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Recent formulation for polyvalent Clostridial vaccine

Roukaia M. Osman, M. M. Fayez, H. A. EL-Helw*, A. EL-Meneisy

Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo, Egypt.

Polyvalent clostridial vaccine has been prepared according to L+ dose of C. perfringens type B and D, C. septicum, C. oedematiens, the optical density of *C. chauvoei*, and flocculation test of C. tetani. The vaccine has been evaluated in guinea pigs, rabbits and sheep. It gave high protective immunity in guinea pigs in challenge test (100% protection), the sera of vaccinated rabbits gave high titers more than the permissible limit. Sera of vaccinated sheep showed high antibody titer and good immune response which revealed that the vaccine able to protect sheep against clostridial diseases. The recent formulation of polyvalent clostridial vaccine is very useful tool for production of highly antigenic multicomponent clostridial vaccine used for control of different clostridial diseases.

Clostridium is one of the largest bacterial genera, ranking the second in size next to *Streptomyces*, classified as Gram-positive endospore-forming obligate anaerobes (Dong *et al.*, 2010). Members of the genus *Clostridium*, including its pathogenic species, are found principally in the soil, and some species are present as a very small component of the bowel flora of human and animals. The clostridia are not normally invasive, but they produce a number of toxins and enzymes that are responsible for their pathogenic effects (Borriello and Aktories, 2005).The severity of clostridial infections is due to organism's motility and the ability to survive in anaerobic environment (Saleh *et al.*, 2009). Most of the clostridial toxins are pore-forming toxins responsible for wide variety of gangrenes and gastrointestinal diseases in humans and animals, which cause significant economic losses to farming industry (Popoff and Bouvet, 2010). Such diseases including enterotoxaemia (*Clostridium perfringens*) ; lamb dysentery (*Clostridium perfringens* types B); pulpy kidney (*Clostridium perfringens* types D); blackleg (*Clostridium chauvoei*); malignant oedema (*Clostridium septicum*); tetanus (*Clostridium tetani*), and black disease (*Clostridium oedematiens* type B) (Roberts, 2000).

Antibiotic treatment of clostridial infections is rarely predictable and often ineffective. Accordingly such infections are generally controlled prophylactically using vaccine compositions containing one or more clostridial bacterins or toxoids (Zemlyakova, 1981). Clostridial toxoids are soluble proteins of relatively low antigenicity and traditionally poor stability (Roberts, 2000). Thus, it is not recommended to use the vaccine in which the antigenic activity of its components is below the minimum doses since the vaccine will fail to produce the antitoxic immunity in vaccinated animals at the protective level (Zemlyakova, 1981). Effective vaccines have been developed for use in sheep (Webster and Frank, 1985); cattle (Stokka *et al.*, 1994) and goats (Uzal and Kelly, 1998; Uzal *et al.*, 1999). The vaccines are considered to reduce the incidence and severity of diseases. The effectiveness of immunization depends on several factors as type of vaccine, route or site of vaccination and adjuvant used (Chirase *et al.*, 2001).

Prevention and control of clostridial infection in bovine and ovine depends mainly on administration of an effective amount of the vaccine comprising of toxoids of *C. perfringens* types B, D; *C. septicum*; *C. tetani*, *C. oedematiens* and high cellular density formalized cultures of *C. chauvoei* with high immunogenic power because immunity to *C. chauvoei* is generally considered to be antibacterial rather than antitoxic (Cortinas *et al.*, 1994).

The currently prepared polyvalent clostridial vaccine in anaerobic Department of Veterinary Serum and Vaccine Research Institute is formulated according to the potency of toxins of strains used, which sometimes did not give the desired immune response in vaccinated animals so the principle objective of this work is to improve the antigenic and immunogenic properties according to L+ dose of each clostridial spp. of polyvalent clostridial vaccine

* Corresponding author. Tel.: +20 010 1022324 ;
E-mail address: hamedelhelw@hotmail.com
(Hamed El- Helw)

to provide strong antitoxic immunity in vaccinated animals.

