later discontinued due to its toxicity (Verma and Jaiswal 1998). The haemolytic activity of the saponin is influenced by the aglycone which binds to cholesterol of cell membranes (Sun *et al.* 2009). In ISCOMs and ISCOMATRIX based delivery system, saponin remains trapped in cholesterol phospholipid matrix. This favours slow release of saponin with reduced toxicity and powerful immunomodulation. Several workers tried to take the advantage of immunomodulating properties of saponin by keeping saponin trapped in the oil of OAV. This has been especially tried against FMD with various degrees of success (Smitsaart *et al*. 2004, Xiao *et al*. 2007). Therefore, in the present study, 420 µg of saponin/ml of vaccine was incorporated in the aqueous phase of OAV and tested for its immunopotentiation against the HS in mice and buffalo calves. Safety of the vaccines was checked in mice before actual trials. No abscess or wound was detected after incorporation of saponin. Kumar *et al.* (2009) observed that saponin incorporation was safe at 300 µg in mice against *Mycoplasma agalactiae*. Saponin content in buffalo calves was 1.26 mg/dose. However, in cattle up to 6 mg against FMD (Smitsaart *et al*. 2004) and in sheep up to 20 mg against *Tenia ovis* (Harrison *et al*. 1999) was observed safe after incorporation in the OAV. Therefore, in the present study, saponin was incorporated in the prescribed limit and our result of safety supported these earlier findings.

Table 1. Physical properties of oil adjuvant vaccine (OAV) and saponified oil adjuvant vaccine (S-OAV)

Particulars	Vaccine groups	
	OAV	S-OAV
Aqueous antigenic phase (%)	50	50
Liquid paraffin (%)	50	50
Vaccine volume/mouse (ml)	0.25	0.25
Vaccine volume/buffalo (ml)	3	3
Antigenic mass/mouse (mg)	0.12	0.12
Antigenic mass/buffalo (mg)	1.44	1.44
Saponin (µg/ml)	0	420
Saponin (µg/mice dose)	0	105
Saponin (mg/buffalo dose)	0	1.26
Viscosity (centipoise)[a]	222.38	215.10

[a]Viscosity was measured in duplicates and is expressed as mean of 2.

The 2 OAV formulations remained stable and did not separate into water and oil phases up to 14 days. Viscosities of the 2 OAV ranged from 215.10 cP to 222.38 cP (Table 1). Preparation of oil adjuvant vaccine needs emulsifier with high hydrophile lipophile balance (HLB) value (Yang *et al*. 2005). Further, its HLB value decides the viscosity of resultant OAV formulations which increases with increase in HLB value of emulsifier (Yang *et al*. 2005). Most reported saponin with adjuvant properties has high HLB value ranging from 8 of soya saponin to 36.3 of QS21 (Song and Hu 2009). This reflects that saponin can serve as emulsifier and will have impact on the viscosity of the OAV. But in the present experiment saponin content was 7% of lanolin (HLB 11), which was too low to have any visible impact. The similar result of viscosity was reflected in our study.

The humoral immune response elicited among different groups of vaccinated mice is presented in Table 2. Both the vaccinated groups observed rise in mean IgG antibody titers from seventh day post vaccination (DPV) onwards. The S-OAV had significantly higher ($P<0.05$) antibody titer on 14th DPV and remained consistently higher compared to OAV or control except on the 30th day when the OAV had significantly higher ($P<0.05$) antibody titer. The result of buffalo calves trial strengthened the finding of mice model, as the group vaccinated with S-OAV vaccine had the higher IgG antibody titers throughout the experimental period in comparison to OAV (Table 3). Application of co-adjuvant for the potency

Table 2. Anti- *Pasteurella multocida* specific Log10 ELISA titres in mice immunized with oil adjuvant vaccine (OAV) and saponified oil adjuvant vaccine (S-OAV)

Vaccine	Days post vaccination					
	0	7	14	21	30	45
Control	2.85±0.05^{a}	2.79±0.04^{a}	2.82±0.06^{a}	2.80±0.03^{a}	2.90±0.02^{a}	2.95±0.07^{a}
OAV	2.87±0.01^{a}	3.03±0.03^{b}	3.23±0.10^{b}	3.75±0.28^{ab}	4.90±0.09^{b}	4.40±0.09^{b}
S-OAV	2.86±0.03^{a}	3.07±0.06^{b}	3.88±0.04^{c}	4.02±0.32^{b}	4.32±0.04^{c}	4.49±0.20^{b}

Values bearing different superscript differ significantly ($P<0.05$) within a column. Values are presented as mean±SE.

Table 3. Anti- *Pasteurella multocida* Log10 ELISA titres of buffalo calves immunized with oil adjuvant vaccine (OAV) and saponified oil adjuvant vaccine (S-OAV)

Vaccine	Days post vaccination								
	0	7	14	21	30	45	60	90	135
OAV*	2.22±0.06[a]	2.69±0.17[a]	3.12±0.28[a]	3.25±0.33[a]	3.34±0.23[a]	3.51±0.18[a]	3.61±0.11[a]	3.59±0.01[a]	3.53±0.08[a]
S-OAV	2.21±0.04[a]	3.24±0.16[a]	3.82±0.23[a]	3.71±0.35[a]	3.99±0.34[a]	4.13±0.28[a]	4.10±0.44[a]	4.09±0.36[a]	3.57±0.21[a]

Values bearing common superscript do not differ significantly within a column. Values are presented as mean±SE; *OAV: served as vaccinated control.

