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Preparation of a Combined Vaccine for Clostridial Diseases and Rabies in Sheep

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Abstract: In the current study, a combined inactivated vaccine was prepared. It was composed of rabies virus and clostridial toxoids *(C. perfringens* type B and D, *C. novyi* type B, *C. septicum* and *C. tetani* with the whole culture of *C. chauvoei*) to protect sheep against such fatal diseases which cause high economic losses. After obtaining completely inactivated clostridial toxins and rabies virus using 0.2% BEI, these antigens were mixed together and alhydragel (patent preparation of 2% aluminum hydroxide gel) was added at a rate of 20% in a manner showing that such mixture contains the protective amounts of the including agents. Each vaccine was tested alone in sheep along with combined one to evaluate if there is any interference between these antigens or not. It was indicated that the rabies vaccine did not interfere with the sheep immunity against the clostridial vaccine. On the other hand, the levels of rabies antibodies were increased when clostridial types were added. So that, because of convenience, time consuming and reduction of stress factors by repeat vaccination of single vaccine, it has become better to employ a mixture of such organisms in a single vaccine to be a combined vaccine.

Key words: Combined Vaccine · Clostridial Diseases · Rabies · Sheep

INTRODUCTION

There are several serious diseases affecting sheep industry dramatically leading to great economic losses. Some infections represent fatal diseases caused by anaerobic bacteria related to genus *Clostridium* including *C. perfringens* type B (causing lamb dysentery) and *C. perfringens* type D (causing enterotoxaemia and pulpy kidney), *C. chauvoei* (causing blackleg) *C. septicum* (causing gas gangrene) *C. novyi* type B (causing black disease) and *C. tetani* (causing tetanus). Clostridial diseases are usually fatal and death occurs rapidly with pulpy kidney, black disease and blackleg, but it takes several days or weeks with tetanus infection. The major factor that controls the effect of such diseases is the satisfactory immunity of animals at risk. The immune response is developed through vaccination [2]. Clostridial vaccines for sheep are prepared as a multicomponent formulation, contains antigens of *C. perfringens* type B, C and D, *C. novyi* type B, *C. septicum, C. tetani* and *C. chauvoei.*

Rabies faces sheep like other farm animals, especially in free yards whereas wild and stray dogs, foxes and wolves that attack sheep and may bite them resulting in rabies infection [1]. It is an acute highly fatal infectious disease caused by a virus of the genus *Lyssavirus* in the family *Rhabdovirideae* which is RNA single stranded virus [3]. It was stated that the majority of warm - blooded animals and human beings are susceptible to rabies [4]. The majority of viral diseases are incurable and vaccination against them is the corner stone in the control and protection of susceptible hosts. Against rabies, many live and inactivated vaccines were developed to control the disease in dogs as well as all farm and wild animals [5].

Because of convenience, time consuming and reduction of stress factors by repeat vaccination of single vaccine, it has become common to employ a mixture of organisms in a single vaccine to be a combined vaccine. So, the present study aimed to prepare a combined vaccine composed of polyvalent clostridial vaccine and

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inactivated cell culture rabies vaccine and this is to evaluate the effect of clostridial vaccine in augmenting the immune response of sheep vaccinated with rabies vaccine.

MATERIALS AND METHODS

Animals:

- Forty five local breed sheep of about 1-1.5 years old were used in the present study. They were screened before the application of experimental work and found to be free from clostridia and rabies antibodies.
- Five hundred weaned Swiss albino mice were used for mouse neutralization test to estimate clostridial antibodies in vaccinated sheep.
- Two hundred mice were used for mouse passive protection test to detect the protective rabies antibody level in experimentally active immunized animals.

The animals were kindly supplied by the Veterinary Serum and Vaccine Research Institute.

Bacterial Strains: *C. perfringens* (type B and D), C. *novyi*, *C. septicum*, *C. chauvoei* and *C. tetani* were isolated and identified using the convenient standard bacteriological and serological methods.

Rabies Virus: Live attenuated virus, (Baby Hamster Kidney) BHK cell culture adapted strain of rabies virus of a titer 10^7 $TCID_{50}$/ml was used. In addition, virulent rabies virus, mice adapted challenge virus strain of rabies (CVS) which was used in mouse passive protection test [6] was available. It had a titer of 10^6 MLD_{50}/ml.

Rabies Antigen: Rabies antigen was prepared according to Brian and Hiller [6] from infected BHK cells. These antigens were used in indirect ELISA to estimate rabies antibodies in sera of vaccinated animals.

All bacterial strains as well as rabies viruses and antigen were kindly supplied by the Veterinary Serum and Vaccine Institute, Abbassia, Cairo.

