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ORIGINAL ARTICLE

Effect of alum co-adjuvantation of oil adjuvant vaccine on emulsion stability and immune responses against haemorhagic septicaemia in mice

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ABSTRACT

Background and Objectives: Haemorrhagic septicaemia (HS), caused by *Pasteurella multocida*, is the most important bacterial disease of cattle and buffaloes in India. Oil adjuvant vaccine (OAV) is the most potent vaccine available for the control of HS. The study aims to evaluate the effect of alum co-adjuvantation of OAV on emulsion stability and immune response.

Materials and Methods: Two different oil adjuvant vaccines viz., standard oil adjuvant vaccine (OAV) and alum precipitated oil adjuvant vaccine (A–OAV) were prepared with *Pasteurella multocida* antigen. Emulsion stability was tested by centrifugation, storage at 37 °C for 3 months and microscopy. Immune responses were evaluated by ELISA antibody titer, CD4, CD8 T cell populations and survival post challenge by *P. multocida* in mice.

Results: The separation of aqueous and oil phase of emulsion by centrifugation and storage test were 0 and 6.76% in A-OAV as compared to 11.00 and 26.39% in OAV, respectively. The mean droplet size was significantly smaller ($p<0.01$) in A–OAV as compared to OAV. The A–OAV recorded higher ELISA antibody titer ($p<0.05$) up to 21st days post vaccination, and higher CD4 ($p>0.05$) and CD8 T cell ($p<0.05$) populations compared to OAV. The A–OAV group conferred 100% protection after challenge with both 100 LD_{50} and 1000 LD_{50} as compared to 100 and 60% respective protection by OAV group.

Conclusion: The results indicates that A–OAV had better emulsion stability, produces higher level of CD4, CD8 T cells and antibody titer with better protection compared to oil adjuvant vaccine.

Keywords: Alum, Emulsion stability, Haemorrhagic septicaemia, Immune response, Oil adjuvant vaccine, *Pasteurella multocida*

INTRODUCTION

In India, haemorrhagic septicaemia (HS) is the most important bacterial disease of cattle and buffaloes and accounts for 46 to 55% of all bovine deaths in the last four decades (1). It is caused by two specific serotypes of *Pasteurella multocida* B: 2 in Asia and E: 2 in Africa.

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Oil adjuvant vaccine (OAV) is the most potent killed vaccine available to control the outbreak of HS in cattle and buffaloes (2, 3) and Foot-and-mouth disease in cloven footed ruminants (4, 5). The OAV represents a water-in-oil emulsion which causes local inflammatory reaction at the inoculation site and retain antigen for longer period by forming depot (5, 6). Alum precipitated vaccine is another important killed vaccine against the HS and is widely used in the field condition. Aluminium adjuvants like alum are powerful immunomodulator and strong Th2 stimulant, a properties desirable for a good vaccine against extracellular pathogens such as *P. multocida* (7). But duration of alum precipitated vaccine is limited to 4–6 months and needs booster doses (8, 9). Thus, there lies a possibility to explore combination adjuvants to have complementary or even synergistic effect. Freund's complete adjuvant (FCA), a gold standard adjuvant, combines depot effect of water-in-oil emulsion and the immune modulator property of *Mycobacterium tuberculosis* (10). The OAV has been co-adjuvanted with saponin and quill A against FMD (11, 12) and with alum against foot rot (13). These co-adjuvanted vaccines were more protective than either of their component adjuvant alone.

Stability of emulsion critically determines the humoral and cellular response, and storage time of the vaccine (14). Several studies have been conducted using salts to improve the stability of emulsion in cosmetic cream and paints (15-17). It has been observed that addition of salts into aqueous phase at a concentration as low as 0.02 M dramtacially improved the stability of emulsion (15). To the best of our knowledge no reports are available with regards to use of salt for improving emulsion stability used as vaccine. Alum and oil based emulsion represents two most successful veterinary adjuvants. However, very little work has been done taking these two adjuvants together. Further, no report is available with regards to effect of alum adjuvantation on emulsion properties of resulting alum precipitated OAV. Thus, in the current study alum was incorporated into aqueous phase of OAV and was compared for its stability with standard OAV. The immune response and challenge study of these vaccines were studied in mice model.

MATERIALS AND METHODS

Laboratory animal. Swiss albino mice of either sex weighing 18 to 20 g were procured from Laboratory Animal Resource section, IVRI, Bareilly, U.P. They were kept for acclimatization for a period of 15 days before start of vaccination trial. All the animal experimentations were carried out under conditions approved by the ethics committee for animal care at IVRI, Izatnagar according to the IAEC guidelines.

