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Development and Evaluation of *Clostridium perfringens* Type D Toxoid Vaccines

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ABSTRACT

The aim of this project was to prepare a toxoid vaccine from the prevailing pathogenic strains of *Type D Clostridium perfringens* strains and evaluate its immune responses in rabbits, goats and sheep. *C. perfringens* were isolated from enterotoxaemia suspected sheep and goats from the endemic areas of Khyber Pakhtunkhwa Province, Pakistan during 2016. The isolates were initially identified through colony characters, Gram staining and biochemical tests. The identified isolates were quantified on blood agar and confirmed through PCR. Toxins were extracted, quantified, formalized and adjuvanted with aluminium hydroxide gel. Safety, sterility and stability of the toxoid were ascertained. Bacterin toxoid of *C. perfringens* type D was procured from Veterinary Research Institute Peshawar. The immunogenicity of toxoid vaccines and bacterin toxoid vaccine were studied and compared initially in rabbits and then in goats and sheep. Experimental trials were conducted on 54 healthy animals of each species and were divided into three equal groups (n=18). The antibody titers were evaluated with indirect haemagglutination test. The results indicated significantly higher ($P< 0.05$) immune titer in animal group vaccinated with toxoid vaccine prepared from pathogenic isolates and provide best protection against challenge infection.

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Authors' Contribution
MAK, AZD, SBK and SGB designed the study. MAK, AZD, SBK, SGB, IH and NUK executed the experimental work and analyzed the samples. IUK, NU, KH and AUK helped in data analysis and article drafting.

Key words
***Clostridium perfringens* type D, Enterotoxaemia, Indirect haemagglutination test, Vaccine.**

INTRODUCTION

Clostridium perfringens type D is the main cause of enterotoxemia (Songer, 2006), responsible for huge economic losses in sheep and goats farming globally due to high fatality rates, treatment costs and decreased productivity (Nillo, 1980; Greco *et al*., 2005). The clostridial organisms live ormally in gastrointestinal tract (GIT) of sheep and goats and other wild ruminants (McClane *et al*., 2006), but the intensity of occurrence is low. Normally these organisms produce toxins in minute quantity which is expelled from the body through normal peristaltic movement or circulating antibodies *C. perfringens* type D is also responsible for producing epsilon toxin that affects various systems of body in small ruminants. Sudden change in feeding or environmental factors may alter the internal environmental conditions of GIT where *C. perfringens* type D may rapidly multiply and produce a huge amount of epsilon toxin resulting in production of disease (Nillo, 1986; Smith and Sherman, 1994). There is a big difference in pathaophysiological mechanism of enterotoxemia in sheep and goat (Blackwell and Butler, 1992). Clinically, enterotoxemia is characterized by sudden onset of death in sheep, while in goats enterotoxemia is reported to be of acute and chronic forms (Fernandez and Uzal, 2003). On postmortem examination, neurological and respiratory lesions are mainly reported in sheep whereas in goats, mainly intestinal lesions are seen (Radad and Khalil, 2011). Among different infectious diseases of small ruminants, enterotoxaemia has been reported to be the most horrifying disease. Reported incidence rate of enterotoxemia in small ruminants is 2-8%, while the case fatality rate may reach 100% (Radostits, 2006). A number of factors including improper vaccination schedule or low-quality vaccine, low hygienic condition, no colostrum fed, fatty animals and low quality of feeding and watering management (Veschi et al., 2006; Khan et al., 2017) may cause the outbreaks of enterotoxaemia. To control this malady, it is necessary to provide quality vaccination and proper feeding

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management (Metre, 2010). *Clostridium perfringens* is a gram positive, anaerobic rod shaped bacteria belonging to genus *Clostridium* and is responsible for production of terminal spores (Hughes *et al*., 2007). *C. perfringens* has been divided into five groups (A –E) based on toxin types such as alpha, beta, epsilon and iota (Sayeed *et al*., 2005). Among different types of toxins of *C. perfringens* type D, epsilon toxin is the most lethal bacterial toxin for small ruminants especially for sheep (Souza *et al*., 2010). The very short clinical course of this disease makes therapeutic interventions challenging and often impossible. Thus, the ideal method of control is vaccination (Chandran et al., 2010; Dela-Rosa et al., 1997; Uzal, 1997; Uzal and Kelly, 1999; Bernath et al., 2004). Toxoid vaccines are widely available commercially and have been used extensively over the past decades for use in domesticated sheep and goats. The vaccines are prepared by toxoiding *C. perfringens* culture filtrate, and are likely to contain a range of proteins in addition to the toxoid. The vaccines are often given with an aluminum hydroxide adjuvant to improve efficacy (Uzal and Kelly, 1999).