Preparation of Clostridial Vaccine According to Bahnemann [7]: Preparation of clostridial cultures and toxins: Each *Clostridium* spp. (mentioned before except *C. tetani*) was subcultured in cooked meat broth and incubated anaerobically at 37°C for 24 hours. The growth and purity of the culture was aerobically and anaerobically tested on blood agar plates. Guinea pig was injected intramuscularly with 0.5ml of the pure culture and with 0.5ml of 5% $CaCl_2$ to increase the pathogenicity of the organism. Guinea pig died within 72 hours was subjected to collection of the heart blood to re-isolate the microorganism. Frequent check of purity and identity was made. An actively growing pure seed culture was transferred to peptone water containing 1% glucose (production media) and incubated at 37°C in water bath for 4 hours. After incubation, culture was inoculated again into peptone water with 2% glucose. The culture was incubated at 37°C for 5 hours. The liquid portion of the culture was siphoned off and centrifuged.

Determination of Minimal Lethal Dose (MLD) of Clostridial Toxins (*C. Perfringens* Type B and D, C. *Septicum*, *C. Chauvoei* and C. *Novyi* Type B): Two fold dilutions of the tested toxin were prepared in saline and 0.1 ml of each dilution was inoculated I/V in a mouse weighing 20 g. 5 mice were used for each dilution. The mice were kept under observation for 3 days and MLDs were determined as the highest dilution of the toxin which killed mice within 2 days. For epsilon toxin, the dilution was starting from 1:10 to 1:1200; for beta toxin from 1:10 to 1:400; for *C. novyi* alpha toxin from 1:10 to 1:50 and from 1:2 to 1:20 for *C. septicum* alpha toxin.

Preparation of *C. Tetani* Culture and Toxin: The culture was prepared in glucose beef heart infusion broth then incubated at 35°C for 48 hours under anaerobic condition and then checked for purity. Only 1 ml of the culture was transferred daily (for 3 successive days) into glucose beef heart broth and incubated at 35°C for 24 hours under anaerobic condition and then checked for purity and sterility. These passages were done for maintenance.

The culture was transferred to Mueller and Miller medium (toxin medium) and incubated at 35°C for 8 hours. From this culture, the main batch of Mueller and Miller medium containing glycine as a stabilizer (5 g/L) was inoculated and incubated for 10 days at 35°C.

Determination of Minimal Lethal Dose (MLD) and Limes Flocculation (LF) Value of Tetanus Toxin: Tenfold dilutions of the crude tetanus toxin were prepared in sterile saline. Only 0.5 ml of each dilution was inoculated

into 5 mice. The route of injection was at the root of the tail toward the right side and then the mice were kept under observation for 4 days. The time of spastic paralysis or death were recorded. The MLD was determined as the smallest dilution of the toxin which kills the mouse within 96 hours. The LF value was determined according to WHO instructions [8].

Inactivation of Clostridial Cultures and Toxins: Each prepared clostridial cultures and toxins were inactivated with 0.2% Binary Ethyleneimin according to Bahnemann [4]. Complete inactivation occurred when 0.2 ml of the filtrate injected I/V in mice failed to kill mice.

Vaccine Formulation: After complete inactivation of each clostridial cultures and toxins by BEI, the polyvalent vaccine was prepared by mixing components which include the toxoids of *C. perfringens* type B and D, *C. novyi* type B, *C. septicum* and whole culture of *C. chauvoie* in equal amounts and then *C. tetani* was added as 25 Lf/dose to the vaccine. Aluminium hydroxide gel adjuvant 2% was added to mixture at the rate of 20% and mixed at 4°C for overnight. After then 10% solution of merthiolate was added to a final concentration of 1:10000 as a preservative.

Evaluation of the Prepared Polyvalent Clostridial Vaccine According to Frerichs and Gray[9]

Sterility Test: Only 1 ml of each prepared toxoid was inoculated into blood agar and cooked meat broth and incubated aerobically and anaerobically at 37°C. Also the prepared toxoid was cultured on Sabouraud's agar and incubated at room temperature. All tubes were observed for bacterial as well as fungal growth. The vaccine is considered sterile if no growth appears in any of the inoculated media within ten days.

Safety Test: Preliminary tests were made by injecting 1 ml of each toxoid I/V into five mice (22 g). All mice survived without showing symptoms of disease. For tetanus toxoid, 1 ml of the prepared toxoid was injected I/P into three mice which were observed for five days. No abnormal reaction was developed.

Potency Test: For determination of the efficacy of *C. chauvoei* vaccine, ten guinea pigs were inoculated S/C with two doses of the vaccine in 3ml amounts with three weeks intervals. Two weeks after the second dose, the animals were challenged by I/M inoculation with the spore suspension of *C. chauvoei* suspended in 5% calcium chloride in saline. The challenge dose was 32 MLD. At the same time, two unvaccinated guinea pigs were used as a control for each spore suspension. The guinea pigs were observed for five days. The potency of the vaccine was estimated in terms of number for infective doses that the vaccinated guinea pigs could tolerate (challenge test for *C. chauvoei*). On the other hand, for other clostridia, ten rabbits 4 months old were vaccinated with two doses of prepared polyvalent inactivated clostridial vaccine, the first dose was 5 ml and injected S/C, followed by 3 ml (second dose) three weeks later. Two weeks after the second dose, rabbits were bled and pooled sera were collected for antitoxin determination. The antitoxin values for *C. perfringens* type B and D, *C. novyi* type B, *C. septicum* and *C. tetani* were determined by mice neutralization test. Antibodies against *C. chauvoei* were determined by plate agglutination test. Ten pooled rabbits' sera without vaccination were used as control.

