Kafkas Universitesi Veteriner Fakultesi Dergisi
ISSN: 1300-6045 e-ISSN: 1309-2251
Journal Home-Page: http://vetdergikafkas.org

Kafkas Univ Vet Fak Derg
27 (4): 475-482, 2021
DOI: 10.9775/kvfd.2020.25732

RESEARCH ARTICLE

Preparation and Evaluation of Alum Precipitate and Oil Adjuvant Multivalent Vaccines Against *Clostridium perfringens* [1]

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[1] *This research work was supported by HEC Pakistan through technology development fund (TDF) and a part of approved project: Code no-TDF-02-028*

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Article ID: KVFD-2020-25732 Received: 11.03.2021 Accepted: 17.06.2021 Published Online: 17.06.2021

Abstract

Enterotoxaemia is one of the hazardous diseases of the livestock. In Pakistan prophylaxis failure is due to the vaccination with type D monovalent vaccine. There is a need to develop a cost effective multivalent vaccine against enterotoxaemia using characterized toxinotypes isolated from field. Indigenously (Punjab, Pakistan) characterized *Clostridium perfringens* toxinotypes A (MW551947.1), B (MW332247.1) and D (MW332258.1) (n=1 each) were used. These toxinotypes were used to produce higher amount of alpha, beta and epsilon toxin units under culture conditions. Colony forming units (CFU) of each bacterium was determined through the standard plate count method and 10^6CFU/mL bacteria were used for vaccine dose. Monovalent, bivalent and multivalent oil adjuvant and alum precipitate vaccines were prepared. Formulated vaccines were passed the stability, sterility and safety test. Bacterin plus toxoid oil adjuvant vaccine produced higher (868.25±3.54 IU/mL) antibody titer at 28th day post vaccination in rabbits and 100% protection was observed after challenge. Multivalent bacterin plus toxoid oil adjuvant vaccine was used in field trials. Increased antibody response was detected after 4 months in sheep (1294.81±1.90 IU/mL) and goats (1091.85±2.51 IU/mL). During the experimental and field trials commercial vaccine did not produced higher antibody titer. Multivalent bacterin plus toxoid oil adjuvant vaccine proved as an excellent candidate for vaccination of animals against *C. perfringens* diseases, and it produced specific and efficient immune response to be used in field.

Keywords: *Alpha, Bacterin, Beta, Epsilon, Toxoid, Vaccine*

Clostridium perfringens'e Karşı Alum Presipite ve Yağ Adjuvanlı Multivalan Aşıların Hazırlanması ve Değerlendirilmesi

Öz

Enterotoksemi, çiftlik hayvanları için tehlikeli hastalıklardan birisidir. Pakistan'daki profilaksinin başarısızlığı tip D monovalan aşıdan kaynaklanmaktadır. Sahadan izole edilen ve karakterize edilmiş toksinotipler kullanılarak enterotoksemiye karşı uygun maliyetli bir multivalan aşı geliştirmeye ihtiyaç vardır. Yöreye özgü (Punjab, Pakistan) karakterize edilmiş *Clostridium perfringens* toksinotipleri, A (MW551947.1), B (MW332247.1) ve D (MW332258.1) (n=1 her biri için) kullanıldı. Bu toksinotipler, kültür ortamında yüksek miktarda alfa, beta ve epsilon toksinlerinin üretiminde kullanıldı. Her bakterinin koloni oluşturan birimleri (KOB) standart plak sayım yöntemiyle belirlendi ve aşı dozu olarak 10^6 CFU/mL kullanıldı. Yağ adjuvanlı ve alum presipite monovalan, bivalan ve multivalan aşılar hazırlandı. Formüle edilen aşılar stabilite, sterilite ve güvenlik testlerinden geçirildi. Bakterin + toksoid yağ adjuvanlı aşı, tavşanlarda aşılamadan sonraki 28. günde yüksek (868.25±3.54 IU/mL) antikor titresine yol açtı ve eprüvasyon sonrası %100 koruma gözlendi. Saha çalışmalarında multivalan bakterin + toksoid yağ adjuvanlı aşı kullanıldı. Aşılamadan 4 ay sonra koyun (1294.81±1.90 IU/mL) ve keçilerde (1091.85±2.51 IU/mL) antikor yanıtında artış saptandı. Deneysel ve saha çalışmaları sırasında ticari aşının daha yüksek antikor titresi üretmediği gözlendi. Hayvanların *C. perfringens* enfeksiyonlarına karşı aşılanmasında multivalan bakterin + toksoid yağ adjuvanlı aşının mükemmel bir aday olduğu kanıtlandı ve bu aşı sahada kullanılmak üzere spesifik ve etkili bir bağışıklık yanıtı üretti.