Materials and methods

Strains. Reference and local strains of *Clostridium* spp. were used for vaccine preparation in Veterinary Serum and Vaccine Research Institute, Anaerobic Research Department. These strains are

C. perfringens type B (6121)
C. perfringens type D (8346)
C. oedematiens type B (9691)
C. tetani Harvard strain (49205)
C. chauvoei local isolate strain
C. septicum (4005)

Swiss White Mice. Five hundred Swiss white mice were used for mice toxicity test, determination of L+ dose of *C. perfringens* types B and D; *C. septicum*, and *C. oedematiens* type B, and determination of antitoxin units in sera of vaccinated animals. These mice were obtained from Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo.

Rabbits. Ten New Zealand rabbits weighted about 2- 2.5kg, were used as a preliminary determination the potency of polyvalent clostridial vaccine.

Guinea pigs. Fifteen guinea pigs weighted about 400- 500g, were used in challenge test for determination of the potency of *C. chauvoei* in polyvalent clostridial vaccine.

Sheep. Ten apparently healthy, one-year-old sheep obtained from Veterinary Serum and Vaccine Research Institute. They were used for evaluation of polyvalent clostridial vaccine.

Vaccine preparation.

Preparation of clostridial cultures and toxins. Clostridial cultures and toxins (*C. perfringens* types B and D; *C. oedematiens* type B; *C. septicum*, and *C. chauvoei*) were prepared according to (Roberts, 2000), in which the organisms of interest are grown in a suitable medium which is generally aqueous solutions of 4% peptone; 0.5% yeast extract; 0.5% sodium chloride; and organs such as muscle or liver were added to media also 1% glucose as a source of carbon and energy, and reducing agents such as Cysteine HCL at concentration of 0.05% to provided anaerobic condition. Organisms are generally incubated at 37°C for 4-96 h depend on organism and rate of growth. *C. tetani* toxin was prepared according to (EL-Helw, 2007).

Determination of dose of each Clostridial species.

Toxicity test for clostridial toxins. The toxicity of each strain and determination of minimal lethal dose (MLD) was done according to (Ernest and Bowmer, 1963) using mouse toxicity tests.

L+ Dose of Toxins. L+ Dose of toxin was determined according to (Barile *et al.*, 1970) for *C. perfringens* types B and D; *C. septicum*, and *C. oedematiens* type B.

Limit of Flocculation (Lf). Limit of flocculation was done according to (WHO, 1997), for determination the potency of tetanus toxin.

Dose for C. chauvoei. It was determined by opacity or absorbency units, these units are based on the optical density (O.D.) of the culture, as measured at wavelength 625 nm according to (Roberts, 2000).

Preparation of polyvalent clostridial vaccine according to L+ dose of each clostridial organism. Clostridial cultures were then inactivated by using 0.5% formalin incubated at 37°C for a period of 5-10 days for complete inactivation of culture. The toxoids of Clostridia spp. except *C. chauvoei* were separated from the medium by filtration using Millipore filter (0.22 µm) and then concentrated by ultra filtration while the inactivated *C. chauvoei* culture was left and used as anaculture.

The formulation of the components of polyvalent clostridial vaccine was mixed together according to L+ dose of *C. perfringens* types B and D; *C. septicum*, and *C. oedematiens* type B. The dose for *C. tetani* was added according to Limit of Flocculation Lf, while the dose of *C. chauvoei* was added according to Opacity Unit. Aluminum Potassium sulfate was added as an adjuvant to the prepared vaccine. Sterility and safety tests were carried out according to the regulation of (British pharmacopoeia, 2009).

Vaccination Schedule.

Guinea pigs. Ten guinea pigs were vaccinated with prepared polyvalent clostridial vaccine in two doses with 21 days apart, the 1st dose (5ml), and 2nd dose (3ml), the vaccine was given subcutaneously, and then guinea pigs were challenged after 14 days from the second dose.