Preparation of Inactivated Cell Culture Rabies Vaccine According to Abelseth [10]

Virus Propagation: Sufficient virus inoculum was inoculated on confluent BHK Roux bottles at a rate of 3 virus/cell Multiplicities of Infection (MOI). After the absorption time (half-an hour), 100 ml of the maintenance media were added for each bottle. The maintenance medium was Minimum Essential Media (MEM) with Hank's salts (G80 Gibco Limited, Scotland, U.K.). It was prepared according to the manufacture directions. MEM was used with 3% new born calf serum. The bottles were incubated at 37°C, until the appearance of complete CPE (usually 72 hours) then they were subjected to 3 cycles of freezing at -70°C and thawing at 37°C and then harvested. Light centrifugation in cooling centrifuge was applied to the harvest and the supernatant fluids represented the virus lot for vaccine preparation (about 1 liter). The virus harvest was tested for sterility. All batches should be free from any bacterial and/or fungal growth.

Titration: It was carried out in BHK21 cells. The batches which gave virus titer less than 10^7, $TCID_{50}$/ml were rejected.

Inactivation of Virus by Bei According to WHO [11]: To the previously prepared virus lot, BEI was added to obtain. The mixture was stirred continuously at 37°C for 12 hours. Samples from the mixture were taken for 3 times

with 30 minutes intervals and tested for the virus infectivity using BHK cells and I/C inoculated weaned mice. Complete inactivation was achieved in the third sample. After then, the virus inactivation was stopped by the immediate addition of cold sodium thiosulphate at a rate of 2%.

Addition of Adjuvant: Patent preparation of 2% aluminum hydroxide gel was added in a ratio of 20%. The mixture of virus and aluminum hydroxide gel was mixed overnight at 4°C. Then centrifuged and the supernatant was tested for non- adsorbed virus in BHK21 cell culture and I/C inoculation in mice. The final pH was adjusted to 7.5. After then, 0.1% thiomersal was added as preservative to the final product.

Evaluation of the Prepared Rabies Vaccine

Sterility Test: According to Sikes and Larghann [12], samples of the prepared vaccine were cultivated in nutrient agar, blood agar, thioglucolate broth and Sabaroud agar. The inoculated media were incubated at 37°C for 7 days except Sabaroud agar which was left for 15 days at 25°C and 37°C. The batch should be free from any contaminants.

Safety Test: *In mice,* according to the OIE [1], about 20 suckling mice were inoculated I/C with 0.03 ml of the tested vaccine, while 10 adult mice via I/P route with 0.5 ml and then observed for 21 days. Mice that died within the 24 hours were discarded and those dead afterwards were checked by virus isolation. The vaccine would be approved as safe if all inoculated mice remained alive. In sheep, according to the OIE [1], about five adult sheep were inoculated S/C with 5 ml of the tested vaccine and then observed for three months. The vaccine would be approved as safe if all inoculated animals remained alive.

Potency Test: It was applied for the inactivated virus using the volumetric method according to Seligmann [13]:

- In this test a reference tissue culture inactivated vaccine was used beside the vaccine under test.
- Tenfold serial dilution of both reference and tested vaccine were done, using phosphate- buffered saline (pH 7.6).
- Groups of 10 mice approximately 4 weeks old were inoculated I/P with 0.5 ml of each diluted vaccine. Two doses of vaccine were given to each mouse on week apart.
- Each control mice (n= 30 - 40) were set aside at the time which the tested mice received the first dose of vaccine.
- All mice (vaccinated and control) were challenged via I/C route 14 days after the first dose of vaccine. The dose was 0.03 ml which contained 50 MLD_{50} of challenge virus strain.
- The challenged mice were recorded for 14 days and the following special equation was applied to estimate the antigenic value (AV) of the vaccine (which must not be less than 0.3).

Preparation of Clostridial and Rabies Combined Vaccine (Vaccine Formulation): After obtaining completely inactivated clostridial toxins and rabies virus using BEI, the antigens were mixed together and patent preparation of 2% aluminum hydroxide gel was added in a ratio of 20% in a manner shoring that such mixture contains the protective amounts of the including agents. The vaccine was mixed well by a high shear mixing equipment.

Sheep Vaccination: The forty sheep were divided into 4 groups (10 animals / group). The design of injection is summarized in the following table:

Blood samples were collected from the experimental animals through the jugular vein puncture under complete aseptic conditions and allowed to form clots at 4°C over night. Serum samples were collected over a period of year to follow up the immunity in the animals. The sera was collected at zero time, 1st WPI, 2nd WPI, 3rd WPI, 4th WPI, 6th WPI, 8thWPI, 12thWPI, 16th WPI, 20th WPI, 24th WPI, 28th WPI, 32nd WPI till 48th WPI. The serum was separated and centrifuged then kept in sterile screw capped vials at -20°C till subjected to serological and chemical examination. Sera were inactivated at 56°C for 20 minutes prior applying serological tests. Serum neutralization test, plate agglutination test and ELISA were used to determine the circulating antibodies against clostridia and rabies.

