

PREPARATION AND COMPARATIVE EVALUATION OF DIFFERENT ADJUVANTED TOXOID VACCINES AGAINST ENTEROTOXAEMIA

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The objective of this study was to prepare and evaluate toxoid vaccines against enterotoxaemia in goats. Pure culture of *Clostridium perfringens* type D was used, already isolated and characterized by Institute of Microbiology, University of Agriculture Faisalabad, Pakistan. Epsilon toxin was extracted; the extracted toxin was subjected to LD₅₀ determination and then with the help of formalin inactivation was done. The resulting toxoids were adjuvanted with two adjuvants including Montanide ISA 206 and Aluminium hydroxide gel (Alum). The pathogenicity and immunogenicity of prepared toxoid vaccines and commercial vaccine procured from VRI, Lahore, Pakistan were studied and compared initially in an experimental trial on rabbits and then in field trials on goat. Field trial was conducted on 72 goats divided into four equal groups (n=18). The Indirect Haemagglutination Test (IHA) was used to evaluate the antibody titer and challenge protection test was performed in rabbits. The results showed that antibody titers were significantly higher ($P < 0.05$) for in group vaccinated with Montanide ISA-206 adjuvanted toxoid vaccine. The geometric mean titer of Montanide group was 203.19, 456 and 670 at 14, 21 and 28 days post vaccination as compared to 181.02, 298.63 and 456.14 of Alum group on same days. Montanide vaccinated group also exhibited highest protection percentage (100%) post challenge as compared to 85.71%, 71.42% and 14.29% of group B, C and D, respectively.

Keywords: *Clostridium perfringens*, Enterotoxaemia, Montanide, Aluminium hydroxide, vaccine

INTRODUCTION

Livestock sector holds a premier place in the economy of the world and also has a significant contribution in the economy of Pakistan. It accounts for 55.1% of the value addition in agriculture sector and 11.5% to Pakistan gross domestic product (GDP), which is higher than the contribution made by crop sector (Khan *et al.*, 2009; Babar *et al.*, 2012). The role of this sector is pivotal in the rural economy of Pakistan as about 40 % rural population of the country is engaged in livestock (Nosheen *et al.*, 2011). The health and wellbeing of livestock is of supreme importance. It is firmly believed that optimum production cannot be achieved without protection from the different livestock diseases particularly enterotoxaemia (Chandran *et al.*, 2010; Wang *et al.*, 2011). The outbreaks of these diseases are disasters for the farmers and may put them out of their business by imposing excessive economic losses. So it is quite necessary to prevent these infectious diseases to ensure wellbeing and prosperity of people by minimizing economic losses. Prophylactic vaccination can be graded as one of the best strategy for controlling infectious diseases.

Sheep and goat farming contribute major part of livestock in Pakistan. In our country it provides milk, meat, mohair, skin and manure. For last many years, it has been observed that the growth of sheep and goat industry decreased (Greco *et*

al., 2005). Among these infectious diseases enterotoxaemia has proven one of the most horrible diseases of small ruminants. Incidence of this disease is 2-8 % but the case fatality rate may go up to 100% (Radostits, 2006). Many factors are responsible for the outbreaks of this disease including improper vaccination poor feeding management (Veschi *et al.*, 2006). Proper vaccination and better feed management is the only way to combat this disease (Metre, 2010).

Clostridium perfringens type D belongs to genus *Clostridium*, which is Gram-positive, rod-shaped and anaerobic bacteria forming sub terminal spores (Brazier *et al.*, 2002; Hughes *et al.*, 2007). *C. perfringens* is divided into five different types (A to E), depending upon the production of major toxins *i.e.* alpha, beta, epsilon and iota (Sayeed *et al.*, 2005). Epsilon toxin of *C. perfringens* type D is considered as one of the most potent bacterial toxins. It is usually lethal for small ruminants especially sheep (Souza *et al.*, 2010). Pakistan has about 28.1 Million sheep and 61.5 Million goats. To vaccinate this huge population large number of vaccine production units are required, presently only a few public sector veterinary vaccine production units are present in the country. These existing units are insufficient to meet requirements of the whole country. At present there is no single toxoid vaccine available against enterotoxaemia in Pakistan. So keeping in view the

importance of the subject present study was conducted for the first time in country for the preparation and comparative evaluation of different adjuvanted toxoid based vaccines against enterotoxaemia.

MATERIALS AND METHODS

Procurement of Microorganism: Pure culture of *C. perfringens* type D was procured from Institute of Microbiology, University of Agriculture Faisalabad. For mass scale production it was grown on blood agar under anaerobic conditions at 37°C for 24 hours.

