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A New Alum Precipitated Oil Formulation Vis-a-Vis Alum Precipitated and Toxoid Enterotoxaemia Vaccines for the Enhanced and Sustained Immune Response against Epsilon Toxin in Sheep

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ABSTRACT

Background: Enterotoxaemia represents one of the major limiting factors in small ruminant farming. Rapid lethal progression of the disease makes treatment impractical in most cases. Thus, suitable immunoprophylactic measures are necessary against this disease. The current study was aimed to test out the ability of novel alum precipitated oil adjuvant vaccine (AOV) for overcoming the difficulties associated with short-term immunity of current enterotoxaemia vaccines.

Methods: A new formulation named, AOV was prepared using a highly toxigenic strain of *Clostridium perfringens* type D. Immunogenicity and protective efficacy of this vaccine in comparison to presently available epsilon toxoid vaccine (TV) and alum precipitated vaccine (APV) was evaluated in the target species, sheep using indirect ELISA, mouse neutralization test (MNT).

Result: Among the three vaccines tested, AOV produced higher and persistent protective antibody titer up to 150 days post-immunization without any booster dose while APV and TV protected only up to 60th and 45th day respectively. All vaccinated animals remained healthy for the whole duration of the study with no systemic or local reactions. The present study delineates the superiority of AOV over presently available TV and APV for the prevention of an extremely lethal disease of sheep and goats.

Key words: *Clostridium perfringens*, ELISA, Enterotoxaemia, Mice neutralization test, Vaccine.

INTRODUCTION

Enterotoxaemia caused by *Clostridium perfringens* type D is worldwide in distribution (Radostitis *et al.* 2007) and represents one of the major limiting factors in small ruminant farming (Tooloei and Masodei 2008). The disease causes considerable loss in the sheep industry due to increased treatment costs, decreased productivity and high fatality rates among the affected animals (Greco *et al.* 2005). Tragically, rapid lethal progression makes treatment impractical in most cases (Aiello 2003). Thus, suitable immunoprophylactic measures are necessary to combat this disease (Rosskopf-Streicher *et al.* 2004). Though the disease can be prevented by epsilon toxoid (Lobato *et al.* 2010) it induces only short term immunity (Bernath *et al.* 2004), so double initial vaccination is currently recommended for both sheep and goats, followed by a booster every year in sheep (Blood *et al.* 1983) and every 3-4 months in goats (Smith and Sherman 1994). Therefore, the present vaccination strategy is not economical and often fails due to the difficulty in tracing the primary immunized animals for a booster dose. Thus, a single-shot enterotoxaemia vaccine would be an enormous benefit for sheep and goat breeders all over the world (Uzal *et al.* 1999) emphasizing the need to develop a new enterotoxaemia vaccine which induces a sustainable prolonged immunity.

The addition of adjuvants helps antigen to elicit an early high and long-lasting immune response with less amount. Aluminium salts are the most common adjuvants (Marrack *et al.* 2009) but provide only a short duration of immunity. However, this duration can be prolonged by adding oil which also helps to cut down the required antigenic mass for effective immunity (Bincy *et al.* 2011). Atthi *et al.* (2001) showed that the oil adjuvanted hemorrhagic septicemia vaccine could protect cattle even after 24 months of post-vaccination. Since the major problem of enterotoxaemia vaccine lies with the duration of immunity, an attempt was made to develop a better enterotoxaemia vaccine using alum and oil as adjuvants and the efficacy was compared with presently available toxoid and alum precipitated vaccines in the target species, sheep.

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MATERIALS AND METHODS

The experiment was conducted in the Biological Products Division, ICAR-IVRI, Izatnagar, India during 2011-2013.

Bacterial cultures

In the present study, a highly toxigenic strain of *C. perfringens* type D was procured from the Division of Biological Standardization, Indian Veterinary Research Institute (IVRI), Izatnagar, Bareilly and was characterized by morphological, cultural and toxigenic properties. The molecular characterization was also done by polymerase chain reaction (PCR) using *etx* specific primers (F- 5′AAG GAT CCA AGT TTA GCA ATC GCA TCA GC3′; R - 5′TAC CTC GAG TTA TTT TAT TCC TGG TGC C3′).