Rabbits. Ten New Zealand rabbits were vaccinated with prepared polyvalent clostridial vaccine in two doses with 21 days apart, the 1st dose (5ml), and 2nd dose (3ml), the vaccine was given subcutaneously, and then rabbit were bled after 14 days from the second dose. Sera were stored at –25°C until used.

Sheep. Ten sheep were vaccinated with prepared polyvalent clostridial vaccine subcutaneously in two doses with 21 days apart, the 1st dose (5ml)

and 2nd dose (3ml). All these animals were maintained under observation. Blood samples were collected on days 0, and 21 after second inoculation. The collected sera were stored at –25°C until used.

Potency test for *C. chauvoei*.

Challenge Test. Fourteen days after the second dose, both vaccinated and non vaccinated guinea pigs were challenged with 0.5 ml of spore suspension of *C. chauvoei* containing guinea pig 10 LD_{50} by the intramuscular route. The spore suspension was diluted with 5% $CaCl_2$ (Micalizzi and De Guzman, 1997).

Plate Agglutination Test. The antibodies titers against *C. chauvoei* in sera of vaccinated rabbits and sheep determined by Plate agglutination test according to (Claus and Macheak, 1972).

Potency test for *C. perfringens* types B and D; *C. septicum*, and *C. oedematiens* type B. The antibody titer of immunized sera were determined by the toxin-antitoxin neutralization test using mice as described by (Rahman and Rahman, 1999). Briefly, toxins purified from *Clostridium perfringens* were used as antigens. Immunized sera prepared against each toxoid were used as antibodies. Immunized sera were incubated at 56°C for 30 min before used for the test. Mice were used as an indicator host. Serum sample was diluted in PBS, pH 7.0 ranging from 1: 2 to 1: 256. 0.3 ml of each toxin (0.1mg/ml) was mixed with 0.3 ml of diluted individual serum sample. The mixture was incubated at room temperature for 1 h. 0.2 ml of toxin-serum mixture was inoculated intravenously into each of 2 mice. To determine the neutralizing titer the highest dilution of sera protecting more than 50% of inoculated mice was recorded (Pal *et al.*, 1990).

Potency test for *C. tetani*. The potency of the final product was tested by determination of antitoxin titer in sera of vaccinated rabbits by toxin neutralization test according to (Barile *et al.*, 1970).

Results and Discussion

The effectiveness of vaccines depends mainly on the quality of specific antigens and the choice of suitable dose of each for increasing their immunogenic enhancing activity.

An effective amount of a clostridial component will be that amount required to generate an amount of circulating antibody sufficient to prevent or reduce clostridial disease symptoms. Such amounts can be expressed using any of several units. For example, effective amounts of clostridial bacterins are usually expressed in terms of opacity or absorbency units (O.U. or A.U., respectively). These units are based on the optical density (O.D.) of the culture, as measured at a suitable wavelength, such as 625 nm. The O.D. value is then multiplied by the volume of the culture in one dose of vaccine as shown in *C. chauvoei*. Effective amounts of toxoid may be measured in terms of L+. An L+ unit of toxoid is equivalent to one units of standard antitoxin, as determined by toxin-antitoxin titration in mice in case of *C. perfringens* types B and D; *C. septicum*, and *C. oedematiens* type B. Effective amounts may also be measured by Flocculation test as shown in *C. tetani* (Roberts, 2000).

The result of L+ dose of *C. perfringens* types B and D; *C. septicum*, and *C. oedematiens* type B was shown in table (1).

Table (1): The L+ dose of *C. perfringens* types B and D; *C. septicum*, and *C. oedematiens* type B:

Organism	L+/ml
C. perfringens type B	100
C. perfringens type D	70
C. septicum	75
C. oedematiens	100

The optical density of *C. chauvoei* was five Opacity Units (O. U.)/ml at 625 nm, and Lf (Limit of Flocculation) of *C. tetani* was 90 Lf/ml.