Anahtar sözcükler: *Alfa, Bakterin, Beta, Epsilon, Toksoid, Aşı*

How to cite this article?

Tariq M, Anjum AA, Sheikh AA, Awan AR, Ali MA, Sattar MMK, Hussain S, Ali T: Preparation and evaluation of alum precipitate and oil adjuvant multivalent vaccines against *Clostridium perfringens. Kafkas Univ Vet Fak Derg,* 27 (4): 475-482, 2021.
DOI: 10.9775/kvfd.2021.25732

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Introduction

Clostridium perfringens is a Gram positive, rod shaped, nonmotile, spore forming pathogenic anaerobic bacteria of human and domestic animals [1]. *C. perfringens* is divided into 5 types as A, B, C, D and E on the basis of major toxins (a, b, e, i) and minor toxins [1,2]. Gas gangrene, necrotic enteritis and enterotoxaemia are the most common symptoms of alpha toxin, which causes hemolysis, platelet aggregation, blood vessels contractions, superoxide generation, cytokine storm and ultimately cause death [3]. Lethal dose 50 (LD_{50}) of the alpha toxin per kilogram of mouse after intravenous injection was 3 mg [4]. Epsilon toxin of *C. perfringens* is the third most dangerous toxin among the clostridial toxins. Its 50% lethal dose (LD_{50}) was 50-110 ng per kg in mice [5]. The epsilon toxin was classified as the potential bio war and bioterrorism agent by the Center of Disease Control and Prevention (CDC) of theUnited States [6]. In sheep and goats, the epsilon toxin is a causative agent of enterotoxaemia [7]. Beta toxin is an etiological agent in necrotizing enterocolitis and also involves in enterotoxaemia [8]. It was reported that this toxin had a LD_{50} as 310 ng/kg [9].

Enteric infections caused by *C. perfringens* in sheep, goats and other ruminants are called enterotoxaemia because of the absorption of the toxins via intestine into the circulation. It is a frequent disease of sheep and goats and its prevalence rates are between 24.13 and 100% [10]. Enterotoxaemia is distributed worldwide and endemic in Pakistan as well [11]. Bacteria are normally present in intestinesin low concentration, however dietary changes (diet rich in carbohydrate) may lead to proliferation of the microorganism and ultimately toxin production [12].

Enterotoxaemia is one of the dangerous infectious diseases of the livestock. This disease has 2-8% incidence rates and 100% case fatality rate [13]. Most of the factors are responsible for the disease outbreak e.g. improper vaccination, change in diet and poor feed management [14]. Vaccination with accurate antigens and improvements on feed management are the only ways to struggle with the disease [15]. At present the disease is being handled by treatment of animals using antibacterial drugs but prognosis is poor. A monovalent clostridial vaccine is being practiced in the country for prophylaxis. This vaccine is prepared using type D which is not providing complete cover. There are genetic differences between field types and vaccine type of Clostridia, the major reason for prophylaxis failure in Pakistan. Dealing with the high economic impact of clostridiosis, prevention of it, is a big challenge for farmers [13].

The development and production of conventional clostridial vaccines involves expensive, time consuming and dangerous processes of detoxification, purification and antigen concentration steps [16]. Furthermore, the continued selection of the toxigenic strains that produce high titers of toxin is necessary [17]. There is a need to develop a cost effective vaccine using indigenously characterized toxinotypes isolates from field. The production of vaccine will help to combat enterotoxaemia in Pakistan. For vaccination of huge number of animals in Pakistan there is need for the development of new production units. Currently existing units are insufficient to fulfill the requirements [18]. An effort was done for preparation of a cost effective multivalent vaccine using prevailing toxinotypes of *C. perfringens* in sheep and goats in Pakistan. An effective vaccine from indigenous *C. perfringens* isolates will be available for sheep and goats to protect them against *C. perfringens* diseases.