Experimental animals

Clinically healthy Swiss albino mice of either sex weighing not less than 18-20 grams were procured from the Lab Animal Resource (LAR) section of IVRI and maintained under standard conditions. The experimental procedures for care and maintenance of live animals were approved by the ICAR-Indian Veterinary Research Institute, Izatnagar, India (No: 4929 dated 29/11/2011).

Clinically healthy sheep without a history of vaccination against clostridial diseases maintained in the animal shed of Biological Products division, IVRI, Izatnagar were used for immunization. This large animal experimentation was approved by CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals, India). Twelve animals were divided randomly into 4 groups as one control and three vaccine groups.

Production of vaccines

Toxoid vaccine (TV)

The production medium (Jayaraman and Mallick 1961) was inoculated with an actively growing seed culture of *C. perfringens* type D and incubated at 37°C for 24th. The culture was then tested for purity and aerobic sterility by microscopic and cultural examination. Subsequently, the pH of the culture filtrate was adjusted to alkaline and trypsinization was done to a final concentration of 0.25% and reincubated at 37°C for one hour. For getting the minimum lethal dose per ml of the product, random samples (2 ml) from each flask were centrifuged and 0.1 ml of various dilution of this supernatant was injected into two mice by intravenous route. The highest dilution producing lethality in both mice was taken as MLD/ml. Culture having above 3000 MLD/ml was formalized (0.5% formalin) and incubated at 37°C for 15 days for detoxification. This was stored at 4°C and used as the TV.

Alum precipitated vaccine (APV)

After adjusting the pH of 100 ml of TV to 6.5, sterile 5% alum solution at 40°C was added to a final concentration of 1% in the vaccine and maintained at room temperature (25°C) overnight with agitation. The product was labeled as APV and stored at 4°C.

Alum precipitated oil adjuvant vaccine (AOV)

Nine parts of sterile liquid paraffin were transferred aseptically to a commercial blender and one part of sterile molten lanolin was added to it. This was mixed for 20 minutes at room temperature as follows. For mixing, a hundred ml of APV was added to the blender (Bajaj, India) and emulsification was carried out at room temperature giving 4 runs of 5 minutes each, with 5 minutes gap between each run at medium speed switch (approx. 10000 rpm) at room temperature. The vaccine was stored at 4°C overnight and re-emulsified on the next day.

Quality testing of vaccines

Sterility, safety and stability testing of all three vaccines were done. For testing sterility, 1 ml of each vaccine was inoculated in 100 ml of nutrient broth and blood agar plate and incubated at 37°C for three days. The safety of the vaccines was tested in six mice and a continuous 10 days observation was made. For stability testing, all three vaccines were kept at 4°C as well as at room temperature for 14 days before immunization.

Immunization experiments

Keeping antigenic mass constant each of all sheep in the designated groups was immunized with a single dose of 2.5 ml TV, 3 ml APV and 5 ml AOV by deep intramuscular route. Serum collection was done on 0, 7, 14, 21, 30, 60, 90, 120, 150 days post-vaccination (DPV) and stored at -20°C until further use.

Immune response studies

Preparation of antigen (Epsilon toxin)

The clear supernatant from the production media was precipitated by 35% ammonium sulfate with stirring overnight. The precipitate after centrifugation was re-suspended in PBS and dialyzed against 0.01 M phosphate buffer (pH 7.4). The dialyzed sample was concentrated using polyethylene glycol-6000. Further purification was done by DEAE-cellulose anion exchange chromatography. The peak fractions were pooled concentrated and checked by SDS-PAGE, Western blotting and toxigenicity studies. Finally, the protein concentration of the formalized toxin was estimated.

Production of hyperimmune serum

Two ml of APV was injected subcutaneously in sheep as the first dose. Thereafter four injections of 3 ml of TV were given at weekly intervals. After the 4th injection, 2 ml of blood was collected to check the antibody titer. The sheep with good titer were finally bled; serum was separated and stored at -70°C until further use.