RESULTS

In the present study, three types of vaccines were prepared. The first type was polyvalent clostridia vaccine containing (*C. perfringens* type B and D, *C. septicum*, *C. novyi C. chauvoei* and *C. tetani*) inactivated by BEI and adjuvanted with aluminum hydroxide gel. The second type was inactivated cell culture rabies vaccine inactivated and adjuvanted with the same inactivator and adjuvant used with clostridial vaccine. The third one was

Table 1: Experimental design for vaccination of sheep

Types of vaccines	Group No.	Weeks post immunization (WPI) 1	2	3	4	6	8	12	16	20	24	28	32
Polyvalent inactivated clostridial vaccine	I	Vaccinated with polyvalent clostridial vaccine with two doses (5ml and3ml with four weeks apart S/C).											
Inactivated rabies vaccine	II	Vaccinated with inactivated rabies vaccine with 3ml S/C. Combined vaccine											
(Rabies andclostridia)	III	Vaccinated with combined vaccine 5ml S/C.											
Unvaccinated	IV	Control											

Table 2: Serum neutralizing clostridial antitoxin titers in vaccinated sheep (groups I and III)

	Sheep group-I (Vaccinated with the polyvalent clostridia vaccine alone)					Sheep group-III (Vaccinated with the polyvalent clostridia vaccine with inactivated rabies vaccine)				
	Antitoxin titer of					Antitoxin titer of				
Weeks Post Immunization	β	ϵ	∝ C.n	∝ C.s	C.t	β	ϵ	∝ C.n	∝ C.s	C.t
0	0	0	0	0	0	0	0	0	0	0
1	0	0	0	0	0	0	0	0	0	0
2	3.6	1.6	1.8	1.2	0.9	2.1	1.2	1.4	1.1	1.3
3	5.9	2.7	2.5	2.7	1.1	5.2	2.4	2.6	2.3	2
4 (2^{nd} dose)	12.2	4.1	3.6	3.1	1.6	10	3.9	3.2	2.9	3.3
6	15.2	7.9	5.1	4.2	3.9	15.4	6.8	5.5	3.2	3.7
8	20.3	10.2	9.6	9.1	5.4	18.1	10.9	8.8	7.4	4.1
12	17.3	8.3	8.5	7.8	5.4	15.9	8.7	6.5	6.4	3.9
16	12.4	7.6	7.4	6.8	3.2	9.4	6.6	5.2	5.3	3.8
20	9.8	6.1	6.7	5.3	2.5	7.8	4.1	4.6	4.1	2.9
24	8.6	5.2	5.1	4.6	2.5	6.9	3.2	3.7	3.2	2.5
28	6.3	4.2	4.2	3.1	1.8	5.2	3.2	3.7	2.9	2
32	5.6	3.1	3.3	2.6	1.8	5.2	2.2	2.9	1.8	1.7

.C.n: *C. novyi*, C.s: *C. septicum*, C.t: *C. tetani*

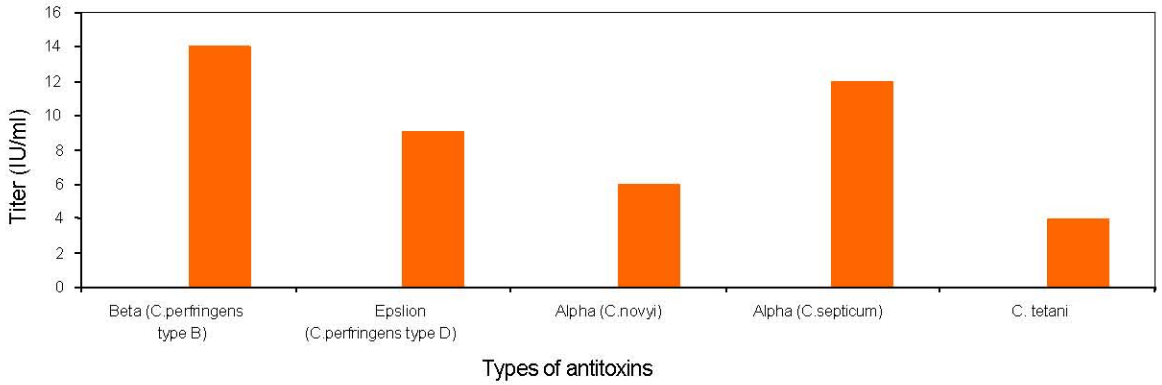

Fig. 1: Antitoxic titers in rabbit sera immunized with inactivated polyvalent clostridial vaccine

a combined vaccine composed of the polyvalent clostridial vaccine and inactivated cell culture rabies vaccine. Each vaccine was examined for sterility and safety.

Potency Tests of Prepared Polyvalent Clostridial Vaccine: Figure 1 summarizes the results of rabbit response against different components of polyvalent clostridial vaccine (*C. perfringens* type B and D, *C. novyi* type B, *C. septicum* and *C. tetani*) by toxin neutralization test. It showed that all the components of the prepared polyvalent inactivated clostridial vaccine resulted in higher immunization levels in rabbit sera.