Culture of bacterial strain. The reference strain of *P. multocida* P52 was obtained from Division of Biological Standardization, IVRI, Bareilly and was maintained on blood agar slants at 4°C during study period. The bacterial biomass was produced by inoculating 3 mL of pure broth culture of *P. multocida* P52 in Roux flax containing 125-150 mL of medium (Nutrient agar 28 g L-1; additional yeast extract 3 g L-1; caesamino acid 3 g L-1) at 37°C for 18 hours. The biomass was harvested by 20 mL of formal saline from each Roux flask. The bacterial culture from each Roux flask was checked for purity by microscopy before pooling. The biomass of the harvest was determined as per methodology of Mishra (18). The harvested culture was washed three times with 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 hours.

Preparation of oil adjuvant vaccines. Two different oil adjuvant vaccines were prepared, viz., standard oil adjuvant vaccine (OAV), served as control vaccine (19) and alum precipitated oil adjuvant vaccine (A–OAV) which served as test vaccine. The OAV was prepared by emulsifying equal proportion of aqueous antigenic phase and sterile liquid paraffin oil with 6% lanolin as emulsifier in a commercial blender (Bajaj, India). Total five cycles of 1.5 min with 5 min interval between each cycle was run at medium speed switch (approx. 10000 rpm) at room temperature. For A–OAV, the aqueous antigenic phase was first precipitated by 20% potassium aluminium sulphate (alum, pH 6.2) by keeping the antigenic solution over magnetic stirrer for overnight. Then equal proportion of alum precipitated aqueous antigenic mass was emulsified with liquid paraffin oil just like OAV. The alum content in the vaccine was adjusted to 8 mg/ ml.

Stability testing of oil adjuvant vaccines. During vaccine preparation, emulsion was tested for stability by drop test (20). The droplet size of both

the emulsions were measured, 50 droplets for each vaccine, using microscope at 400× magnification and Progress C3 camera (Jenoptik AG, Jena, Germany) and dedicated software. Immediately after vaccine preparation, stability of emulsions were tested by centrifugation at 10,000 rpm (6700×g) for 10 min at 4°C (Eppendorf, 5417R centrifuge) by taking 1 ml vaccine in triplicate. Further, both the emulsions were kept at 4°C and 37°C for 3 months and tested for phase separation (21). The percentage phase separation was determined by the following formulae

% phase separation by centrifugation = 100×(total separated volume/total volume of emulsion)

% phase separation after 3 months = 100×(height of separated phase (cm)/height of total emulsion (cm)

Vaccines were checked for sterility by inoculation on blood agar plate and tested for safety in mice as per standard protocol (22).

Immunization schedule in mice. Three different groups, each consisting of 40 mice, were made for immune response studies. First and second groups were intramuscularly inoculated with 0.25 ml of OAV and A– OAV respectively. Third group were inoculated with 0.25 ml of sterile phosphate buffer saline which served as un-inoculated control. Sera samples were collected from the retro-orbital sinus at weekly interval starting from day 0 to 45 post vaccination and were stored at –20°C till analysis.

Estimation of ELISA antibody titer. Serum antibody titer of each treatment mice group was measured by single dilution ELISA (23). In brief, ELISA plates (Nunc, Denmark) were coated by 100µl of sonicated *P. multocida* antigen (30µg/ml) prepared in carbonate-bicarbonate buffer (pH 9.6) and incubated at 4°C for overnight. The plates were washed three times with PBS–T. Subsequently plates were blocked by 5% skimmed milk powder at 37°C for 1 hour. After washing, plates were incubated with 1:400 dilution of test sera in triplicate (37°C for 1 hour). After washing, plates were incubated with 1:5000 dilution of goat anti-mice HRPO conjugate (Bangalore Genei, India) at 37°C for one hour. The o-phenylenediamine (OPD) was used as substrate and reaction was stopped after 10 minutes by addition of 0.5N H2SO4. The absorbance was recorded at 492 nm in ELISA reader (Bio-Rad, USA). Absorbance value was transformed in ELISA titer by the regression equation of single dilution; ELISA titer = 2.676+0.27×(P/N), where P is the absorbance value of test sera and N is the absorbance value of negative sera.