Material and Methods

Indigenously characterized *C. perfringens* (field isolates from Punjab, Pakistan) toxinotypes, A (MW551947.1), B (MW332247.1) and D (MW332258.1) (n=1 each) were procured under the project TDF 02-028, from the Institute of Microbiology University of Veterinary and Animal Sciences (UVAS), Lahore, Pakistan. These toxinotypes were used for preparation of multivalent toxoid and bacterin + toxoid clostridia vaccine(s). Type A, B and D isolates were previously confirmed on the basis of the 16SrRNA gene polymerase chain reaction (PCR) after sequence analysis and sequence submission to GenBank NCBI, these (MW551947.1, MW332247.1 and MW332258.1) accession numbers were received.

Vaccine Preparation

Vaccine was prepared by following the method described by Saadh et al.[18] with minor modifications. One Liter reinforced clostridial medium (RCM) broth (6.5 pH) was inoculated separately with bacterial inoculum (10% v/v) (concentration was adjusted as McFarland No. 1) of *C. perfringens* type A, B and D. The RCM broth was supplemented with glucose (0.2%), vitamin mixtures, mineral mixture (0.2%), tween 80 (0.3%) along with sodium chloride (0.75%) and sodium acetate (0.3%). Inoculated culture flasks were incubated at 37°C for 24 h in an CO_2 incubator with ~80-90% CO_2. Following the incubation, representative (100 mL) volumes were taken from broth culture. It was centrifuged at 8000 rpm for 10 min. Supernatant was separated and purified trypsin (10 µg per milliliter) was added for epsilon toxin activation and incubated for 60 min at 37°C. After the incubation, it was stored at 4°C. Alpha and epsilon toxins hemolytic units per milliliter (HU/mL) were quantified through a hemolytic system by using washed 1% sheep RBCs [19] and beta toxin cytotoxic units per milliliter (CU/mL) were estimated on BHK 21 cell line following the method of Nagahama et al.[20]. Bacteria colony forming units (CFU) were counted by the standard total plate count method [21]. Bacteria and toxins were inactivated using formaldehyde (0.4% v/v). After 2 weeks of incubation, inactivation was checked by inoculating the bacteria on blood agar and injecting 0.5 mL of each toxin into a healthy rabbit. Oil (Montanide ISA

70) and alum adjuvant (20% stock solution) was added into the inactivated culture and toxins up to 50% and 3% concentration, respectively. After proper mixing, vaccine mixtures were filled in sterile labelled bottles and stored at 4°C. Each vaccine was prepared with standard CFU per mL of bacterin and toxins units quantified through assays. Toxin units and bacterial CFU per vaccine dose is represented in *Table 1-A* and *B*.

Evaluation of Vaccine

Vaccine(s) were evaluated for sterility, safety and stability according to the OIE manual of diagnostic tests and vaccines for terrestrial animals (chapter 1.01.08, 2018) [22]. For sterility testing fluid thioglycolate medium (FTM) with 0.5% beef extract and soybean casein digest medium (SCDM) were used for bacterial (37°C) and fungal (25°C) growth, respectively and the media were incubated for 14 days. At intervals during the incubation and after 14 days of incubation, inoculated media tubes were examined for the evidence of microbial growth. For safety testing of vaccine, recommended dose of vaccines was administered intramuscularly (IM) and subcutaneously (SC) to rabbits. For adverse localized reaction, the rabbits were observed for 30 days. For stability testing, vaccine vials were stored at 4°C. Emulsions were stored for 6 months without any significant loss of potency. Efficacy testing was conducted in rabbits following the method of Saadh et al.[18]. Oil and alum adjuvant vaccines were injected to rabbits (n=5 for each vaccine) intramuscularly (IM) and subcutaneously (SC), respectively.

Experimental Trials in Rabbits

All animals experiments were approved by the institutional Ethical Review Committee for the Animals, University of Veterinary and Animal Sciences, Lahore, Pakistan and carried out according to the International Ethics Law and Regulations. All efforts were made to minimize the animal sufferance.