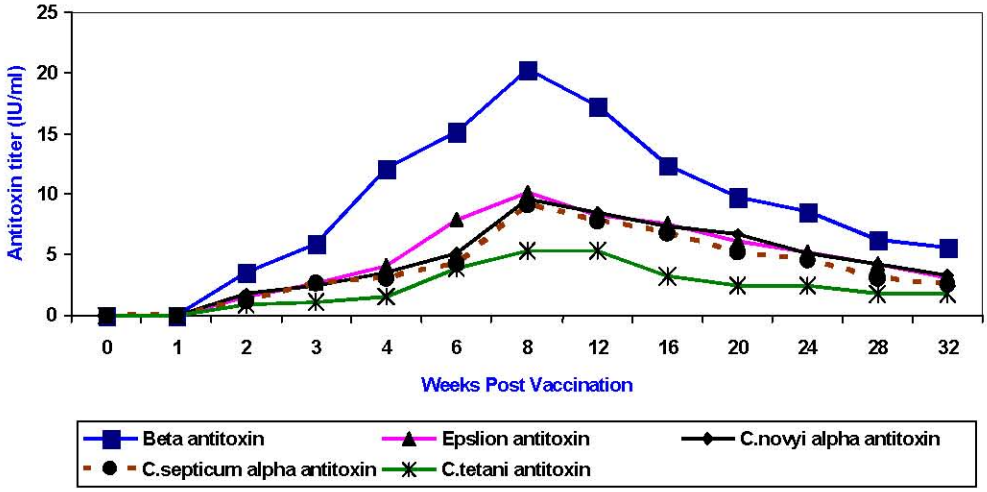

Fig. 2: Serum neutralizing clostridial antitoxin titers in vaccinated sheep (group I)

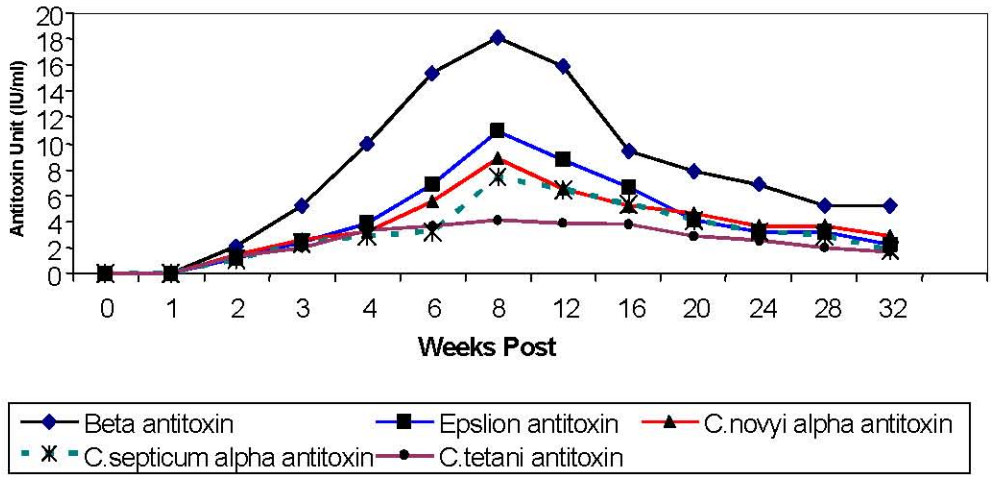

Fig. 3: Serum neutralizing clostridial antitoxin titers in vaccinated sheep (group III)

Table 3: *C. chauvoei* agglutinating titers in sheep sera with polyvalent clostridial vaccine and inactivated rabies vaccine (group I and III)

Weeks Post Immunization	Agglutinating titers of sera from Sheep group-I (Vaccinated with only polyvalent clostridial vaccine)	Agglutinating titers of sera from Sheep group-III (Vaccinated with both polyvalent clostridial vaccine and inactivated rabies vaccine)
0	0	0
1	0.022	0.01
2	0.12	0.1
3	0.16	0.12
4 (2^{nd} dose)	0.31	0.27
6	0.38	0.29
8	0.41	0.36
12	0.54	0.42
16	0.53	0.42
20	0.61	0.46
24	0.69	0.55
28	0.60	0.50
32	0.53	0.35

Table 4: Rabies neutralizing antibody titer in different vaccinated sheep (group II and III)

	Serum rabies neutralizing antibody titer*								
Sheep groups	1WPI	2WPI	3WPI	4WPI	2MPI	3MPI	6MPI	9MPI	12MPI
Group-II	8	16	32	64	64	64	64	64	64
Group-III	16	32	64	128	128	128	128	128	128

*Antibody titer = the reciprocal of the final serum dilution which neutralized and inhibited the CPE of 100 $TCID_{50}$ of rabies virus

WPI: weeks post immunization

MPI: month post immunization

Table 5: Rabies neutralizing antibody titer as estimated by mouse passive protection test

	Mean mouse neutralizing rabies antibody titer*			
Sheep groups	1WPV	2WPV	3WPV	4WPV
Group-II	8	16	32	64
Group-III	16	32	64	128