Estimation of CD4 and CD8 T cells populations in mice. Estimation of CD4 and CD8 T cell lymphocyte population was conducted on blood samples 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) (BD FACS Calibur; BD Bio-Sciences) and fluorescence activity was plotted on the X-axis and the cell numbers on Y-axis.

Challenge study with *Pasteurella multocida* P52. Median lethal dose (LD_{50}) of *P. multocida*, strain P_{52} was calculated according to the method of Reed and Muench (24). Each vaccinated and control group of mice were divided in two sub-groups, each with five mice and were challenged with two different doses; 100 LD_{50}, and 1000LD_{50} by *P. multocida* (P_{52} strain). Challenged mice were observed for 7 days and number of mice died and mortality pattern was carefully recorded.

Statistical analysis. One way ANOVA was performed to compare means of serum titers and CD4 and CD8 T lymphocyte cell population. If results were significant, ANOVA was followed by Tukey test (25). Phase separation and droplet size of the emulsion was measured by paired sample T test. All the statistical analysis was done using Statistical Package for the Social Sciences version 17 (SPSS 17; SPSS Inc, Chicago, IL, USA).

RESULTS

Stability of oil adjuvant vaccines. Stability of vaccines measured by droplet size, %phase separation using centrifugation test, and 3 months storage at 4 and 37°C are presented in Table 1, Figs 1 and 2. Alum precipitation of aqueous antigenic phase (A–OAV) significantly ($p<0.01$) improved the stability of emulsion as compared to OAV. After centrifugation, the A–OAV recorded zero phase separation against 11% in OAV. Similarly, at 37°C the A–OAV had 6.36% partial phase separation against 26.39% clear phase separation in OAV. The mean droplet size was significantly smaller ($p<0.01$) in A–OAV (2.81±0.19 µm) compared to OAV (15.38±1.47 µm) and it ranged between 0.5 to 5.8 µm and 0.9 to 41.3 µm respectively (Fig. 3). Both the vaccines were found safe as no mortality was recorded after injection.

Table 1. Stability of standard oil adjuvant vaccine (OAV) and alum precipitated oil adjuvant vaccine (A–OAV)

Particulars	OAV	A–OAV	Significance
Droplet size (µm)	15.38 ± 1.47^{a} (0.9–41.3)	2.81 ± 0.19^{b} (0.5–5.8)	**
PS (Centrifugation)	11.0 ± 0.58^{a}	0 ± 0^{b}	**
PS (Incubation at 37°C, 3 months)	26.39 ± 1.39^{a}	6.76 ± 1.35^{b}	**
PS (Incubation at 4°C, 3 Months)	3.05 ± 0.28^{a}	0.0^{a}	NS (0.058)

The values bearing different superscript within a row differ significantly at $p<0.01$. Droplet size within parenthesis is presented as range. Centrifugation was done at 6700 × g at 10,000 rpm for 10 minutes. PS, % Phase separation. **, $p<0.01$; NS, Not significant.

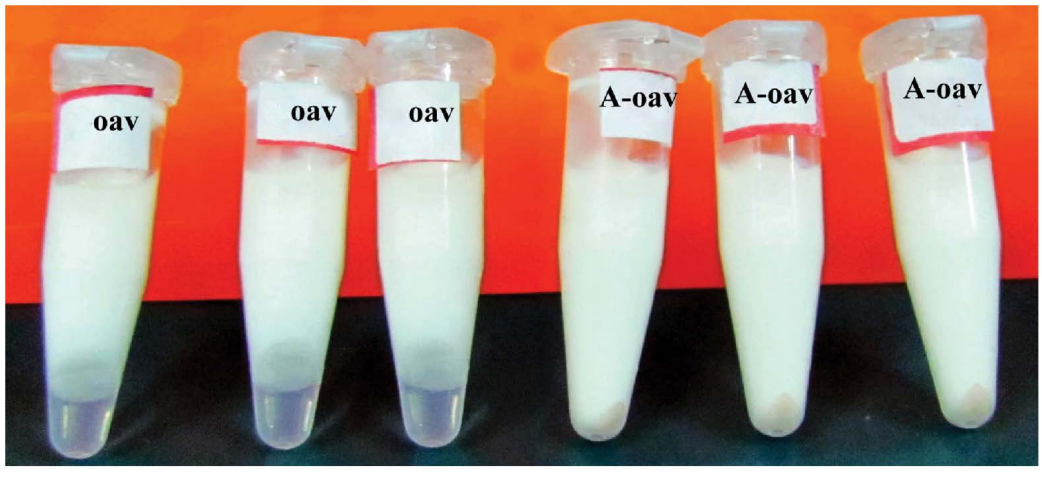

Fig. 1. Stability test by centrifugation at 6700 × g at 10,000 rpm for 10 minutes. OAV, standard Oil adjuvant vaccine; A–OAV, Alum precipitated oil adjuvant vaccine.