In Pakistan, there is a huge goat (70.3 millions) and sheep (29.8 Million) population (GoP, 2010). To minimize the losses in these animal population of small ruminants proper immunization is required to improve the health status of these animals. This project was designed to develop an effective vaccine to control future losses from *C. perfringens* type D in small ruminants.

MATERIALS AND METHODS

The study protocol was approved by the Animal Ethical Committee (Reference No. 5121, dated 09.03.2016).

Bacterial cultures

Pathogenic isolates of *C. perfringens* type D were obtained from intestinal samples taken from enterotoxaemia suspected dead sheep and goats from district Mardan of Khyber Pakhtunkhwa province, Pakistan. The samples were cultured on TCA media (HiMedia Laboratories Pvt. Ltd., India) in anaerobic condition at 37°C for 24 h. The isolates were initially identified by colony morphology, Gram staining and biochemical tests kit (remel RapID ANA II system test kit Lenexa, USA). Pathogenic strains of *C. perfringens* were identified having more than 104–107 CFU/g colony count on blood agar (Kalender *et al*. 2005). *C. perfringens* type D isolates were confirmed by PCR using genomic DNA isolated from the pathogenic strains, cpa and etx specific primers
F- 5-TGC TAA TGTTAC TGC CGT TGA TAG-3;
R- 5-TGC TAA TGT TAC TGC CGT TGA TAG-3 and F-5-ATT AAA ATC ACA ATC ATT CAC TTG-3;
R-5-CTT GTG AAG GGA CAT TAT GAG TAA-3 (Khan *et al*., 2017).

Extraction and quantification of toxin

Selected strain of *C. perfringens* type D was grown on Robertson's Cooked Meat medium anaerobically for 24 h. The supernatant of the liquid culture was recovered through centrifugation in temperature controlled centrifugation machine for 10 min at 8000 rpm and stored at 4°C (Tahir *et al*., 2013).

For quantification of toxin the extracted toxin was serially diluted tenfold using phosphate buffered saline (pH 7.3). The dilutions so achieved were 1:10, 1:20, 1:40, 1:80, 1:160, 1:320, 1:640, 1:1280, 1: 2560 and 1:5120. 0.5 ml of the each diluted toxin was given intraperitoneally to a group of five rabbits. Rabbits were observed for mortality for 24-72 h. The titers were calculated through Reed and Muench (1938) method and expressed as LD_{50} units/ml.

Toxoid preparation

C. perfringens type D was cultured in 5000 mL capacity flask in Robertson's Cooked Meat broth. The flasks were sealed with liquid paraffin and incubated at 37°C for 24 h. The growth in flasks was tested for purity. Purified (0.1%) trypsin (Sigma USA) was added to the flask containing type D culture. Formalin (0.4%) was added to the flask having *C. perfringens* type D and was stored at room temperature for 14 days. Inactivated toxoids were adjuvanted with equal volume of aluminum hydroxide gel and were preserved in sterile vials at 4°C.

Safety, sterility and stability testing

For safety, inactivation of toxin was tested by injecting 2 mL of toxoid intraperitoneally to 3 healthy rabbits. Inactivation of the toxin was confirmed with no mortality or morbidity for 24-72 h of the inoculation. For sterility testing toxoids were inoculated on different media *i.e.* nutrient agar, nutrient broth, Tarrozi and Sabouraud's agar culture media to check the presence of any contamination. Stability of the toxoid was tested after keeping the toxoids at different temperatures *i.e.* 25, 35 and 45°C for 45 days (Raana, 2007).