Indirect ELISA

The proper dilution of the antigen and serum was determined using the chequer-board method (Briggs and Skeeles 1984). Sera of sheep grown up in a clostridia-free environment was used as negative sera. 1: 10000 dilution of rabbit anti-sheep horseradish peroxidase (Sigma-Aldrich) was used as the

secondary antibody. Titre of the individual serum sample was expressed in positive/negative ratio (P/N ratio). Statistical analysis was done using the program SPSS 17.

Mouse neutralization test (MNT)

In MNT, 1ml of pooled sera from each group was mixed with 1ml of epsilon containing 300 MLD and kept at 37°C for half an hour. 0.2 ml of the mixture was given intravenously to two mice. 0.1 ml of the 300 MLD/ml toxin was injected into another two mice as control (British Pharmacopoeia 1993).

RESULTS AND DISCUSSION

Characterization of *Clostridium perfringens* type D

Clostridium perfringens type D showed typical morphological and cultural properties. Briefly, the organism appeared as large rectangular gram-positive bacilli, arranged singly, in short chains and sometimes in bundles. The organism was capsulated and non-motile. The organism was able to grow under microaerophilic conditions. The organism produced rich growth in Robertson's cooked meat broth at 37°C within 24-48 h. Molecular characterization yielded the desired 997bp product (Fig 1). The toxin titer was 3000 MLD/ ml.

Quality testing of vaccines

During purity testing of vaccines, it was found that the growth in the production flask was free from any contamination. Final vaccines inoculated in different media did not show any growth denoting their sterility. The sterility test in mice ensured the safety of all the prepared vaccines. AOV did not show the separation of different phases (oil and water) when kept at 4°C and room temperature indicating its stability.

Immune response studies

Vaccinated animals remained healthy for the whole duration of the study and all recorded normal weight gains. Moreover, none of the immunized animals showed untoward reactions at the injection site.

Preparation of antigen (Epsilon toxin)

In DEAE cellulose anion exchange chromatography, the fractions from tubes No. 5 to 15 were found to contain good concentrations of epsilon toxin (Fig 2). So these fractions were pooled. The protein concentration of the pooled fractions was 4.51 mg/ml. On SDS-PAGE analysis of purified toxin, a single band of 32 KDa was obtained (Fig 3). In Western blotting hyperimmune sera raised in sheep against TV and APV could detect the purified epsilon toxin (Fig 4).

Indirect ELISA

Optimal dilutions for antigen, sera and conjugate were found as 1:200, 1:100 and 1:10000 respectively. ELISA results are given in Table 1-4. AOV produced significant and persistent differences in titers. The highest P/N value was observed in AOV on the 28th DPV (1.74±0.11) followed by APV (1.66±0.05). There was a gradual rise from 7th (1.26±0.06) to peak value on 28th day (1.74±0.11) after which it almost remained persistent up to 150th DPV (1.43±0.15) although there was a minor reduction. TV produced an early high P/N value on the 21st day (1.59±0.19) followed by a fast reduction on subsequent days. APV showed a gradual increase in P/N value from the 14th day (1.35±0.11) to peak value on the 28th day (1.66±0.05) after that it showed decreasing trend and the P/N value on the 150th day was 1.07±0.06.

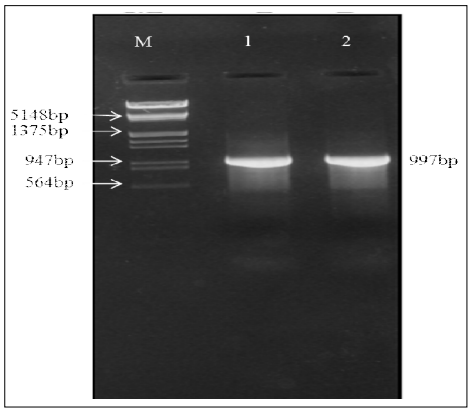

Fig 1: PCR amplification of *etx* gene. Lane 1, 2: Amplified *etx*, Lane M: DNA ladder (λ DNA/ *Eco*RI + *Hin*dIII double digest).