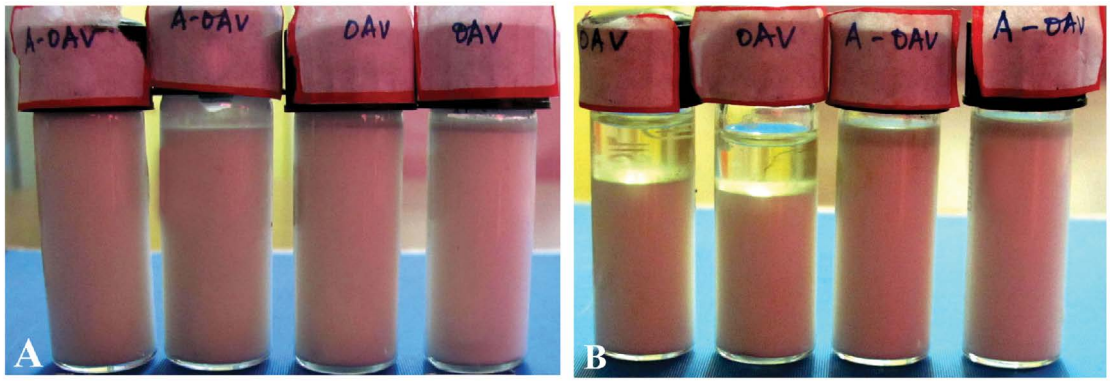

Fig. 2. Phase separation in standard oil adjuvant vaccine (OAV) and alum precipitated oil adjuvant vaccine (A–OAV) kept for 3 months at 4oC (A) and 37oC (B).

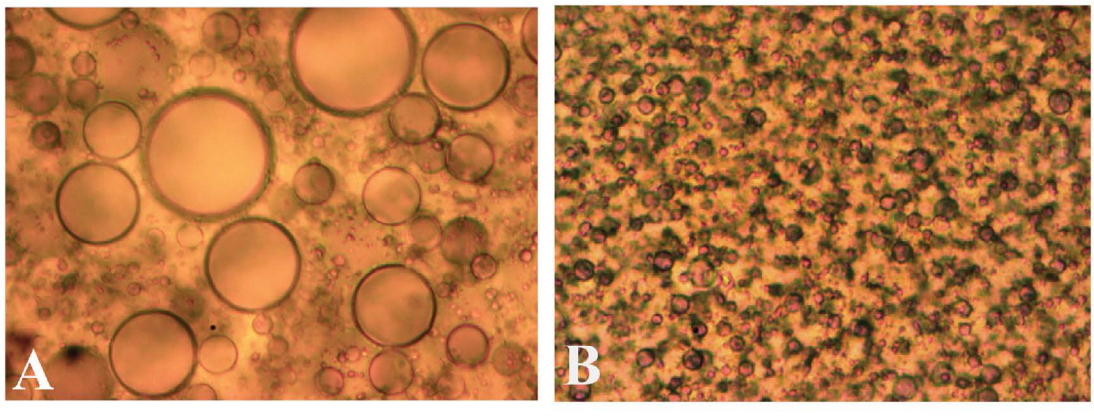

Fig. 3. Microscopic view (400 ×) of standard oil adjuvant vaccine (OAV) (A) and alum precipitated oil adjuvant vaccine (A-OAV) (B). The droplet size varied between 0.9 to 41.3 µm in OAV and between 0.5 to 5.8 µm in A–OAV.

Serum ELISA antibody titer. The Log10 ELISA titer in all the three groups ranged between 2.79±0.04 to 4.90±0.09 (Fig. 4). A rise in mean Log10 ELISA antibody titer was recorded from seventh day post vaccination (DPV) on ward in both the vaccinated groups. The antibody titer was significantly higher ($p<0.05$) in A–OAV during 7 to 21st DPV compared to both unvaccinated and vaccinated control (OAV) groups. But, on 30th DPV the OAV group had significantly higher ELISA titer ($p<0.01$) compared to A–OAV. Thereafter significant drop in ELISA titer was observed in OAV group on day of challenge ie 45th DPV.