Experimental trials were conducted in rabbits (n=70, 1 kg) according to the report of Saadh et al.[18] with some modifications. A total of 14 groups of rabbits were constituted according to the type of vaccine and control *(Table 2)*. Each group contained 5 rabbits. One mL of each vaccine was injected to rabbits and blood was collected at day 0 before the vaccination, 14th, 21st and 28th day after the vaccination. Serum was separated and anti-toxin (antibody) titer (IU/mL) was determined by an indirect enzyme linked immune sorbent assay (ELISA). Commercially available imported vaccine and sterile saline solution was injected to positive control and negative control groups, respectively. Grouping of rabbits for vaccine experimental trials was represented in *Table 2*.

Challenge Test

The immunized rabbits were challenged with a particular toxinotype of *C. perfringens* broth culture containing toxin (having double amount of toxin without formalin inactivation) post vaccination at 28th day and they were kept under observation for three days for mortality. Multivalent bacterin and toxoid oil adjuvant vaccine found to be the most effective in this experiment and selected for field trials in sheep and goat.

Field Trials in Sheep and Goats

Field trials were conducted in sheep and goats (n=80, 40/each). These sheep and goats were screened for the presence of any parasitic infestation and each of them was divided into two groups (Goats A, Goats B, Sheep A and Sheep B). One mL of vaccine was injected subcutaneously to Goat A and Sheep A group (n=20 each). Similarly a commercially available imported vaccine was injected to Goat B and Sheep B group (n=20 each) by the same route. Blood of the animals was collected before and after the vaccination. Anti-toxin (antibody) titer was measured by ELISA and continuously monitored till the 6-9 months with an interval of one month [18]. Indirect ELISA for anti-bodies detection was performed following the method of Bentancor et al.[23]. Optical density (OD) was measured immediately after the addition of the stop solution at 450 nm.

Statistical Analysis

Data obtained from antibody titer was analyzed through one way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMR) using statistical package for the social sciences (SPSS) version 20.0.

Results

Titers of toxins (in culture supernatant) produced under culture conditions (as mentioned above) were 2048.22±0.11 HU/mL (alpha toxin) (MW551947.1), 1052.46±0.18 CU/mL (beta toxin) (MW332247.1) and 512.25±0.06 HU/mL (epsilon) (MW332258.1) observed. These toxins titers were used in vaccine formulation. Toxins units per vaccine dose were presented in *Table 1-A* and *B*. Alpha toxin inactivation test result represented that toxin titer was 64.89 HU/mL after one week. Epsilon toxin inactivation result represented that toxin titer decreased to 35.54 HU/mL. After 14 days, titers of the alpha and epsilon toxins were observed zero. For the beta toxin inactivation, cytotoxicity assay represented that the beta toxin titer after 1 week was 45.67 CU/mL and zero at 14 days post inactivation. Colony forming units of *C. perfringens* types A, B and D were calculated by anaerobic plate count. Bacterial CFU was adjusted to 10^6CFU/mL. Bacteria were inactivated by 0.4% formalin (v/v) and after 36 h of the incubation the inactivated bacterial culture was inoculated on Perfringens agar base. No growth was observed indicating complete inactivation of the bacteria by formalin. *C. perfringens* toxoid and bacterin plus toxoid vaccines were prepared using formulations as already

Table 1 A. *Formulation of toxoid vaccines (oil adjuvant and alum precipitate)*

Sr. No	Vaccines	Antigen (toxins)	Toxins Units/ Vaccine Dose	Volume of Antigen Containing CFS(µL)/ Vaccine Dose	Volume of Oil Adjuvant (µL)/ Vaccine Dose	Volume of Alum Adjuvant (µL)/ Vaccine Dose	Volume of PBS (µL) in Alum Vaccine/ Vaccine Dose	Volume of Single Dose (µL)
1	Monovalent Toxoid (oil adjuvant vaccine)	Alpha	1024.11±0.05	500	500	-	-	1000
2	Monovalent Toxoid (alum precipitate vaccine)				-	150	350	
3	Bivalent Toxoid (oil adjuvant vaccine)	Alpha + Epsilon	1024.11±0.05 + 256.12±0.03	500	500	-	-	
4	Bivalent Toxoid (alum precipitate vaccine)				-	150	350	
5	Multivalent Toxoid (oil adjuvant vaccine)	Alpha + Beta + Epsilon	1024.11±0.05 + 256.12±0.03 + 526.23±0.09	500	500	-	-	
6	Multivalent Toxoid (alum precipitate vaccine)				-	150	350	