Raising of hyper-immune sera: Six healthy rabbits of homogenous nature were purchased from local market and housed in laboratory animal house of Institute of Microbiology under standard animal welfare conditions. Commercially available enterotoxaemia vaccine of Veterinary Research Institute (VRI) Lahore, Pakistan was administered in increasing dose at alternate days for 10 days. The rabbits were bled at day 14 post last injection, serum was separated through centrifugation and stored at 4°C till further use. The procured antigen was confirmed as *C. perfringens* Type D through serum neutralization test by using hyperimmune serum.

Extraction of toxin: A loopful inoculum of *C. perfringens* type D was subjected into 10 ml of thioglycollate broth (Oxoid UK) and incubation was carried out at 37°C for 18 hours. The resultant broth was inoculated into 100 ml of Duncan and Strong medium (Oxoid UK) and cultured for 4 hours under same conditions. The whole 110 ml culture was inoculated into 1 liter of Duncan and Strong medium (Oxoid UK) and incubated for 24 hours at 37°C aerobically. Then the culture was centrifuged in temperature controlled centrifugation machine at 8000 rpm for 10 minutes and the supernatant was collected. Purified Trypsin (Sigma USA) was added in the supernatant to a final concentration of 10 µg per ml and incubated at 37°C for 30 minutes. The supernatant fluid with Trypsin was aliquoted and refrigerated at 4°C till further use.

Quantification of toxin: The extracted toxin was serially tenfold diluted using phosphate buffered saline (pH 7.3). Twenty five homogenous rabbits were purchased from local market and divided into five equal groups. Each group was given 0.5 ml of each dilution intraperitoneally (I/P) using 25 gauge needle. Rabbits were observed for mortality for three days and the titers were calculated through Reed and Munch method and expressed as LD₅₀ units/ml (Reed and Muench, 1938).

Toxoid and vaccine preparation: Formalin was added @ 0.4% in to the toxin and incubated at room temperature for two weeks. The complete inactivation of toxin was confirmed by injecting 1 ml of undiluted toxoid intraperitoneally in rabbits. Inactivated toxoid was divided in two aliquots; one aliquot was adjuvanted with equal volume

of Montanide ISA 206 and homogenized for one hour by magnetic stirrer. Similarly, equal volume of aluminium hydroxide gel and toxoid were mixed and homogenized. The products were collected in sterile vials and labeled accordingly. Vaccines were stored at 4°C till further use.

Stability and sterility testing: Stability of the vaccines was tested by keeping them at different temperatures *i.e.* 25, 35 and 45°C for 45 days. For sterility testing toxoid vaccines were inoculated on different bacterial (Trypticase soy broth) and fungal (Sabouraud's agar) culture media to check the presence of any contamination.

Experimental trials in rabbits: Twenty eight rabbits of homogenous nature were purchased and divided into four equal groups *i.e.*, A, B, C and D. rabbits of group A and B were injected with 1 ml of Montanide ISA 206 adjuvanted toxoid vaccine and aluminium hydroxide gel adjuvanted toxoid vaccine, respectively. Group C was kept as positive control and injected with commercially available vaccine of Veterinary Research Institute (VRI) Lahore, Pakistan. Group D served as negative control. The blood was drawn and serum was separated from each group before vaccination, at days 14, 21 and 28 post injections for the presence of antibodies. The antibody titer for each group was measured through indirect haemagglutination (IHA) test. Then these rabbits were challenged with 1 ml of potent epsilon toxin of *C. perfringens*. The rabbits were observed for three days for any morbidity and mortality. The protection percentage was calculated in each group.

Field trial in goats: Seventy two goats were selected for field trial. These animals were tested for the presence of any parasitic infestation through stool sample screening test and divided into four equal groups *i.e.* A, B, C and D. Group A was given Montanide ISA-206 adjuvanted toxoid vaccine while group B was injected with aluminum hydroxide gel adjuvanted toxoid vaccine. Group C was kept as positive control and injected with commercially available vaccine Veterinary Research Institute (VRI) Lahore, Pakistan. Group D served as negative control.

Determination of humoral immune response: The immunogenicity of injected vaccines was determined through antibody titers using indirect haemagglutination (IHA) test. Blood was drawn before vaccination, at days 14, 21 and 28 post vaccination from each group and serum was separated. The sonicated antigen of *C. perfringens* type D was used for sensitization of human blood group 'O' erythrocytes in the presence of 1% glutaraldehyde as coupling agent. The Geometric Mean Titers (GMTs) of each group before and at different days post vaccinations were calculated and compared statistically.