The formulation of polyvalent clostridial vaccine according to L+ dose of *C. perfringens* types B and D; *C. septicum*, and *C. oedematiens* type B, the optical density of *C. chauvoei* and Flocculation test of *C. tetani* was shown in table (2).

As a rule, the antitoxin level in the blood of immunized animal is a very good index of immunity, as estimated by the number of antitoxin units (Tizard, 2004). So Potency test of the polyvalent clostridial vaccine was done in guinea pigs, rabbits and sheep. The results are expressed as International Units (IU) of antitoxin per ml of serum. The immunogenic response of the different antigens comprising the multi-component clostridial vaccine is best examined independently.

The results obtained from Potency test of the polyvalent clostridial vaccine in guinea pigs showed that the *C. chauvoei* protected 100% of vaccinated guinea pigs as ten of ten vaccinated guinea pigs survived 5 days after challenge test

Table (2): The formulation of polyvalent clostridial vaccine.

Organism	% of organism in formula of vaccine	Amount (ml) in formula of vaccine for 5ml dose	Amount (ml) in formula of vaccine for 3ml dose
C. perfringens type B	24%	1.2	0.72
C. perfringens type D	30%	1.5	0.90
C. septicum	14%	0.7	0.42
C. oedematiens type B	14%	0.7	0.42
C. chauvoei	10%	0.5	0.3
C. tetani	8%	0.4	0.24

Table (3): Potency of the polyvalent clostridial vaccine in rabbits.

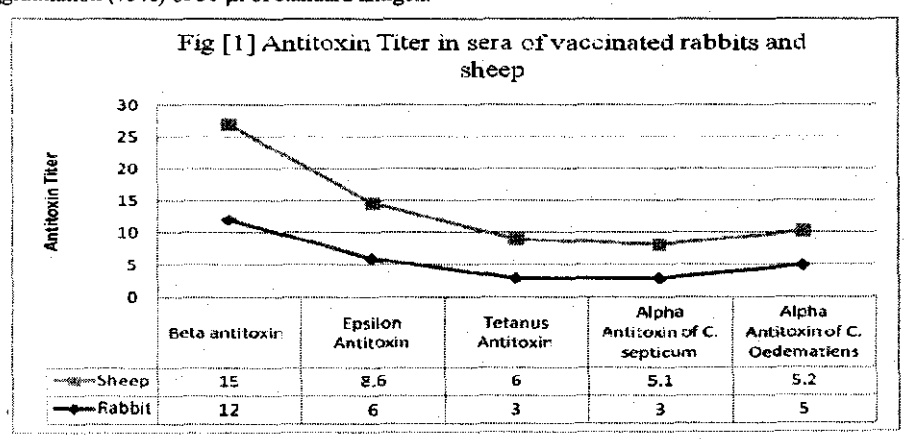
	Antitoxin titers in sera of vaccinated rabbits expressed as (IU/ml)					[1]Agglutination titer of *C. chauvoei* (µl/ml)
	Beta toxin C. perfringens B	Epsilon toxin C. perfringens D	Tetanus toxin of *C. tetani*	Alpha toxin of *C. septicum*	Alpha toxin of C. oedematiens type B	
Mean	12	6	3	3	5	0.02

[1]Agglutination titer is defined as the number of micro liters (µl) of serum required to provide definite agglutination (75%) with 30µl of standard antigen.

Table (4): Potency of the polyvalent clostridial vaccine in sheep.