*Antibody titer = the reciprocal of the final serum dilution which protects mice against 100MLD_{50}

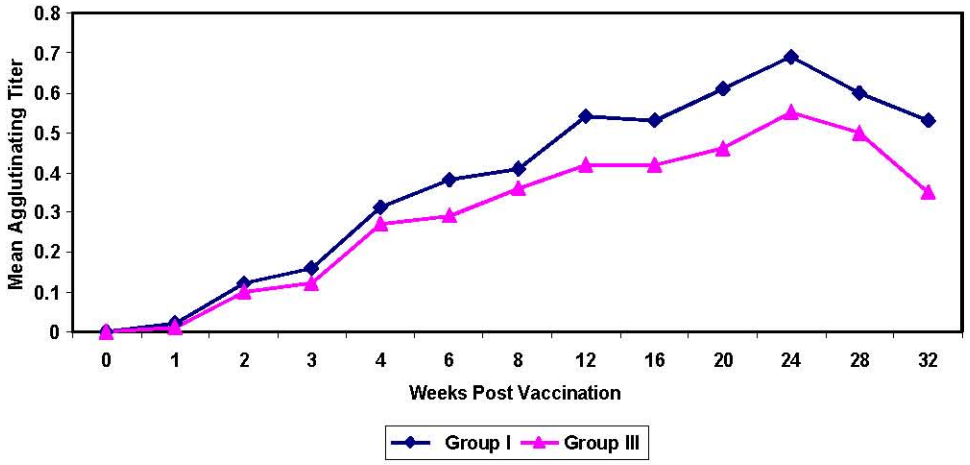

Fig. 4: *C. chauvoei* agglutinating titers in vaccinated sheep (groups I and III)

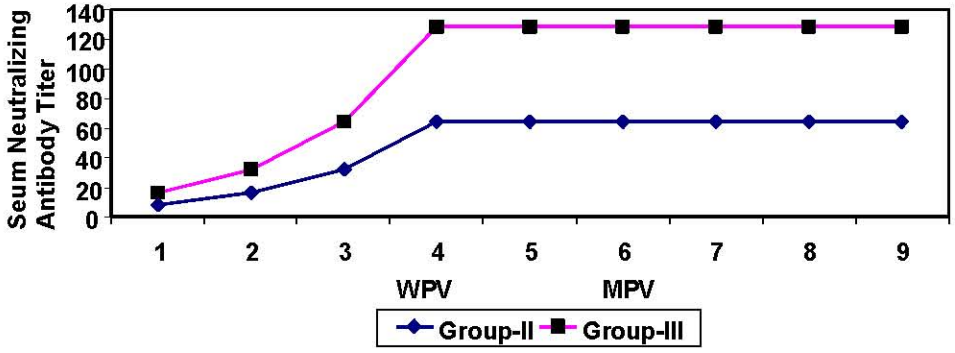

Fig. 5: Rabies neutralizing antibody titer in vaccinated sheep (groups I and III)

Potency Tests of Prepared *C. Chauvoei* Vaccine: Results revealed that the tested vaccine showed higher degrees of protection in guinea pigs when challenged with 32 MLD *C. chauvoei* spores suspension. Total survived were 10 guinea pigs out of 10 (100% protection). The two unvaccinated guinea pigs which were used as a control died after receiving one MLD of the spore's suspension within 48 hours.

The Immune Response of Sheep Against Clostridial Vaccine (Group I and III): The pre-vaccinated sera were tested for presence of clostridial antitoxin titers and no sample had a detectable titer to any of the toxin tested. On the other hand, post-vaccinated serum samples were collected over a year to follow up the immunity in the animals against β and ∈ toxins of *C. perfringens*, ∝ toxins of *C. novyi*, ∝ toxins of *C. septicum* and *C. tetani* toxins. The sera were collected from groups I and III at zero time, 1st WPI, 2nd WPI, 3rd WPI, 4th WPI, 6th WPI, 8thWPI, 12th WPI, 16th WPI, 20th WPI, 24th WPI, 28th WPI and 32nd WPI till 48th WPI.

***C. Chauvoei* Antibody Titers in Vaccinated Sheep Sera (Group I and III):** The following table and figure show the results of agglutinating titers for *C. chauvoei* component in sheep sera immunized with the polyvalent clostridial vaccine and combined (clostridial and rabies) vaccine.

The Immune Response of Sheep Against Rabies Vaccine (Group Ii and III): SNT indicated that the induced rabies antibodies reached their peak levels in all vaccinated animals by the 4th week post vaccination (64 and 128) in group II and III, respectively and still unchanged until the 12th month post vaccination. In addition, it was found that the results of mouse passive protection test were similar to those of SNT as tabulated in table 5. The challenge test which evaluates the potency of rabies vaccine is forbidden particularly in farm animals to avoid the public health hazard according to WHO [11].

DISCUSSION

The multicomponent clostridial vaccine which contained six antigens was prepared to produce high yield of antibodies. It contained the toxoids of *C. perfringens* type B and D, *C. novyi* type B, *C. septicum* and *C. tetani* with the whole culture of *C. chauvoei* which was inactivated by 0.2% BEI and 20% aluminum hydroxide gel was used as an adjuvant.

