Indian Journal of Animal Sciences **83** (10): 1014–1017, October 2013/Article

Improved immunogenicity and protection against enterotoxaemia in sheep by saponin and aluminium hydroxide gel co-adjuvanted vaccine

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Received: 14 January 2013; Accepted: 14 May 2013

ABSTRACT

Immuno-prophylaxis is the most effective control measure for enterotoxaemia, a devastating disease of small ruminants caused by *Clostridium perfringens* type D. Realizing the problems associated with short term immunity of current enterotoxaemia vaccines, a new formulation using saponin and aluminium hydroxide gel as adjuvants (ASV) was made. Immune response generated in the principal target species, sheep was evaluated in comparison with saponified vaccine (SV) and presently available toxoid vaccine (TV) by indirect ELISA, mice neutralization test (MNT) and standard tube agglutination test (STAT). Out of the 3 vaccines tested, ASV generated the maximum protective immune response with persistent high titers of neutralizing antibodies as denoted by ELISA and STAT. Furthermore, MNT revealed that ASV was able to create protection up to 11 months while it was only for 30 days in TV and SV. This is the first report of a vaccine which offers protection for 11 months against enterotoxaemia in sheep.

Key words: *Clostridium perfringens*, Enterotoxaemia, ELISA, Sheep, Vaccine

Clostridium perfringens type D, the principal cause of enterotoxaemia, is responsible for wide spread ovine and caprine mortality leading to heavy economic losses to small ruminant farming throughout the world (Titball 2009). Owing to the rapid and lethal progression, treatment is having little value making vaccination as the most effective control measure (Greco *et al*. 2005). Even though the disease can be well prevented by epsilon toxoid, double initial dose is currently recommended for both sheep and goats, followed by a booster every year in sheep and every 3–4 months in goats (Smith and Sherman 1994). The difficulty in tracing animals for booster dose and requirement of heavy input of resources necessitate the development of a safe and reliable enterotoxaemia vaccine that can produce a long lasting protective immune response with persistent high titers of neutralizing antibodies (Rai *et al*. 2012). The most common adjuvants in veterinary vaccines are aluminium salts (Gupta *et al*. 1995) and saponin (Mastelic *et al*. 2010). Aguilar and Rodriguez (2007) stated that simultaneous use of 2 or more adjuvants with different mechanism of action produces a greater immune response than the sum of their individual effects. In the light of above facts, our objective was to develop a better enterotoxaemia vaccine through the synergistic effect of saponin and aluminium hydroxide gel.

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MATERIALS AND METHODS

Healthy Swiss albino mice of either sex weighing not less than 18–20 g, procured from the institute, were housed in hygienically controlled environment. Animal ethics committee of Indian Veterinary Research Institute, approved this study. Apparently healthy sheep were procured commercially and maintained in animal shed of the division with *ad lib*. feed and water. The experimental procedures involving large animals were carried out according to the recommendations and the approval of CPCSEA.

Bacterial strain: A highly toxigenic strain of *C. perfringens* type D was procured from the Division of Biological Standardization, IVRI, Izatnagar and characterized by morphological, cultural and toxigenic properties. Molecular characterization was done by *etx* specific primers (Frd- 5'AAG GAT CCA AGT TTA GCA ATC GCA TCA GC-3', Rev 5'-TAC CTC GAG TTA TTT TAT TCC TGG TGC C-3').

Preparation of vaccines: The production medium (Jayraman and Mallick 1961) was inoculated with actively growing seed culture and incubated at 37° C for 24 h. The growth was tested for purity and aerobic sterility followed by trypsinization to a final concentration of 0.25% and re-

incubation at 37°C for 30 min. Mouse minimum lethal dose (MLD) per ml was deliberated by using serial dilutions of bacteria free clear toxin. Afterwards, the culture was inactivated by formalinization (final concentration 0.5%) with subsequent incubation at 37°C for 14 days. The final product was tested for sterility, toxicity and stability and used as TV. For the preparation of SV, 0.85ml of the sterilized (by filtration) saponin solution in saline (10 mg/ml) was mixed with 30ml of TV at room temperature for 4 h. ASV was prepared by mixing 45ml of TV, 37ml of sterilized 3% aluminium hydroxide gel, 3ml of the filtered saponin (10mg/ml) and 15ml of sterile distilled water. The complete mixture was then agitated slowly at room temperature for 4. All the 3 vaccines were then tested for sterility and stability. Safety testing was done by injecting 0.5ml of each vaccine i/p into 6 mice (Schedule F1, Drugs and Cosmetics Act, GoI 1979).