Preparation of antibodies

C. perfringens culture was centrifuged at 2,000 rpm for 30 min at 4°C. The sediment was washed with phosphate buffer saline (0.15 molar, pH 7.2). Bacterial suspension was sonicated (Ultrasonic Homogenizer (MODEL 300VT (BioLogics, Inc. Manassas, Virginia 20109, United States of America) twice for 5 min at 20 kHz and 105W, for

sensitization of human RBCs of blood group O for indirect haemagglutination test (IHA). Gluteraldehyde at 1% was mixed with sensitized human RBCs of blood group O (Tahir *et al*., 2013).

Measuring antibody titer

For measuring antibodies titer the collected serum was heated to 56°C for 30 min to eliminate any non-specific protein. 50 µL of normal saline was added into the first well of 96 well U-bottom titter micro plates (Flow Labs, UK). Serum sample (50µL) was added into the 1st well. Two fold dilutions were made by removing 50 µL from first well and added to second well and mixed well. Similarly, two fold dilutions were made until 11th well. From 11th well, 50 µL was removed and discarded. The last well was kept negative control. 50µL of 1% gluteraldehyde fixed sensitized human RBCs of blood group O were added in all wells. The plate was incubated for 2 h at room temperature with number of times gentle tapping in order to ensure even suspension of RBCs. Haemagglutination was noted at every well bottom. The end point dilution having agglutination was reciprocal of dilution to be taken as antibody titer. Antibody titer was calculated by noticing the agglutination well number and comparing in the standard table. Geometric mean titer was calculated by taking geometric mean of antibody titers (Tahir *et al*. 2013).

Efficacy testing

Efficacy of toxoid was testing in rabbits, sheep and goats. For evaluation C. perfringens type D toxoids, fifty four homogenous healthy rabbits were used for immunogenicity trial, divided into three groups (A, B and C)each of 18 rabbits. Animals of group A were inoculated with 1 ml of aluminum hydroxide gel adjuvanted C. perfringens type D conventional toxoid (CPCT), Group B was kept as positive control by inoculating C. perfringens type D bacterin toxoid (CPBT) of the commercially available enterotoxemia vaccine, prepared in Veterinary Research Institute (VRI) Peshawar, Pakistan while Group C was kept as negative control. The blood was collected on days 0, 7, 14 and 28 of the vaccination for determination of immune titer. Immunized rabbits were challenged with ten times the dose of potent LD_{50} on day 28. The rabbits were observed for three days for any mortality. Protection percentage was calculated upon number of rabbits surviving in each group. Vaccines provoking highest antibody titers were regarded as better than the other.

For evaluation of *C. perfringens* type D toxoids in goats and sheep, thirty six healthy goats were divided into three groups (A, B and C) each of 18 goats. Animals of group A were inoculated with 1 ml of aluminum hydroxide gel adjuvanted *C. perfringens* type D conventional toxoid (CPCT), Group B was kept as positive control by inoculating *C. perfringens* type D bacterin toxoid (CPBT) of the commercially available enterotoxemia vaccine, prepared in Veterinary Research Institute (VRI) Peshawar, Pakistan while Group C was kept as negative control. The blood was collected on days 0, 7, 14 and 28 of the vaccination for determination of immune titer. A protocol with similar specification was adopted for evaluation the immune titer in healthy sheep against *C. perfringens* type D.

Statistical analysis

For statistical design the means of antibody titers among different groups in vaccine therapy experiment was analyzed by one way ANOVA at the level of 95% confidence interval ($P<0.05$) using SPSS 21 version of computer statistical program.

RESULTS AND DISCUSSION

Table I shows the comparison of antibody titers of rabbits, sheep and goats. In rabbits, CPCT vaccine was found with significantly higher immune titer throughout the trial following which were CPBT and unvaccinated (UV) groups. The maximum Geometric mean titer in CPCT was found to be 436.0 on 28th day and CPBT indicated 226.67 while –ve control indicated baseline antibody titer on 28th day post vaccination. The rabbits of both groups vaccinated with CPCT and CPBT were evaluated for protection % on 28th day post accination. Groups of rabbits received CPCT exhibited 100% protection from the ten times higher dose of potent LD_{50} challenge while protection percentage for groups of rabbits received CPBT was 70%, served as positive control and -ve control groups indicated 20% protection percentage respectively. Antibody titer among trial groups on 28th day was compared to check the difference of immune response produced in different vaccine groups. Trial showed significant difference ($P<0.05$) among all the groups at 28th days post inoculation.