Challenge protection test: Protection percentage was determined by administering ten times the dose of potent LD₅₀ epsilon toxin. The rabbits were observed for three days for any mortality. After three days protection percentage was calculated based upon number of rabbits surviving in each

group. Group A exhibited 100% protection from the challenge while protection percentage for group B, C and D was 85.71, 71.42 and 14.29%, respectively.

Statistical analysis: Geometric mean titer of all the groups was calculated using Perkins method (Perkin, 1958). Later on two factor ANOVA was applied and comparison was conducted through Tukey's test.

RESULTS AND DISCUSSIONS

Vaccination is the most important and valuable aid to overcome the infectious diseases. Inactivated whole cell bacteroid vaccines have been most extensively used for this purpose but they have certain draw backs including administration of high dose, local inflammatory reactions and short term immunity (Chandran *et al.*, 2010). In recent few years toxoid vaccines have emerged as effective alternate to bacteroid vaccines. This study was also a similar effort to prepare toxoid vaccine against *Cl. Perfringens* type D enterotoxaemia as for the first time in Pakistan.

Pure culture of *Cl. perfringens* was identified and confirmed by using different morphological, biochemical and cultural test. In our study, we observed that these were Gram positive rods, beta hemolysis in blood agar, sub-terminal spore and production of gelatinase and lecithinase. Similar findings have been depicted by Shome *et al.* (2006) and Das *et al.* (2012).

Humoral immune response was calculated through antibody titer determination by IHA in experimental trial as well as field trial. Experimental trial was performed in laboratory animal house Institute of Microbiology, University of Agriculture Faisalabad, Pakistan. The serological response increased significantly ($P < 0.05$) at different days in Groups A, B and C. The antibody titer of group D was non-significantly different along the days (Table 1). Group A produced the highest mean antibody titer of all the groups but the difference was statistically non-significant ($P > 0.05$) for groups B and C while significant ($P < 0.05$) for group D. The geometric mean titer at the start of trial was 1.64, 1.49, 1.49 and 1.64 for groups A, B, C and D, respectively. Group A exhibited a sudden increase in titer with a GMT of 172.28,

312.07 and 689.10 at days 14, 21 and 28 post vaccination. Similarly, group B also showed a significance increase in titer having 141.32, 312.07 and 624.14 GMT on days 14, 21 and 28 after vaccination. Geometric mean titer of group C was relatively lower as it was 128, 282.65 and 565.29 on same days. In challenge protection test, group A exhibited 100% protection from challenge infection while protection percentage of groups B, C and D was 85.71%, 71.42% and 14.29%, respectively as shown in Table 2.

Field trials were performed in different villages of District Faisalabad, Pakistan. The findings were similar to those of experimental trial. The goats of group A vaccinated with Montanide adjuvanted vaccine showed the highest mean antibody titer (1.999 ± 0.129) followed by group B vaccinated with Alum adjuvanted vaccine (1.907 ± 0.11) and group C vaccinated with commercial bacteriod vaccine (0.171 ± 0.031) (Table 3 and Table 4). The control group has significantly lower mean antibody titer (0.171 ± 0.031) than the vaccinated groups. Similarly, the increase in antibody titer was significant ($p < 0.05$) in group A, B, C whereas it was non-significant ($p > 0.05$) in control group.

The results showed that toxoid vaccines are more efficacious against enterotoxaemia as both the toxoid vaccines gave higher geometric mean titer as compared to commercial bacteroid vaccine. Similarly among toxoid based vaccines Montanide ISA 206 showed significantly higher titer. Montanide ISA 206 is a double oil emulsion so vaccines having it as adjuvant possess lower viscosity. These vaccines have the ability to boost both short as well as long term immune response (Sutmoller *et al.*, 2003). Other water based or single oil emulsions usually do not show long short term humoral immune response (Cox and Coulter, 2005). Water-in-oil-in-water emulsions usually induce humoral immune response for both short and long term (Aslam *et al.*, 2012). The antigen present in aqueous phase interacts with the immune system to produce immediate immune response. On the other hand antigen present in the internal oil phase persists for longer period of time and is responsible to maintain immune response for longer period of time.