CFS: *cell free supernatant;* ***PBS:*** *phosphate buffer saline*

Table 1 B. *Formulation of bacterin plus toxoid vaccines (oil adjuvant and alum precipitate)*

Sr. No	Vaccines	Antigen				Volume of Antigen/ Vaccine Dose	Volume of Oil Adjuvant (µL)/ Vaccine Dose	Volume of Alum Adjuvant (µL)/ Vaccine Dose	Volume of PBS (µL) in Alum Vaccine/ Vaccine Dose	Volume of Single Dose (µL)
		Toxin Units/Vaccine Dose		Bacteria CFU/ Vaccine Dose						
1	Monovalent Bacterin and Toxoid (oil adjuvant vaccine)	Alpha	1024.11±0.05	Type A	10^6	500	500	-	-	1000
2	Monovalent Bacterin and Toxoid (alum precipitate vaccine)						-	150	350	
3	Bivalent Bacterin and Toxoid (oil adjuvant vaccine)	Alpha + Epsilon	1024.11±0.05 + 256.12±0.03	Type A + Type D	10^6 + 10^6	500	500	-	-	
4	Bivalent Bacterin and Toxoid (alum precipitate vaccine)						-	150	350	
5	Multivalent Bacterin and Toxoid (oil adjuvant vaccine)	Alpha + Beta + Epsilon	1024.11±0.05 + 256.12±0.03 + 526.23±0.09	Type A + Type B + Type D	10^6 + 10^6 + 10^6	500	500	-	-	
6	Multivalent Bacterin and Toxoid (alum precipitate vaccine)						-	150	350	

CFU: *colony forming units;* ***CFS:*** *cell free supernatant;* ***PBS:*** *phosphate buffer saline*

described *(Table 1B)*. Vaccines were proved to safe as there was no localized reaction observed at site of the injection in rabbits. Vaccines remained stable after storage at 4ºC. Vaccines were pure, white, and stick to glass like paint. Emulsions did not display the signs of cracking. All vaccines passed the sterility test. No growth of bacteria and fungi was observed in fluid thioglycolate broth and soya bean casein digest medium, respectively after 14 days of incubation at 37ºC and 25ºC.

Vaccine experimental trial was conducted in groups of rabbits and serum samples were collected as already described in materials and methods section. The cutoff OD value of antibody detected by ELISA was calculated 0.124±0.03. Optical density values greater and less than this cutoff value were considered positive and negative, respectively. The anti-toxin titer in rabbit sera at day 0 was lower and ranged from 2.67±1.08 to 5.89±1.02 IU/mL. Non-significant differences (P>0.05) were observed among the anti-toxin titer of all groups at day 0 of the experimental trial. At day 14th of the experimental trial, a higher anti-toxin titer (75.86±2.95 IU/mL) was observed in multivalent toxoid vaccine (alum based) followed by (71.86±2.29 IU/mL) of monovalent toxoid vaccine (alum based). There were significant differences (P<0.05) observed among the anti-toxin titers of different groups at day 14th of the experimental trial. At day 21st of the experimental trial, a higher anti-toxin titer (181.97±4.57 IU/mL) was observed in multivalent toxoid vaccine (oil based). Meanwhile, the positive control depicted (8.71±1.63 IU/mL) anti-toxin titer. There were significant differences (P<0.05) observed among the anti-toxin titers of different groups at day 21st of experimental trial. At day 28th of the experimental trial, a higher anti-toxin titer (868.25±3.54 IU/mL) was observed in multivalent bacterin and toxoid vaccine (oil based) followed by (851.14±3.72 IU/mL) of multivalent toxoid vaccine (oil based). There were nonsignificant differences (P>0.05) observed among the anti-toxin titers

Table 2. *Humoral immune response in different experimental groups of rabbits at different time intervals measured by ELISA*