In sheep the maximum geometric mean titer in CPCT was found 756.0 and CPBT indicated 326.5 on 28th day while –ve control indicated baseline antibody titer on 28th day in sheep (Table I).

Vaccination trial in goats indicated significant difference ($p<0.05$) among three groups on days 14, 21 and 28th post vaccination. The maximum geometric mean titer in CPCT group was found 444.67 and CPBT indicated 263.33 while the -ve control group indicated baseline antibody titer on 28th day post vaccination against *C. perfringens* type D.

Table I.- Geometric mean titer comparisons of *C. perfringens* type D vaccines in rabbits, sheep and goats.

Days	Vaccine groups		
	CPTC	CPBT	-ve control
Rabbits			
0	1.67±3.2 [a]	1.67±3.2 [a]	1.67±3.2 [a]
7	62.33±5.2 [a]	54.33±6.6 [b]	2.00±3.9 [b]
14	184.67±15.2 [a]	75.0±6.2 [b]	2.00±3.9 [c]
21	311.83±50.7 [a]	166.0±12.1 [b]	1.67±3.2 [c]
28	436.0±86.6 [a]	226.67±41.3 [b]	2.00±3.9 [c]
Sheep			
0	1.67±3.2 [a]	1.67±3.2 [a]	2.00±3.9 [a]
7	89.0±7.8 [a]	70.0±6.1 [b]	1.67±3.2 [c]
14	205.17±47.2 [a]	109.50±9.9 [b]	2.00±3.9 [c]
21	373.33±53.3 [a]	184.67±15.2 [b]	2.00±3.9 [c]
28	658.0±22.1 [a]	326.5±66.1 [b]	1.67±3.2 [c]
Goats			
0	2.00±3.9 [a]	2.67±5.2 [a]	2.67±5.2 [a]
7	64.83±7.8 [a]	59.50±10.1 [b]	2.67±5.2 [b]
14	135.9±98 [a]	94.17±22.5 [b]	2.00±3.9 [c]
21	369.33±30.5 [a]	172.33±15.2 [b]	2.67±5.2 [c]
28	444.67±69.3 [a]	263.33±46.6 [b]	2.67±5.2 [c]

CPCT, *Clostridium perfringens* conventional toxoids; CPBT, *Clostridium perfringens* bacterin toxoids made in VRI Peshawar. Different superscripts indicate significant difference ($P<0.05$).

The results of our study indicated that toxoid vaccines made from prevalent isolates were more efficacious against enterotoxaemia as the toxoid vaccine gave higher geometric mean titer as compared to commercial bacterin toxoid vaccine. Similar findings have been shown by Shome *et al*. (2006), Das *et al*. (2012) and Greco *et al*. (2005). Humoral immune response was calculated through determination of the antibody titer by indirect haemagglutination in small ruminants which is similar to the study conducted by Tahir *et al*. (2013) in rabbits and small ruminants by IHA in their study. The use toxoid vaccines for the protection against *C. perfringens* infection in domesticated sheep and goats are widely available commercially and have been used extensively over the past decades. The vaccines are prepared by toxoiding *C. perfringens* culture filtrate, and are likely to contain a range of proteins in addition to the toxoid. The vaccines are often given with an aluminum hydroxide adjuvant to improve efficacy (Uzal and Kelly, 1999). Similarly, in our study toxoids were prepared from prevalent pathogenic strain and adjuvant with aluminum hydroxide for better protection. The toxoid adjuvanted with oil persists for longer period in body tissues and maintains an immune response for longer period of time in comparison of water based toxoid. The immunogen present in aqueous phase produce immediate immune response while immunogen present in oil phase persists for longer period and is responsible for immunity for the longer period (Sutmoller *et al*., 2003; Aslam *et al*., 2012). The short course of the disease mostly makes therapeutic interventions impossible therefore necessitate further research in the area of enterotoxaemia vaccine development focusing on the protection and duration of immunity (Aiello, 2003), to combat with the present lengthy and costlier schedule of the present vaccination strategy (Monika *et al*., 2011).

CONCLUSION

Conventional toxoid vaccine developed from local pathogenic isolates was found highly immunogenic, produced significantly higher ($P< 0.05$) immune titer and provide best protection against challenge infection in rabbits.

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Statement of conflict of interest

All authors declare no conflict of interest.

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