Experimental design: Animals were randomly divided into 4 groups (4 sheep/group) as TV, SV, ASV and control groups. Keeping antigenic mass constant, each group was immunized with single dose of 2.5ml TV, 2.5ml SV, and 4ml of ASV respectively by deep i/m route. A regular observation was made for any untoward effects every day after the vaccination. Serum was collected from all the animals on 0 day and weekly for 1 month and thereafter monthly up to 12 months.

Preparation of antigen: The method of Rai *et al*. (2012) was followed for the purification of epsilon toxin from the pure culture of *C. perfringens* type D and used as antigen in indirect ELISA. The whole cell antigen prepared by re-suspending the formalized bacterial pellet in normal saline to give turbidity equivalent to Brown's opacity tube No.2 was used as antigen for STAT.

Serology: The method of Uzal *et al*. (1997) was followed for the analysis of immune response by indirect ELISA. The proper dilution of antigen (stock- 1mg/ml), serum and conjugate was determined using chequer board method (Uzal *et al*. 1997). ELISA results were expressed as positive/negative ratio (P/N ratio). MNT was carried out for accessing antitoxic units in the pooled sheep sera as per British Pharmacopoeia (1993). In STAT, serial 2-fold dilutions of sera were made in normal saline to which 0.5 ml of formalinized plain antigen was added. After shaking, overnight incubation was done at 37°C. STAT titre was expressed as the reciprocal of the highest dilution showing 100% clearing. Statistic analysis of results of all 3 tests was done using SPSS 10 software. One-way ANOVA was used to compare the means between groups with a value $P < 0.05$ set to represent a significant difference and a value $P < 0.01$ set to represent a highly significant difference.

RESULTS AND DISCUSSION

Realizing the need for an improved vaccine against enterotoxaemia a new formulation was made in the present study by peeping into the synergistic effect of aluminium hydroxide gel and saponine co-adjuvantation.

Experimental animals and bacterial cultures: All vaccinated animals remained healthy and showed no adverse effects for the whole duration of the study. During characterization of the strain, typical morphological, cultural and molecular (Fig.1A) properties were observed. The toxin titer was 2000MLD/ml.

Quality testing of prepared vaccines: All prepared vaccines were found sterile, safe in mice as well as stable at 4°C, room temperature and at 37°C for 14 days.

Antigens: Elution pattern of epsilon toxin is given in Fig. 1B. Analysis of purified toxin by SDS-PAGE, showed a single band of 32 KDa (Fig. 1C). The protein concentration was 1 mg/ml.

Serology: As humoral immune response against epsilon is the key aspect for protection in enterotoxaemia (Kerry and Craig 1979) and the efficacy of the vaccines were evaluated by monitoring humoral immune response. MNT is the gold standard test for measuring protective anti-epsilon titres (WHO 1985). The highest MNT value was obtained with ASV in which mice survived even after 11th month dpv, demonstrating the ability of ASV for inducing a longer protective immunological response by a single dose. Conversely, in both TV and SV even 90th dpv, serum could