Rabbits Groups (n=5)	Type of Vaccine	Adjuvant	Anti-Toxin (Antibody) Titer (IU/mL)			
			Day 0	Day 14th	Day 21st	Day 28th
A	Monovalent Toxoid	Alum	3.39±1.02[a]	71.86±2.29[d]	6.76±1.38[a]	7.41±1.62[a]
B		Oil	3.89±1.01[a]	28.18±2.09[b]	30.90±2.69[c]	457.09±6.16[b]
C	Bivalent Toxoid	Alum	5.89±1.02[a]	64.56±2.45[c]	17.37±3.16[b]	9.33±1.35[a]
D		Oil	4.68±1.01[a]	57.54±1.86[c]	109.65±4.17[d]	776.25±3.09[b]
E	Multivalent Toxoid	Alum	4.57±1.01[a]	75.86±2.95[d]	32.36±2.82[c]	17.78±3.02[a]
F		Oil	4.57±1.01[a]	37.15±2.75[b]	181.97±4.57[e]	851.14±3.72[c]
G	Monovalent Bacterin plus Toxoid	Alum	2.69±1.02[a]	60.26±2.04[c]	11.48±2.29[b]	5.89±1.54[a]
H		Oil	3.24±1.00[a]	20.42±1.99[b]	87.54±2.51[d]	363.08±5.62[b]
I	Bivalent Bacterin plus Toxoid	Alum	5.13±1.01[a]	51.28±2.29[c]	13.80±2.95[b]	7.41±1.31[a]
G		Oil	2.95±1.00[a]	29.51±2.63[b]	93.32±3.98[d]	616.59±2.81[b]
K	Multivalent Bacterin plus Toxoid	Alum	3.63±1.00[a]	58.88±2.81[c]	25.70±2.63[c]	14.12±2.81[a]
L		Oil	2.88±1.01[a]	45.71±1.77[b]	144.54±4.36[e]	868.25±3.54[c]
M	Positive Control	Alum	4.57±1.01[a]	17.15±1.61[b]	8.71±1.63[a]	5.48±2.95[a]
N	Negative Control	----	2.67±1.08[a]	2.37±1.09[a]	2.32±1.04[a]	2.29±1.01[a]

[a,b,c,d,e] *Values with these different superscripts in column differ significantly (P<0.05) and with same differ non-significantly (P>0.05); IU/mL: international units per milli liter*

of multivalent toxoid and monovalent bacterin plus toxoid oil adjuvant vaccines at day 28th of the experimental trial. Positive control revealed 5.48±2.95 IU/mL anti-toxin titer. There were significant differences (P<0.05) observed among the anti-toxin titers of different groups at day 28th of the experimental trial. Humoral immune responses in different experimental groups of rabbits were presented in *Table 2*.

There was a 100% protection in rabbits (multivalent toxoid and bacterin plus toxoid vaccines groups) after the challenge. In case of multivalent, bivalent and monovalent oil adjuvant vaccines 98%, 95% and 85% protection was observed, respectively.

Multivalent bacterin plus toxoid vaccine (oil based) produced a higher anti-toxin titer in experimental trial thus selected for the field trial in sheep and goats. The anti-toxin titer was determined by indirect ELISA *(Table 3)*. The anti-toxin titer in sheep and goats at day 0 was lower and ranged from 39.98±2.43 to 47.01±2.93 IU/mL. Non-significant differences (P>0.05) were observed among the anti-toxin titer of all groups at day 0 of the field trial. At one month, a higher anti-toxin titer (654.59±3.12 IU/mL) was demonstrated by the sheep A group. A slightly decreased anti-toxin titer (642.23±3.01 IU/mL) was observed in the goat A group. The goat B and sheep B groups produced relatively lower anti-toxin titer (474.76±1.23, 577.91±2.44 IU/mL). There were significant differences (P<0.05) observed among the anti-toxin titers of different groups at one month of the field trial. At two months, a higher anti-toxin titer (887.07±4.03 IU/mL) was demonstrated by the sheep A group. A slightly decreased anti-toxin titer (859.84±1.81 IU/mL) was observed in the goat A group. The goat B and sheep B groups produced relatively lower anti-toxin titer (589.76±1.30, 741.20±1.35 IU/mL). A higher anti-toxin titer was observed in the Sheep A (1294.81±1.90 IU/mL) at four) months of the field trial followed by the sheep A (1192.54±1.79 IU/mL) at five) months of the field trial. A lower anti-toxin titer was observed in the goat A (445.32±0.72 IU/mL) at nine months of the field trial. The anti-toxin titer of the commercial vaccine under study was higher (948.70±1.59 IU/mL) at three months of the field trial in the sheep B whereas, the lower anti-toxin titer (131.54±1.32 IU/mL) was observed in the goat A at nine months of the field trial. There were significant differences (P<0.05) among the anti-toxin titers of different groups throughout the course of the field trial *(Table 3)*.