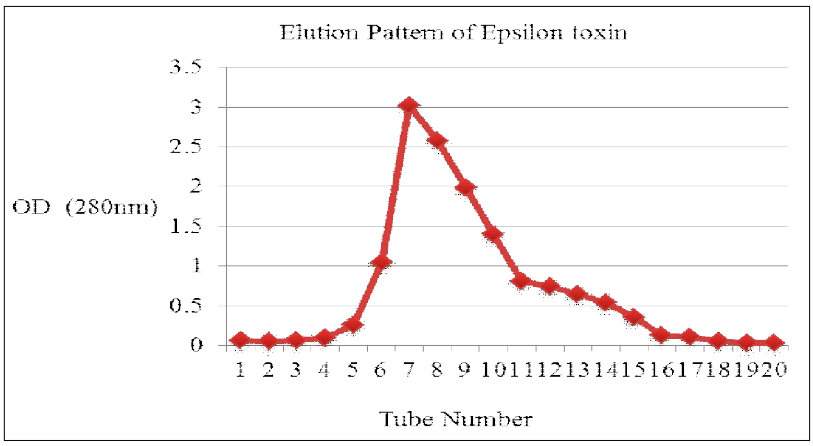

Fig 2: Elution pattern of epsilon toxin.

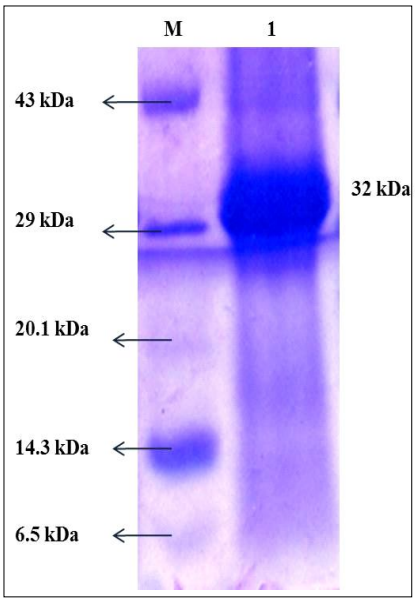

Fig 3: SDS-PAGE analysis of purified epsilon. Lane 1-3: Epsilon having 32kDa molecular weight, Lane M: Protein Marker.

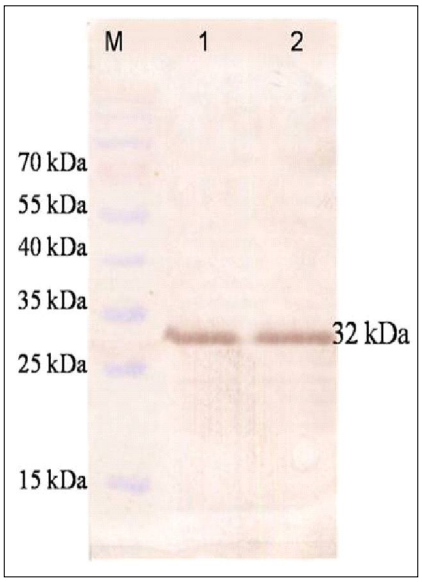

Fig 4: Western blotting of purified epsilon. Lane 1, 2: Purified Epsilon, Lane M: Protein Marker.

Mouse neutralization test (MNT)

MNT results of different vaccines are presented in Table 5. AOV showed positive MNT for the longest duration compared to other vaccines. In this case, mice did not die even after 150th DPV. APV and TV showed the death of mice on the 90th day and 60th day respectively.

Vaccination remains the main prophylactic measure against enterotoxaemia (Rosskopf-Streicher *et al.* 2004), the disease-causing large economic losses in small ruminant farming (Veschi *et al.* 2008). However, a lengthy immunization schedule and difficulty in tracing the animal for booster dose necessitate further research focusing on the protection and duration of immunity. The attempts to improve vaccine efficacy using liposome formulation have not been successful (Uzal *et al.* 1999). Although incomplete Freund's adjuvant was successful in enhancing immune response to epsilon toxin (Uzal and Kelly 1998), this causes strong side effects such as abscess and granuloma thus recommended only for laboratories. Kumar *et al.* (2009) showed that there was no significant difference between alum precipitated and aluminium hydroxide gel adsorbed enterotoxaemia vaccines in sheep. Bincy *et al.* (2011) reported that the duration of immunity provided by alum precipitated oil adjuvant hemorrhagic septicemia vaccine was longer with less antigenic mass. Keeping these facts a new formulation alum precipitated oil adjuvant enterotoxaemia vaccine was made and compared with presently available toxoid and alum precipitated vaccines in the target species, sheep. As humoral immune response against epsilon is the key factor for protection against enterotoxaemia (Kerry *et al.* 1979), the humoral response was assayed.