Table 1. Comparison of mean antibody titer \pm SE (\log_{10}) of all vaccinated groups under experimental trials

Group	Days				Mean \pm SE
	1	14	21	28	
A	0.215 \pm 0.086e	2.236 \pm 0.090cd	2.494 \pm 0.086b	2.838 \pm 0.090a	1.946 \pm 0.201A
B	0.172 \pm 0.090e	2.150 \pm 0.079d	2.494 \pm 0.086b	2.795 \pm 0.086a	1.903 \pm 0.201A
C	0.172 \pm 0.090e	2.107 \pm 0.093d	2.451 \pm 0.079bc	2.752 \pm 0.079a	1.871 \pm 0.198A
D	0.215 \pm 0.108e	0.172 \pm 0.090e	0.215 \pm 0.056e	0.129 \pm 0.061e	0.183 \pm 0.039B
Mean \pm SE	0.194 \pm 0.044D	1.666 \pm 0.171C	1.914 \pm 0.192B	2.129 \pm 0.225A	

Group A was injected with Montanide ISA 206 adjuvanted toxoid vaccine. Group B was given Alum adjuvanted toxoid vaccine. Group C and D were injected with Commercial vaccine and Normal saline respectively. Means sharing similar letters in a row or in a column are statistically non-significant ($P < 0.05$). Small letters represent comparison among interaction

means and capital letters are used for overall mean. Groups A, B and C showing statistically significant ($P < 0.05$) increase in antibody titer on days 14, 21 and 28.

Table 2. Geometric Mean Titer of all vaccinated groups under experimental trial

Group	Days				Mean GMT
	1	14	21	28	
A	1.64	172.28	312.07	689.10	88.30
B	1.49	141.32	312.07	624.14	79.97
C	1.49	128.00	282.65	565.29	74.25
D	1.64	1.49	1.64	1.35	1.52
Mean GMT	1.56	46.39	81.98	134.50	

Rabbits of group A (Montanide adjuvanted vaccine) exhibited highest geometric mean titer followed by group B (Alum adjuvanted vaccine), group C (Commercial vaccine) and group D (negative control) were injected with Commercial vaccine and Normal saline, respectively.

Table 3. Comparison of mean antibody titer \pm SE (\log_{10}) of all vaccinated groups under field trials

Group	Days				Mean \pm SE
	1	14	21	28	
A	0.201 \pm 0.064gh	2.308 \pm 0.064def	2.659 \pm 0.061ab	2.826 \pm 0.065a	1.999 \pm 0.129A
B	0.234 \pm 0.067g	2.258 \pm 0.061ef	2.475 \pm 0.052cd	2.659 \pm 0.050ab	1.907 \pm 0.119B
C	0.284 \pm 0.079g	2.191 \pm 0.068f	2.392 \pm 0.051cde	2.525 \pm 0.065bc	1.848 \pm 0.113B
D	0.268 \pm 0.084g	0.251 \pm 0.061g	0.134 \pm 0.056gh	0.033 \pm 0.023h	0.171 \pm 0.031C
Mean \pm SE	0.247 \pm 0.036D	1.752 \pm 0.108C	1.915 \pm 0.126B	2.011 \pm 0.139A	

Means sharing similar letters in a row or in a column are statistically non-significant ($P < 0.05$). Small letters represent comparison among interaction means and capital letters are used for overall mean. Group A showing higher titer ($P < 0.05$) overall and at different days as compared to other three groups.

Table 4. Geometric Mean Titer of all vaccinated groups under field trial

Group	Days				Mean
	1	14	21	28	
A	1.59	203.19	456.14	670.40	99.66
B	1.71	181.02	298.63	456.14	80.63
C	1.92	155.18	246.33	335.20	70.47
D	1.85	1.78	1.36	1.08	1.48
Mean	1.76	56.47	82.20	102.58	

Rabbits of group A (Montanide adjuvanted vaccine) exhibited highest geometric mean titer followed by group B (Alum adjuvanted vaccine), group C (Commercial vaccine) and group D (negative control) were injected with Commercial vaccine and Normal saline, respectively.

All the animals were in good health status before the start of trials. No adverse reactions were observed after vaccination as well. However animals injected with Montanide ISA-206 adjuvanted toxoid vaccine developed local inflammation. These inflammatory lumps were about 3-5 cm in diameter and subsided completely within one week. These findings were quite similar to those observed by earlier scientists (Uzal *et al.*, 1999).

Conclusion: From above results it is concluded that toxoid vaccines are more effective against Enterotoxaemia than bacterin. Further among toxoid vaccines Montanide ISA-206 adjuvanted vaccine provided better results than alum adjuvanted toxoid vaccine so Montanide adjuvanted toxoid vaccine may be recommended for field use. As little or no

data is available about toxoid vaccines in Pakistan hence further investigation are necessary on different aspects of this subject.

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