Sheep No.	Antitoxin titers in sera of vaccinated sheep expressed as (IU/ml)					[1]Agglutination titer of *C. chauvoei* (µl/ml)
	Beta toxin C. perfringens B	Epsilon toxin C. perfringens D	Tetanus toxin of *C. tetani*	Alpha toxin of *C. septicum*	Alpha toxin of C. oedematiens type B	
1	15	10	4	5	6	0.02
2	12	10	4	5	6	0.01
3	18	10	8	5	5	0.01
4	15	8	6	4	5	0.005
5	15	8	6	6	5	0.005
6	15	8	6	6	5	0.005
7	15	8	6	5	5	0.01
8	15	8	8	5	5	0.01
9	15	8	6	5	5	0.01
10	15	8	6	5	5	0.005
Mean	15	8.6	6	5.1	5.2	0.009

[1]Agglutination titer is defined as the number of micro liters (µl) of serum required to provide definite agglutination (75%) of 30 µl of standard antigen.



and five non-vaccinated guinea pigs were died. These results agree with (British pharmacopeia, 2009) which mentioned that not less than four out of five of control group receiving the highest challenge dose(10 LD_{50}) die from *C. chauvoei* infection within 72h and none of the vaccinated animals die within five days of challenge.

The minimum antitoxic titer of the pooled serum from tested rabbits as specified by (British pharmacopeia, 2009) are 5 IU/ml for *C. perfringens* types D; 2.5 IU/ml for *C. septicum*; 3.5 IU/ml for *C. oedematiens* type B and 2.5 IU/ml for *C. tetani*.

The results of potency of the polyvalent clostridial vaccine in rabbits as shown in table (3) revealed that the average titers for *C. perfringens* types B and D; *C. tetani; C. septicum*, and *C. oedematiens* type B were all more than the permissible limit according to (British pharmacopeia, 2009).

The titers of antitoxin of different clostridial spp. in sheep sera illustrated in table (4) revealed that the response of individual sheep to the same antigenic stimulus showed conspicuous variation. This was probably related to the effect that might be produced by even minor variations in management, as nutritional deficiencies are known to influence quantitatively antibody response (Sheffy and Gilmartin, 1963). All sheep gave high titers, which revealed high immune response to polyvalent clostridial vaccine.

The results of (Fig.1) showed that sheep are preferable for quality control assessments because of their sensitivity in antitoxin response to clostridial vaccines. Furthermore the vaccines are being tested for efficacy and safety in one of species in which they will be used commercially. The original reason for choosing laboratory animals for testing veterinary biological products was purely economic but it has been cheaper to purchase and maintain sheep at pasture than it has been to buy or breed rabbits and maintain them under laboratory conditions. Moreover sheep can be sold on completion of the test, whereas rabbits are usually killed. These obtained results confirmed the results of (Webster and Frank, 1985).

From the above mentioned results, it could be concluded that determination of L+ dose of *C. perfringens* types B and D; *C. septicum*, and *C. oedematiens* type B, and formulation of the components of polyvalent clostridial vaccine according to it, is a very useful mean for production of highly antigenic multi-component clostridial vaccine used for control of clostridial diseases.

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تركيبة حديثة للقاح الكلوستريديا الجامع

تم استخدام تركيبة جديدة في إنتاج لقاح الكلوستريديا الجامع تعتمد على (L+ dose) لكل من عترات الكلوستريديا المكونة اللقاح. عند تقييم اللقاح في كل من خنازير غينيا والأرانب البوسكات والأغنام أظهرت النتائج ارتفاع ملحوظ في الاستجابة المناعية لكل من العترات المستخدمة. حيث أعطي نسبة حماية عالية في خنازير غينيا باستخدام اختبار التحدي للكلوستريديم شوفياي وصلت إلي ١٠٠ % . وأعطت نسبة أجسام مناعية عالية لكل من عترات الكلوستريديا في مصل الأرانب البوسكات والأغنام المحصنة بهذا اللقاح اعلي من المعدلات المثالية المطلوبة. لهذا ينصح باستخدام هذه التركيبة الجديدة في إنتاج لقاح الكلوستريديا الجامع للوقاية من أمراض الكلوستريديا المختلفة.