The gold standard test for measuring the protective antibody titers against epsilon toxin is the toxin neutralization test in mouse (MNT). Ripley (1983) and Rahman *et al.* (2001) had used MNT to estimate immune response against enterotoxaemia vaccination. However, apart from the ethical considerations on the use of live animals, this suffers many other disadvantages such as variation in animal sensitivity, cumbersomeness, slowness and higher cost (Kozaki *et al.* 1979; Henderson 1984). Thus a simple *in-vitro* test is necessary for vaccine manufacturing laboratories, animal health authorities and researchers (Uzal *et al.* 1997). So indirect ELISA (Uzal *et al.* 1997; Bentancor *et al.* 2009) and competitive ELISA (Wood 1991) were proposed as alternative methods to measure anti-epsilon titers. Thus, an indirect ELISA was also used in the present study along with MNT to monitor the immune response.

During MNT, AOV showed a positive reaction up to the last period of observation *i.e.* 5 months DPV indicating the ability for producing a satisfactory level of protective immune responses from 7 to 150 days after inoculation with a single dose. APV elicited better protection than TV though it was less than AOV revealing the synergistic role of adjuvants in inducing better immunity. TV showed protection up to 45th DPV which is in contradiction to the earlier reports of Jayaraman *et al.* 1971; Walker *et al.* 1992 and Bernath *et al.* 2004 who have reported protection only for 28 days with toxoid. Rahman *et al.* (2001) reported protection only up to 21 days after epsilon and beta toxoid vaccination. But Uzal *et al.* (1998) had reported that toxoid vaccines were giving variable immune responses.

In ELISA, the anti-epsilon antibody level was the highest among the AOV group followed by APV and TV. The AOV produced the highest ELISA titer and it persisted for a longer time than TV and APV. Though TV produced an early peak titer on the 21st day compared to the other vaccines, there

was a rapid fall in titer on subsequent days. The titer on the 120th day itself was less than the 7th day thus revealing the reason for the short-term immunity provided by TV. This fast rise and fall of antibody titer revealed the rapid dispersion of free antigen from injection sites. In the case of AOV, there was a gradual rise in titer from 7th (1.26±.06[a]) to peak value on 28th day (1.74±0.11) which was slightly decreased at the 150th DPV. APV elicited a better and persistent immune response than TV though it was less than AOV. This is in agreement with the observation of Kumar *et al.* (2009) who observed the enhancing effect of alum on anti-epsilon production. The gradual increase and decrease of antibody titer in the APV and AOV group might be due to the slow and gradual antigen release from the depot site which is the mechanism of adjuvanticity of aluminium compounds proposed by Edelman 1997. By the comparison between ELISA and MNT results it was found that a P/N value of 1.26 or more is required to neutralize 150 MLD/ml of epsilon toxin.

Another important consideration when assessing adjuvanted vaccines, particularly for use in meat-producing animals, must be the degree of local reaction at the injection site. Surprisingly, in the present study, the immunized sheep did not show any untoward reactions at the injection site

Table 1: Anti-epsilon ELISA titres (P/N ratio) of sheep immunized with TV.

Animal	DPV (Days post vaccination)								
	7	14	21	28	45	60	90	120	150
1	1.320	1.380	1.516	1.212	1.040	1.018	1.005	1.005	0.971
2	1.228	1.250	1.300	1.198	1.117	1.070	1.026	1.005	1.001
3	1.294	1.693	1.945	2.022	1.721	1.615	1.502	1.402	1.244
Mean±SE	1.280±0.03[a]	1.441±0.13[a]	1.590±0.19[a]	1.480±0.27[a]	1.290±0.22[a]	1.200±0.19[a]	1.18±0.16[a]	1.140±0.13[a]	1.070±0.09[a]

[a] represents no significant difference.