Discussion

C. perfringens is an anaerobic bacterium that produces several toxins. Of these, alpha, beta, and epsilon toxins are responsible for causing the most severe *C. perfringens*-related diseases in farm animals. The best way to control these diseases is through the vaccination [24]. Entero-toxaemia is an important disease of sheep, and this disease causes severe economic losses to sheep farmers [25]. The development and production of conventional clostridial vaccines involves expensive, time consuming and dangerous processes of detoxification, purification and antigen concentration steps [16]. Alternatively, the use of recombinant vaccines against the clostridial infections has yielded promising results in other animal species [26]. Furthermore, the continued selection of the toxigenic strains that produce high titers of toxin is necessary [17].

Table 3. *Humoral immune response in different experimental groups of sheep and goat at different time intervals measured by ELISA*

Time Period	Anti-Toxin Antibody Titer (IU/mL)			
	Multivalent Bacterin and Toxoid Vaccine (Oil Based)		Commercial Vaccine	
	Goat A	Sheep A	Goat B	Sheep B
Day 0	45.49±2.64^{a}	47.01±2.93^{a}	42.99±1.58^{a}	39.98±2.43^{a}
Month1	642.23±3.01^{c}	654.59±3.12^{c}	474.76±1.23^{a}	577.91±2.44^{b}
Month2	859.84±1.81^{c}	887.07±4.03^{c}	589.76±1.30^{a}	741.20±1.35^{b}
Month3	943.83±3.17^{b}	987.20±1.25^{b}	886.87±1.01^{a}	948.70±1.59^{b}
Month4	1091.85±2.51^{b}	1294.81±1.90^{c}	601.91±1.79^{a}	633.51±1.16^{a}
Month5	1014.87±2.13^{b}	1192.54±1.79^{c}	435.03±1.71^{a}	474.92±1.36^{a}
Month6	826.83±2.55^{b}	928.11±1.19^{c}	324.55±2.44^{a}	386.28±1.23^{a}
Month7	674.56±1.85^{b}	776.67±2.87^{c}	201.42±2.10^{a}	212.66±1.83^{a}
Month8	587.33±1.27^{b}	623.74±3.05^{c}	165.34±1.10^{a}	178.45±1.34^{a}
Month9	445.32±0.72^{b}	457.50±1.77^{b}	131.54±1.32^{a}	142.65±1.24^{a}

^{a,b,c,d,e} *Values with these different superscripts in column differ significantly (P<0.05) and with same differ non-significantly (P>0.05); IU/mL: international units per milli liter*