CD4 and CD8 T cells populations in mice. The result of CD4 and CD8 T cells in mice post-immunization for different treatment groups are presented in Table 2. The level of CD8 T cells were significantly higher ($p<0.05$) in A–OAV as compared to OAV. Similarly the level of CD4 T cells was higher in A–OAV group (11.65±0.35%) compared to OAV (8.77±0.58%). However, CD4 and CD8 T cells populations did not differ significantly between the OAV and unvaccinated control.

Challenge trial with *Pasteurella multocida* P52. The protection conferred in two vaccinated groups and unvaccinated control after challenge with 100 and 1000 LD_{50} of *P. multocida* strain P52 are presented in Table 3. Mice from both the vaccinated groups had 100% protection after challenge with 100 LD_{50} against 0% in control. However, upon challenge with 1000LD_{50}, the A–OAV conferred 100% pro-

tection against 60% by OAV.

Table 2. Percentage population of CD4 and CD8 T cells in blood of mice immunized with oil adjuvant vaccine (OAV) and alum precipitated oil adjuvant vaccine (A–OAV) on 21st days post immunization.

Vaccine groups	% CD4 T cells	% CD8 T cells	Ratio of CD4 CD8 T cells	% increase of CD4 T cells compared to control	% increase of CD8 T cells compared to control
Control	8.55±1.51[a]	3.26±0.56[ab]	2.61	00	00
OAV	8.77±0.58[a]	2.76±0.37[b]	3.18	2.57	–15.34
A–OAV	11.65±0.35[a]	5.21±0.55[a]	2.23	36.26	59.82

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

Table 3. Protection in different treatment groups of mice after challenge with 100 and 1000 LD50 of Pasteurella multocida on 45th days post immunization

Treatment groups	100 LD_{50}		1000 LD_{50}	
	Survived Challenged	% survival	Survived Challenged	% survival
OAV*	5/5	100	3/5	60
A–OAV	5/5	100	5/5	100
Control**	0/5	00	0/5	00

Abbreviations: OAV, Oil adjuvant vaccine; A–OAV, Alum precipitated oil adjuvant vaccine
*OAV served as vaccinated control ** Unvaccinated control.

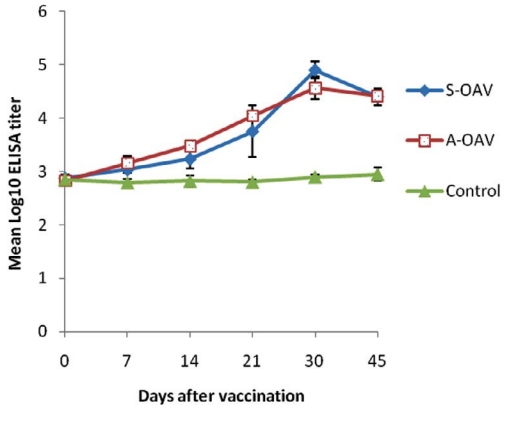

Fig. 4. Mean Log10 ELISA titer (Mean ± SE) of mice immunized with standard oil adjuvant vaccine (OAV) and alum precipitated oil adjuvant vaccine (A–OAV). The OAV served as vaccinated control while control is unvaccinated control. ELISA titer was measured by regression equation of single dilution ELISA at 1: 400 dilutions of sera. ELISA titer = 2.676+0.27*(P/N), where P is the absorbance value of test sera and N is the absorbance value of negative sera.

DISCUSSION

Oil adjuvant vaccine (OAV) is considered as the most potent vaccine against HS which provides minimum 1 year protection (19, 26). Water-in-oil ratio determines the stability and viscosity of oil adjuvant vaccine (27, 28). At present commercial OAV against HS is formulated in the water to oil ratio of 50:50 (19). Therefore, the OAV in the present study served as vaccinated control. Alum is widely used as adjuvants in human and animal vaccines due to its great safety record (8). Alum precipitated vaccine against HS contains 1% alum i.e., 10 mg/ml of vaccine. The present A–OAV vaccine contained 8 mg alum/ml of vaccine which is equivalent to 1.36 mg elemental aluminum/cattle dose. Though, no upper limit safety guideline is available with regards to aluminium for cattle, in human vaccine 1.25 mg and 0.85 mg aluminium per vaccine dose is recommended in Europe and USA, respectively (8). Therefore, aluminium level used in the present study was within the widely used limit. Moreover, at this dose, mice didn't record any visible toxicity.