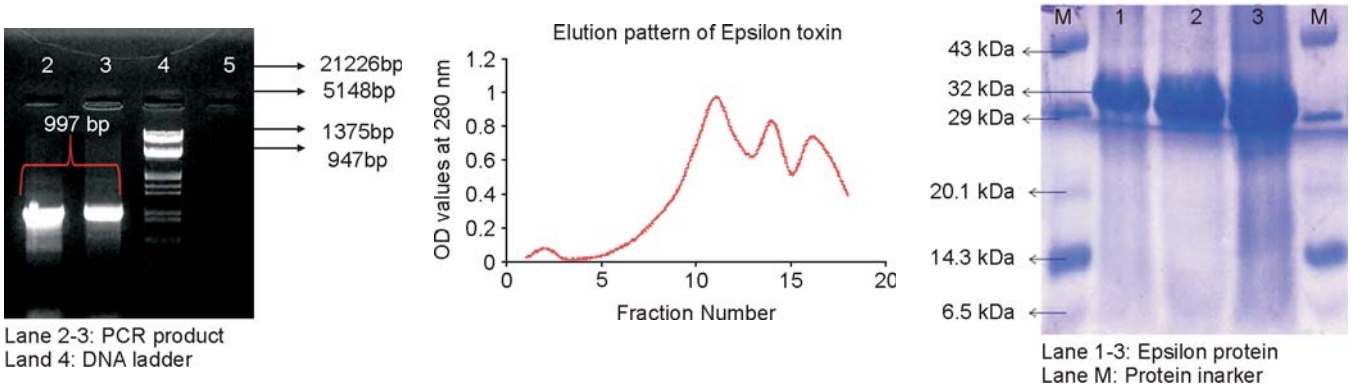

Fig. 1. A–C: **A.** PCR amplification of *etx* gene. **B.** Elution pattern of epsilon. **C.** SDS-PAGE analysis of purified epsilon.

Table 1. Anti- epsilon toxin ELISA titers of sheep immunized with different adjuvant vaccine

Vaccine	Days post vaccination										
	7	14	21	28	60	90	120	150	180	210	240
TV	1.48 ±0.21^{abc}	1.85 ±0.31^{ab}	1.98 ±0.27^{a}	1.77 ±0.26^{ab}	1.54 ±0.19^{abc}	1.43 ±0.15^{abc}	1.28 ±0.10^{bc}	1.05 ±0.12^{bc}	1.03 ±0.12^{c}		
SV	1.42 ±0.40	1.52 ±0.44	1.47 ±0.37	1.39 ±0.27	1.29 ±0.22	1.17 ±0.16	1.13 ±0.17	0.97 ±0.10	0.82 ±0.23		
ASV	1.51 ±0.22^{abc}	2.10 ±0.29^{ab}	2.33 ±0.19^{ab}	2.50 ±0.15^{a}	2.44 ±0.08^{abc}	2.16 ±0.08^{bc}	2.07 ±0.08^{bc}	1.73 ±0.18^{bc}	1.68 ±0.17^{bc}	1.62 ±0.15^{c}	1.56 ±0.12^{c}

a, b and c represents significant difference level 1, 2 and 3.

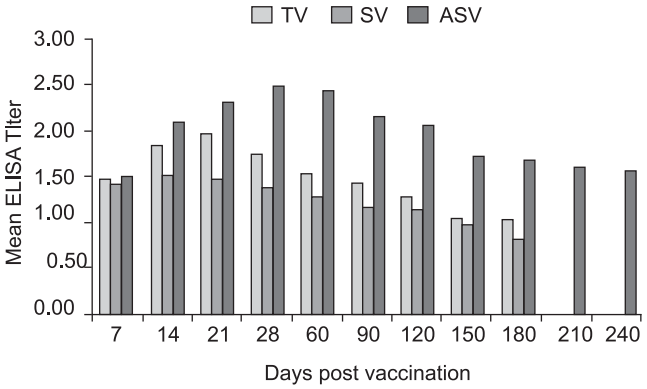

Fig. 2. Mean P/N ratio against different vaccines.