In the present study, toxoid and bacterin + toxoid oil and alum adjuvanted vaccines were prepared. Experimental and field trials were performed on rabbits, sheep and goats, respectively. 0.2 mg of *C. perfringens* type D epsilon toxoid expressed by *Escherichia coli,* was administered to rabbits, goats, sheep, and cattle to evaluate the potency of the vaccine [27]. Higher antibody titers in rabbits were 776.25±3.09 and 616.59±2.81 IU/mL in the bivalent toxoid and bacterin plus toxoid oil adjuvant vaccines, containing epsilon toxin respectively. These antibody titers were higher than that of evaluated by Morcrette et al.[27] in the rabbit (40 IU/mL) serum pools. The Type B bacterin plus toxoid vaccine potency titer in sheep (1294.81±1.90 IU/mL) and goat (1091.85±2.51 IU/mL) was higher than as reported by Morcrette et al.[27] in goat, sheep, and cattle serum pools, 14.3, 26 IU/mL respectively. In vaccinated ewes, titer was peaked at 1st week with 15 IU/mL. The titer was dropped after lambing [28]. Commercial enterotoxaemia vaccine evaluation was done in goats and a great majority of the vaccinated animals had titers below the protective level, arbitrarily set at 0.25 IU/mL, by day 98 [29]. In the present study, antibody titers at 28th day post vaccination in rabbits and after 4 months post vaccination in sheep and goats were observed to be higher than as observed by de la Rosa et al.[28] and Uzal et al.[29] among rabbits, sheep and goats. The serological response to monovalent epsilon toxoid alum hydroxide adjuvant vaccine against *C. perfringens* type D enterotoxaemia was evaluated in goats. Mean antibody titers was 0.6 and 1.1 IU/ mL at 40th day after the first vaccination, 1.8 IU/mL at 40th day after the booster dose, respectively [30]. These titers were not in agreement with the titer observed in goats after 1st and 2nd month post vaccination with multivalent bacterin plus toxoid vaccine. The potency value of the recombinant epsilon toxoid with aluminum hydroxide as an adjuvant in sheep was determined >5 IU being protective. Further, the use of this construct in a combination vaccine against sheep pox resulted in the sheep being protected against enterotoxaemia [25]. Epsilon -beta fusion toxin was used as vaccine candidate to evaluate potency in rabbits. There was 6 and 10 IU/mL epsilon and beta antitoxin titer observed in rabbits [31]. ELISA test represented that antibody titer against multivalent toxoid vaccine was observed greater than titers observed by Langroudi et al.[31]. Recombinant trivalent vaccine (alpha, beta and epsilon) given to cattle, sheep, and goats and generated respectively, 5.19±0.48, 4.34±0.43, and 4.70±0.58 IU/mL against alpha toxin, 13.71±1.17 IU/mL (for all three species) against beta toxin, and 12.74±1.70, 7.66±1.69, and 8.91±2.14 IU/mL against epsilon toxin. These levels were above the minimum recommended by the international protocols. Vaccines represent an interesting alternative for the prevention of *C. perfringens* related intoxications in farm animals [24]. Moreira and colleagues study results were in contrast to the present study that there were increase in antibody titer in sheep and goats up to 4 months post vaccination. Recombinant monovalent alpha toxoid (200 mg/dose) vaccine potency evaluated against the yellow lamb disease in rabbit and sheep. This vaccine induced 13.82 IU/mL antitoxin in rabbits. In sheep, antibody titer was 4 IU/mL after 56 days of the vaccination [32]. In the present study, monovalent toxoid alum and oil adjuvant vaccine antibody titers in rabbits were observed higher at 14th and 28th day post vaccination, respectively, in contrast to titers observed by Ferreira et al.[32]. The rTA and rTB proteins produced and tested, induced an immune response (9.6 and 20.4 IU/mL, respectively) and can be regarded as candidates for the development of a commercial vaccine against *C. perfringens* type A and C induced diarrhea in pigs [19]. Present study observations were in agreement with that Montanide vaccinated groups exhibited a highest protection percentage (100%) post

challenge [18]. At 31st day post vaccination experimental units, rabbits were challenged with double dose of antigen and there was 100% protection against multivalent toxoid and bacterin plus toxoid oil adjuvant vaccines. Antibody titers of the bivalent and trivalent oil adjuvant vaccine (Montanide ISA 70 oil adjuvant) were in contrast to Saadh et al.[18] observations because at 28th day antibody titer was observed higher. In the present study, antibody titers results post vaccination at 28th days were also not in agreement with the results observed by by Hu et al.[19] and Moreira et al.[24].

For effective vaccine production against *C. perfringens* diseases, selection of toxigenic strains that produce high titers of toxin is necessary. *C. perfringens* toxinotype A, B and D produced higher amount of a, b and e toxins can be used at industrial scale for antigen production. Multivalent bacterin plus toxoid oil adjuvant vaccines produced a specific and efficient immune response during the experimental and field trials; proved an excellent candidate to use in the field to vaccinate animals to protect against *C. perfringens* diseases.

Acknowledgment

Authors are thankful to the Higher Education Commission (HEC) of Pakistan for funding the research.

Conflict of interest

There is no conflict of interest.

Funding

Higher Education Commission (HEC) of Pakistan, funded the research (technology development fund (TDF-02-028).

Author's Contributions

MT, TA and AAS conducted the research on vaccines preparation. Vaccine evaluation and experimental trials were conducted by AAA, MAA and ARA. Field trial of vaccine on sheep and goat was performed by MMKS and SH.

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