Stability of emulsion critically determines the humoral and cellular response, and storage time of the vaccine (14). In the present experiment alum precipitation of aqueous antigenic phase prior to emulsification significantly improved ($p<0.05$) the stability of emulsion as measured by phase separation by centrifugation test and incubation at 37°C for 3 months (Figs. 1, 2 and Table 1). Similarly, the mean droplet size was significantly smaller ($p<0.01$) in A–OAV as compared to OAV. Several studies have been conducted using salt to improve the stability of emulsion in cosmetics (15-17). The salt of calcium, magnesium, sodium, potassium have been found to enhance the stability of water-in-oil emulsions (16, 17). It has been speculated that addition of salt increases the refractive index of aqueous phase (16). This leads to reduced attractive forces between the water droplets resulting into reduced coalescense and increase in stability of emulsion (16, 17). Alum, being a salt of potassium and aluminum might have worked in similar manner to improve the stability of water-in-oil emulsion. However, to our knowledge no published report is available which describe the emulsion stabilizing properties of alum. In developing countries like India refrigeration facility is not available in the remote villages. The alum precipitation of OAV will help to store the vaccine at room temperature for longer period without affecting its stability. This may also help to reduce the dose of emulsifier.

The co-adjuvantation of oil adjuvant vaccine by alum (A–OAV) significantly increased ($p<0.05$) the IgG antibody titer as compared to OAV from 7th days post vaccination (DPV) onwards and was consistently higher except on 30th DPV. In several trials, OAV found to act synergistically with other co-adjuvants like *Mycobacterium tuberculosis* in Freund complete adjuvant (10), alum (13) and saponin (11, 30). Thorley and Egerton (13) reported that alum co-adjuvantation of OAV in *Bacteroides nodosus*, enhanced the antibody titer by 3 to 5 times as compared to OAV. The CD4 T cells, helper T cells, are responsible for antibody based humoral immunity while CD8 T cells are responsible for cellular immunity. Their estimation help to explain the vaccine functioning. In the present experiment, both CD4 and CD8 T cell population were higher in alum precipitated OAV (A–OAV) compared to OAV. Alum works by forming antigen depot at the injection site and generate strong humoral immune response with increased CD4 T cell population (3, 29). Thus early higher IgG titer in the A–OAV group probably resulted from the synergistic effect of humoral response of alum and OAV. In the present experiment, FACS analysis was done on 21st DPV. The level of CD4 T cells in OAV group was not significantly different from the unvaccinated control. Therefore, it seems that OAV takes longer time to sensitize the immune cells compared to A–OAV.

In the present experiment, higher ELISA antibody titer was observed in OAV group on 30th DPV with significant reduction on 45th DPV. Earlier, Richards *et al*. (14) reported higher antibody titer in unstable emulsion as compared to stable emulsion. Alum precipitation made the emulsion more homogeneous with smaller droplet size as compared to OAV. Therefore, it seems that relatively better depot effect of A–OAV lead to sustained antibody titer compared to OAV. In the present experiment, the study period was limited for 45 days. During the period we observed the sustained antibody titer in A–OAV group in contrast to OAV. This demands the duration of immunity study for A-OAV in target host of HS such as cattle and buffaloes.

In present study, 100% protection was observed by both the vaccines after challenge at 45 days post vaccination by 100 LD_{50}. This result is in consonance with earlier findings that observed OAV as a potent vaccine (3, 26). However, the A–OAV group had better survival compared to OAV after challenge with 1000 LD_{50}. Thorley and Egerton (13) observed higher protection against foot rot in alum precipitated OAV as compared with OAV alone after challenge with *Bacteroides nodosus*. Therefore, our finding is in line with their observation. However, on day of challenge, no significant difference in ELISA antibody titer was observed between A–OAV and OAV vaccinated groups. Earlier, Chandrasekaran *et al*. (26) reported predominance of IgG1 and IgG2 isotypes after vaccination with oil adjuvant vaccine. Alum

adjuvant is known for CD4 T cells mediated class switching (31). Therefore, it seems that higher level of CD4 T cells (31) and improved emulsion stability (14) in A–OAV group resulted in better protection compared to OAV. However, the detail mechanism of higher protection in A–OAV group needs to be further explored.

In conclusion, alum precipitation of aqueous antigenic phase of oil adjuvant vaccine improved the stability of emulsion, enhanced the antibody titer and protection in mice. The A–OAV group was more homogeneous with smaller droplet size resulting in consistent antibody titer as compared to OAV group in mice model. But the vaccine needs to be further explored in the target host such as cattle and buffaloes for its duration of immunity and protection.

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