Table 2: Anti-epsilon ELISA titres (P/N ratio) of sheep immunized with APV.

Animal	DPV(Days post vaccination)								
	7	14	21	28	45	60	90	120	150
4	1.141	1.339	1.435	1.700	1.660	1.431	1.331	1.193	1.186
5	1.481	1.547	1.673	1.721	1.457	1.421	1.194	1.147	1.047
6	1.156	1.166	1.506	1.548	1.406	1.381	1.092	0.994	0.991
Mean+SE	1.26±0.11[abc]	1.35±0.11[bcd]	1.54±0.07[de]	1.66±0.05[e]	1.51±0.07[de]	1.41±0.02[cd]	1.20±0.07[abc]	1.11±0.06[ab]	1.07±0.06[a]

[a, b, c, d] and [e] represents significant difference level 1, 2,3, 4 and 5.

Table 3: Anti-epsilon ELISA titres (P/N ratio) of sheep immunized with AOV.

Animal	DPV (Days post vaccination)								
	7	14	21	28	45	60	90	120	150
7	1.248	1.274	1.416	1.645	1.716	1.656	1.472	1.228	1.228
8	1.305	1.416	1.660	1.964	1.665	1.630	1.934	1.822	1.723
9	1.202	1.229	1.447	1.620	1.630	1.624	1.533	1.330	1.330
Mean ± SE	1.26±0.06[a]	1.31±0.06[a]	1.51±0.08[ab]	1.74±0.11[b]	1.67±0.02[b]	1.63±.01[ab]	1.63±0.14[ab]	1.46±0.18[ab]	1.43±0.15[ab]

[a] and [b] represents significant difference level 1 and 2.

Table 4: Anti-epsilon ELISA titres (P/N ratio) of sheep immunized with different vaccines.

Vaccines	DPV (Days post vaccination)								
	7	14	21	28	45	60	90	120	150
TV	1.28±0.03[a]	1.441±0.13[a]	1.590±0.19[a]	1.480±0.27[a]	1.290±0.22[a]	1.200±0.19[a]	1.18±0.16[a]	1.140±0.13[a]	1.070±0.09[a]
APV	1.26±0.11[abc]	1.35±0.11[bcd]	1.54±0.07[de]	1.66±0.05[e]	1.51±0.07[de]	1.41±0.02[cd]	1.20±0.07[abc]	1.11±0.06[ab]	1.07±0.06[a]
AOV	1.26±0.06[a]	1.31±0.06[a]	1.51±0.08[ab]	1.74±0.11[b]	1.67±0.02[b]	1.63±.01[ab]	1.63±0.14[ab]	1.46±0.18[ab]	1.43±0.15[ab]

[a, b, c, d] and [e] represents significant difference level 1, 2,3, 4 and 5.

Table 5: Mice neutralization test (MNT).

Vaccines	DPV (Days post vaccination)								
	7	14	21	28	45	60	90	120	150
TV	ND	ND	ND	ND	ND	D	D	D	D
APV	ND	ND	ND	ND	ND	ND	D	D	D
AOV	ND	ND	ND	ND	ND	ND	ND	ND	ND

*ND- Not died, **D- Died, ***In all tables DPV- Days post vaccination.

maybe because of the deep intramuscular administration. On contrary to this, some authors (Green *et al.* 1987; Stokka *et al.* 1994; Mamak and Aytekin 2009) observed adverse reactions following enterotoxaemia vaccination.

CONCLUSION

The findings of this study, thus, unfolded the synergistic role of adjuvants in enterotoxaemia vaccination along with the development of oil adjuvant alum precipitated enter otoxaemia vaccine which can give better and prolonged immune response without any side effects. However, a better understanding of the high level of protection observed in AOV needs further exploration on a large scale and on various molecular determinants. Further studies are also required for increasing the epsilon toxoid antigenic mass and reducing the dose of oil-based vaccine so that vaccine load can be reduced to 1 ml of final formulation with equivalent immune response. The present limitations of the current vaccines can be very well resolved with this formulation and field application of such vaccine may reduce the incidence of disease to the lowest possible level. Such a study can lead to the further development of these better vaccines so that these can be finally transferred to the hands of farmers.

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