Effective combinations cannot be prepared by simple addition of compounds with the same dilutions, because the dose at such time would be extremely large and would provoke excessive reaction. Thus, the preparation of satisfactory multicomponent vaccine was not feasible until the immunogenic value of each constituent could be increased sufficiently in a sufficient dose. This could be reached by concentrating the toxoid components by subjecting the antigens to some forms of chemicals or by ultrafiltration through millipore membrane filter [14].

The obtained results of immunization value of polyvalent clostridial vaccine indicated that all components of the prepared polyvalent inactivated clostridial vaccine had an antitoxin levels in immunized rabbit sera exceeding the international standard levels which are 10, 5, 3.5, 2.5 and 1.5 IU for β (*C. perfringens* type B), ∈(*C. perfringens* type D), ∝ (*C. novyi*), ∝ (*C. septicum*) and *C. tetani* toxins, respectively [11].

For the immunization power of *C. chauvoei*, the results revealed that the challenged Guinea pigs with 32 MLD *C. chauvoei* spore suspension gave 100% protection (10/10). The two unvaccinated guinea pigs (control) died after receiving one MLD spore suspension within 48 hours; these results are parallel to what is recorded in WHO Manual [11] and OIE [1].

Since rabies is one of the most fatal and dangerous disease for animals and human beings, the systemic control of rabies plays an important role in countries where rabies is endemic and considered of public health hazard. Therefore, vaccination of domestic and pet animals against rabies plays a great role among all sanitary measures taken to control this world wide scourge [1].

In the present study, a cell culture rabies vaccine inactivated with 0.2% BEI was prepared and 20% aluminum hydroxide gel were added as adjuvant. Since, the inactivated vaccines are more stable and easier to be handled in field, the BHK21 cell lines are the best cells yielding the highest rabies titers [15]. Thus, studies were conducted to develop inactivated T.C. rabies vaccine in such cell line. The current study described the investigations done to find the most immunogenic, economic and stable inactivated T.C rabies vaccine for use in animals.

To obtain the most suitable rabies vaccine of high antigenicity titer and consequently of efficient immunogenicity, virus should be propagated in the BHK21 cell line [16-18]. Therefore, in the current study the ERA rabies virus strain was prepared at 3 virus/cell MOI and pH 7.2-7.6 for serial passages on the BHK21,cell line. The virus was increased progressively with increasing the number of passages with a ratio of 0.3 - 1.2 Log / passage, until reaching the optimum virus titer 10^8 TCID50 / ml on the passage No. 9, after which no increase in virus yield on BHK21 [19].

One of the measures to increase the effectiveness of inactivated vaccine is to select the best inactivator and adjuvant. So in the present study, BEI (alkylating agent) was used in the inactivation of rabies virus. BEI was used by cyclization of 2- Bromoethylamine hydrobromide in

alkaline solution (sodium hydroxide) at different concentration, 0.5, 1, 1.5 and 2%. Aluminum hydroxide gel adjuvant at 20% concentration was also used for virus inactivation. The results were confirmed by inoculation of mice which were observed for 15 days for any signs of rabies [20].

Potency test in mice using NIH test on the prepared inactivated rabies vaccine was equal to 2 which is higher than the minimum requirement of antigenic value which was equal to 0.3. These results are in agreement and accordance to WHO [22]. According to WHO [11] challenge test which evaluates the potency of rabies vaccine is forbidden especially in dogs and farm animals to avoid the public health hazard.

In the current study, a combined inactivated vaccine was prepared. It was composed of rabies virus and clostridial toxoids *(C. perfringens* type B and D, *C. novyi* type B, *C. septicum* and *C. tetani* with the whole culture of *C. chauvoei*) to protect sheep against such fatal diseases which cause high economic losses. After obtaining completely inactivated clostridial toxins and rabies virus using 0.2% BEI, these antigens were mixed together and alhydragel (patent preparation of 2% aluminum hydroxide gel) was added at a rate of 20% in a manner showing that such mixture contains the protective amounts of the including agents. Each vaccine was tested alone in sheep along with combined one (Table 1 experimental design) to evaluate if there is any interference between these antigens or not. The trend of using various combinations containing viral and bacterial vaccines for the animals either simultaneously or in mixed form has been successfully applied [22].

The results of the mean serum neutralizing titers for beta antitoxin ranged between 3.6-20.3 IU/ml. The titer was gradually increased starting from the 2nd WPI till reached the peak (20.3 IU/ml) at the 8thWPI. It is higher than the recommended level [23] which is 10 IU/ml). Then, the titer started to decrease till reached 5.6 IU/ml at the 32nd WPI. Sterne *et al.* [24] indicated that a level of 0.5 IU/ml of beta antitoxin of *C. perfringens* type B is sufficient to protect sheep against lamb dysentery.

In case of epsilon antitoxin, the titers ranged between 1.8-10.2 IU/ml with a peak of 10.2 IU/ml at the 8thWPI as well. Up to 16 weeks, the antitoxin titer was 7.6 IU/ml, which is more than the adequate standard for protection (5 IU/ml) specified by Hulse [25] and the British Veterinary Codex [23].