Table 4. Percentage population of CD4 and CD8 T cells in blood of mice immunised with oil adjuvant vaccine (OAV) and saponified oil adjuvant vaccine (S-OAV) on 21st days post immunisation

Vaccine group	% CD4 T cells	% CD8 T cells	Ratio CD4/ CD8	% increase of CD4 T cells	% increase of CD8 T cells
Control	8.55±1.51[a]	3.26±0.56[a]	2.61	-	-
OAV	8.77±0.58[a]	2.76±0.37[a]	3.18	2.57	–15.34
S-OAV	13.24±0.72[b]	4.20±0.38[a]	3.15	54.85	28.83

Values bearing different superscript differ significantly ($P<0.05$) within a column. Population of CD4 and CD8 T cells are presented as mean±SE.

improvement of oil adjuvant vaccine started with the formulation of Freund complete adjuvant which combines the immunomodulatory properties of *Mycobacterium tuberculosis* with depot effect of water-in-oil emulsion (Freund 1951). This combination adjuvant generates strong Th1 and Th2 responses (Sun *et al*. 2009). In the present formulations saponin was incorporated as co-adjuvant at 105 µg/mice dose. The S-OAV group consistently had high level of antibody titers in both mice and buffalo calves and high level of CD4 T and CD8 T cell population in comparison to other groups. Saponin has long been known as a powerful immunomodulator with strong Th1 and Th2 response (Sun *et al*. 2009). It seems that better immune response by S-OAV was the synergistic effect of strong depot and inflammatory effect of oil, and immunomodulating power of saponin. Although, the combination of saponin with oil is not common, the synergism between them for immunopotentiation was reported against foot-and-mouth disease (Smitsaart *et al*. 2004, Xiao *et al*. 2007), *Fasciola hepatica* (Martinez-Fernandez *et al*. 2004) and *Tenia ovis* (Harrison *et al*. 1999). Till date, no report is available with regards to use of saponin for improvement of immune response of OAV against any bacterial disease. Therefore, better humoral response by saponified OAV against *Pasteurella multocida* support these earlier findings and signify its use in bacterial vaccines.

The CD4 T cells or helper T cells are mainly responsible for antibody based humoral immunity; while, CD8 T cells or cytotoxic T cells are responsible for cellular immunity. The estimation of their cell number quantifies the immune response. The saponification of oil adjuvant vaccine (S-OAV) significantly ($P<0.05$) increased the CD4 T cell and CD8 T cells as compared to control and OAV (Table 4). Xiao *et al*. (2007) observed the synergistic response by quil A and OAV over CD4 T cells subsets i.e., Th1 and Th2. Thus high CD4 and CD8 T cells population by S-OAV are in agreement with the earlier report. The OAV is widely used as veterinary vaccine due to its strong and sustainable humoral response but in the present study the level of CD4 T cells were not as high as expected. No parallel report is available regarding CD4 and CD8 T cell population with respect to the OAV. Therefore, it needs further work for better elucidation.

In the present trial, vaccinated and control group were challenged with 2 different LD_{50} doses i.e., 100 and 1000 LD_{50} (Table 5). A 100% protection was observed by both the vaccines after challenge at 45 DPV by 100 LD_{50}. This result is in consonance with earlier report (OIE 2008). However, upon direct challenge with 1000 LD_{50} the S-OAV group had 80% protection as compared to 60% protection by OAV. Okerman and Devriese (1987) observed the ineffectiveness of OAV by higher challenge dose against *P. multocida* in similar multiple dilution challenge test. The ELISA antibody titer is correlated with protection against haemorrhagic septicaemia in mice (Dawkins *et al*. 1990) and

Table 5. Level of protection by different vaccine formulations after challenge on 45th days post vaccination in mice

Treatment	100 LD_{50}		1000 LD_{50}	
	Challenged/ survived	Protection %	Challenged/ survived	Protection %
Control	5/0	00	5/0	00
OAV	5/5	100	5/3	60
S-OAV	5/5	100	5/4	80

buffalo calves (Chandrasekaran *et al*. 1994). The vaccine, S-OAV synergised the depot effect of oil adjuvant vaccine and immunomodulator properties of saponin resulting in better protection. The group had high ELISA antibody titer from seventh DPV onwards in buffalo calves. This makes it appropriate for emergency vaccination in case of HS outbreak. Verma and Jaiswal (1997) reported that both humoral and cell mediated immunity is required for protection of multiple emulsion vaccine against *Pasteurella multocida* in calves. The S-OAV increased both CD4 T cells and CD8 T cells and thereby augmented both humoral and cell mediated arm of immune response leading to better protection observed by the group. Therefore, based on the results of mice model and buffalo calves we recommend further testing of saponin and oil adjuvant vaccine combination for its possible use as emergency vaccination against haemorrhagic septicaemia.

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