not neutralize 300MLD of the toxin. Nevertheless, MNT is considered to be far away from ideal immunological test due to the disadvantages such as variation in animal sensitivity, high cost, cumbersomeness, and requirement of large number of animals and specialized technicians (Sobrinho *et al*. 2010). Thus, there is a need to define and develop alternative strategies to measure the protective anti-epsilon titer. Consequently, indirect ELISA and STAT, 2 simple immunological tests were also exploited in the present study. In ELISA, optimum dilutions of the antigen, serum and conjugate were found to be 1: 400, 1: 400 and 1: 10, 000 respectively. Controls showed no sero-conversion throughout the experiment. Antibody titers at the start were essentially same in all groups ($P > 0.05$) (Table 1; Fig. 2). Among all vaccines, TV produced an early high titre on14th day (1.85±0.31) with peak value on 21st day (1.98±0.27) followed by a sudden fall subsequently. Antibody titre produced by SV showed almost similar trend with the peak titre on 14th day (1.52±0.44). In case of ASV there was a gradual increase in titre from 14th day (2.10±0.29) to peak value on 28th day (2.50±0.15) then it receded gradually (11th month after immunization). When ELISA and MNT results compared, it was found that P/N value of 1.44 or more is required to neutralize 150MLD/ml of toxin. This showed that the results of ELISA are comparable to MNT in detecting anti epsilon antibodies. The correlation coefficient between ELISA and STAT results (Table 2) was found to be 0.419 and 0.424 for TV and ASV respectively, indicating the suitability of this simple test for getting a preliminary idea about sero-conversion. Thus, the present study indicated that ELISA and STAT can be used in lieu of MNT. However, ELISA test is more feasible as it is more sensitive (Kumar *et al.* 2009).

In our study, TV and SV showed almost similar trend in the development of antibody with early high titre on 14th day and rapid fall on subsequent days, revealing the demand for booster dose associated with these vaccines. Subsequent to the report of Moreno *et al*. (1999) on the synergestic effect of QS–21(purified saponin) and alum in malaria vaccine, there was an increasing interest in the use of saponin as adjuvant (Rivera *et al*. 2003). Their unique capacity to stimulate balanced TH1/TH2 immune response and cytotoxic T-lymphocytes against exogenous antigens makes them ideal for use in various veterinary vaccines (Mastelic *et al*. 2010). Keeping in view of these data, a new formulation using saponin and aluminium hydroxide gel as combined adjuvant (ASV) was made in the present study and evaluated in sheep. We got the maximum protective immunological response

Table 2. Anti- epsilon toxin log $_{10}$ STAT titers of sheep immunized with different vaccine

Vaccine	Days post vaccination								
	7	14	21	28	60	90	120	150	180
TV	0.25±0.25^{b}	1.68±0.19^{a}	1.68±0.08^{a}	1.60±0.25^{a}	1.10±0.65^{ab}	0.95±0.60^{ab}	0.88±0.54^{ab}	0.80±0.48^{ab}	0.25±0.25^{b}
SV	1.60±0.12^{ab}	1.98±0.33^{a}	1.98±0.31^{a}	1.10±0.65^{abc}	0.80±0.52^{abc}	0.48±0.48^{bc}	0.40±0.40^{bc}	0.00±0.00^{c}	0.00±0.00^{c}
ASV	1.83±0.08^{abc}	2.20±0.17^{a}	2.28±0.14^{a}	2.28±0.08^{a}	2.05±0.15^{ab}	1.75±0.15^{abc}	1.60±0.30^{bc}	1.30±0.30^{c}	1.30±0.30^{c}

a, b and c represents significant difference level 1, 2 and 3.

with persistent high titers of neutralizing antibodies in ASV group. Most strikingly, ASV could create protection up to 11 months while it was only for 60 days in enterotoxaemia vaccine (TV). This might be the depot effect of saponin and aluminium hydroxide gel on the injection site to stimulate the antigen presenting cells favorably (Rivera *et al*. 2003).

In conclusion, our findings suggested that the newly formulated ASV against enterotoxaemia can offer high titre of protective antibodies for prolonged period in sheep. Thus, the present study on the immunogenicity and protective efficacy of ASV in sheep is beneficial for sheep and goat breeders. Further, large scale field trials of this vaccine are warranted.

ACKNOWLEDGEMENTS

The authors are grateful to the Director and Joint Director (Academic and Research) of the Institute for providing the research facilities to carry out the investigation.

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