Results recorded also in Table 2 and Figures 2 and 3 summarize the mean alpha antitoxic values of *C. novyi* type B that have been obtained with vaccinated sheep (groups I and III).The high level of *C. novyi* alpha antitoxin at eight weeks was clear (9.6 IU/ml), this was above the standards recommended by the British Veterinary Codex [23] which is 3.5 IU/ml. During 16 weeks, the antitoxic titer remained high and this agrees with Forsyth and Wynne-Jones [26].

Regarding to the response of sheep to the *C. septicum* toxoid in group I and III, it would appear that the toxoid was capable of producing an encouraging degree of antitoxin titer in serum following the administration of the second dose. It is worthy of emphasis that the results revealed significant amounts of antitoxin at the 12th week more than the recommended amount for protection which is 0.5 IU/ml as it had been considered by Oxer *et al.* [27] as the minimum protection level.

When the mean titers were calculated for *C. tetani* toxoid, it was clear that the titers ranged between 0.9-5.4 IU/ml and also the antibodies reached their peak at the 8th week. Steven *et al.* [28] revealed similar results.

Statistical analysis of these results using t-test [29] revealed that there was a significant difference ($p = 0.05$) in the all clostridial antitoxin titers in sera of sheep vaccinated with polyvalent inactivated clostridial vaccine alone (group I) and combined vaccine (group III) which was detected by MNT.

The circulating neutralizing antitoxin in group III (vaccinated with combined vaccine), as shown in the previous results, was more or less in the same way of response. The combination of polyvalent clostridial vaccine and rabies one did not interfere or affect the immune response. The peak of beta, epsilon (*C. perfringens*), alpha (*C novyi.*), alpha (*C. septicum*) and *C. tetani* antitoxin was at the 8th WPI in which the mean titers were 18.1, 10.9, 8.8, 7.4 and 4.1 IU/ml, respectively. So, it is concluded that the titers of antibodies were protective in both groups I and III till the 20th WPI.

Plate agglutination test was used in evaluating the *C. chauvoei* component of the multicomponent vaccine prepared in the current study. Table No. 3 and Figure 4 showed the results of agglutination test for *C. chauvoei* component in sheep sera (groups I and III). It was clear that there is a detectable level of *C. chauvoei* agglutinating titers in the examined sera after the primary dose of vaccination for both vaccines with no significant difference in immunizing values. The mean agglutination titers began to increase after booster dose (4th week) after primary vaccination and was still higher than the minimum protective level determined by Claus and Macheak [30] at the 24th week (6 months).

Statistical analysis of these results using t-test, indicated that there was no significant difference in agglutinating antibody titer of *C. chauvoei* of sheep sera vaccinated with polyvalent clostridial vaccine (group I and group III) using plate agglutination test.

Table 4 and figure No. 5 show the results of the serum neutralizing antibodies in case of rabies vaccine alone (group II). The antibody levels ranged from 8 to 64 and remained fixed up to 12 months post vaccination (MPV), while the same table and figure showed that the use of combined vaccine doubled the titers of antibodies (16 to 128) at the same periods of time. The peak was reached at the 4th WPI and the stationary phase remained during all the experiment time (12 MPV). These criteria are in agreement with Khodier [31]. In this respect, the WHO [11] regulated the challenge test which evaluates the potency of the rabies vaccine which is forbidden, especially in farm animals to avoid the public health hazard. In addition, it was found that the results of mouse passive protection test, which was carried out to avoid the challenge of vaccinated sheep, were similar to these of SNT. These results are parallel to the findings of Larghi and Nebel [20], Sikes *et al.* [32] and Bass *et al.* [33]; who stated that the cell culture inactivated rabies vaccine is safe for all animal species and clarified that the protective neutralizing antibody titer should not be less than 1: 5. However, group-III showed higher levels of anti-rabies antibodies, the thing which could be attributed to the presence of clostridia toxoid in the combined vaccine which acts as immune stimulant as stated by Guirguis *et al.* [34].

From the mentioned results, it was indicated that the rabies vaccine did not interfere with the sheep immunity against the clostridial vaccine i.e. the time of antibody release was not affected in comparison with the vaccination of clostridial vaccine alone. In addition, titers ranged between 3-20 (more than the minimal protection limits) when TNT technique was adopted against neutralizing clostridia antitoxin titers in vaccinated sheep against β, ∈, (*C.perfrigens*) ∝ (*C. novyi*), ∝ (*C. septicum*) and *C. tetani* antitoxins. On the other hand, the levels of rabies antibodies were increased when clostridial types were added. The titers of rabies antibody may be increased due to immuno-stimulus-effect of clostridial toxoids. This fact is true since the group II of sheep vaccinated with inactivated rabies vaccine adjuvanted with aluminum hydroxide gel was used alone; the protective titer reached its peak at 4th week post vaccination and continued at the same level till 12th month post vaccination. The titer of antibodies against rabies was doubled in group III which was vaccinated with combined vaccine and the criteria of duration were the same.

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