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EFFICACY OF FREEZE-DRIED INACTIVATED VACCINE AGAINST RABBIT ENTEROTOXAEMIA

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ABSTRACT

Clostridium (C.) perfringens type A is a leading cause of enterotoxemia in rabbits, significantly affecting outcomes in rabbit farming. It results in a high mortality rate, particularly in weaned rabbits. Vaccination is the most effective control method for this disease. The study focused on preparing and assessing a rabbit bloat vaccine of inactivated toxoid of *C. perfringens* type A in a lyophilized form that is reconstituted at the time of vaccination using saponin that acts as an adjuvant. The immune response and keeping quality of the prepared vaccine were evaluated in rabbits every 6 months till 24 months of storage at 4 ^{o}C and compared to a local vaccine prepared from an inactivated toxoid of *C. perfringens* type A using aluminium hydroxide gel as an adjuvant. The results showed that all vaccinated rabbits had protective antibodies against *C. perfringens*, and the results from tested rabbits achieved 4 IU/ml for *C. perfringens* types A α toxin. It was also found that the lyophilized vaccine prepared from the toxoid of *C. perfringens* type A gave a higher antitoxic titer than the local vaccine, which lasted protected for up to 24 months; in contrast, the local vaccine lasted only up to 12 months. It was clear that using the lyophilization method not only affects the potency of the vaccine but also increases the duration of preservation of the vaccine and reduces the time spent during vaccine transportation. Also, using saponin as an adjuvant helps in stimulating the immune response.

Keywords: Enterotoxaemia vaccine, Lyophilization, Saponin.

INTRODUCTION

C. perfringens type A greatly affects rabbit farms with substantial losses and high mortality rates, especially in weaned rabbits. It results in bloat, severe diarrhoea, and enterotoxemia (El-Bakey *et al.*, 2018). The word ‘enterotoxemia’ refers to a toxemia of intestinal origin that occurs when toxins produced in the intestine are absorbed into the bloodstream (Popoff 1998, Diab *et al.*, 2003, and Pawaiya *et al.*, 2020). Enterotoxemia is considered one of the most serious enteric diseases affecting rabbits, with economic importance and financial disability. It produces the most prevalent flash infection, which can lead to epidemics with up to 50% mortality rates (McDevitt *et al.*, 2006, and Hussain *et al.*, 2022). Treating infections caused by toxins is highly intolerable; therefore, vaccination against toxins is the standard process to combat infection (Saadh *et al.*, 2022).

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By activating natural, non-specific defenses and then developing adaptive immune responses to combat invading microbes, vaccination has proven to be an efficient and economically viable means of preventing infectious diseases over time. (Look *et al.,* 2009).

The process of freeze-drying vaccines is very significant. Numerous vaccines are sensitive to changes in temperature, posing difficulties for storing and distributing them. Creating products that can withstand fluctuations in temperature can improve the stability, effectiveness, and distribution of vaccines, as well as prevent product wastage resulting from improper storage conditions. Furthermore, lyophilized forms may offer extended product expiration dates (Michelle *et al.,* 2016, and Payton *et al.,* 2022).

Vaccines need optimal adjuvants containing immunopotentiators and delivery systems in order to offer prolonged protection. Saponin has the ability to stimulate not only cell-mediated immune response but also improve the production of antibodies, and only a small dose is needed to activate their adjuvant properties (Rajput *et al.,* 2007). Saponins can play a multifaceted role in coordinating an efficient and sophisticated antigen-specific immune response (Wang, 2021).

This study was carried out to evaluate the impact of lyophilization by preparing rabbit bloat vaccine in a lyophilized form and adding saponin as an adjuvant to prolong the shelf life of the vaccine and increase its immunogenicity.

MATERIALS AND METHODS

Ethical approval

This study was approved by the Agricultural Research Center Institutional Animal Care and Use Committee (ARC-IACUC). It was reviewed and supervised by the Ethical Committee of the Veterinary Serum and Vaccine Research Institute (VSVRI) (ARC-VSVRI-22).

Strain

C. perfringens type A strain local isolate from rabbits obtained from Anaerobic Bacterial Vaccine Research Department, Veterinary Serum and Vaccine Research Institute (VSVRI), Abbasia, Cairo, Egypt. Cooked meat media was used for rehydration of *C. perfringens* type A. Production medium was used to produce *C. perfringens* type A α toxin (El-Helw *et al.,* 2017).

Laboratory Animals

1. Swiss white mice

Two hundred (200) Swiss white mice weighing approximately 18-20 g were taken from the farm of VSVRI. These mice were utilized to determine the minimum lethal dose (MLD) of *C. perfringens* type A toxin and to titrate antitoxin levels in the sera of vaccinated animals using the serum neutralization test (SNT).

2. Rabbits

Thirty-five Bosket rabbits weighing 2–2.5 kg were kept in batteries in a well-ventilated, sterile place, where they were fed commercial pellets and provided with clean water. Be sure that they are free from *C. perfringens*. They were used for determination of the potency of the prepared vaccine.

Reagents

1. Formalin

It was used for inactivation of *C. perfringens* culture at a concentration of 0.5% (Gadalla *et al.,* 1969) .

2. Saponin

It was supplied by ACROS Co. (New Jersey, USA) and used as a diluent for vaccines and as an adjuvant for freeze-dried vaccines. A saponin concentration of 1 mg/dose was added accordingly (Samuilenko, 1982 and Nehal *et al.,* 2017).

Stabilizer

It consists of casitone 2%, sodium glutamate 1%, sucrose 4%, and gelatin 0.2% according to a modified method by Angus 1984 and Behroozikhah *et al.,* 2009.

Preparation of lyophilized C. perfringens type A vaccine

The vaccine was prepared from the *C. perfringens* type A lyophilized strain, according to El-Helw *et al.*(2017). Bacterin was inactivated using formalin (0.5%) at 37˚C for one day and separated using an ultra-filtration system (Millipore Corporation, USA). The inactivated toxoid was divided into 2 parts: the first part was a local liquid vaccine mingled with aluminium hydroxide gel adjuvant (vaccine No. 1), and the other part was mingled with the same quantity of stabilizer and divided into vials (2 ml/vial) and subjected to the lyophilization process (vaccine No. 2). This vaccine formulation was reconstituted using saponin (1 mg/dose), as an adjuvant at vaccination (Atwa *et al.*, 2020).

Quality control of the prepared experimental vaccines

1.Sterility test

The vaccines, once tested, were immediately used to ensure that they were free from any contaminants, such as aerobic and anaerobic bacteria and fungi, accordingly (WOAH Terrestrial Manual 2023). This was done through direct inoculation in two types of media, including fluid thioglycollate medium with the addition of 0.5% beef extract (FTMB) incubated at 30-35^{o}C and soybean casein digest medium (SCDM) incubated at 20-25^{o}C. All media were incubated for 14 days to detect slower-growing microorganisms, with recommended daily reads for detection of any microbial growth/turbidity.

2. Safety test

The safety of the vaccines was evaluated by administering double the recommended dose of each vaccine subcutaneously to 5 rabbits lacking antibodies. The vaccines were monitored for possible adverse reactions, both locally at the site of injection and systemically through monitoring any body temperature changes or generalized abnormal changes in the rabbits according to the directions of WOAH (OIE) Terrestrial Manual 2022.

Experimental design

Thirty 4-week-old hybrid Bosket rabbits were separated into three groups, each consisting of 10 rabbits, as follows*:*

Group (1): was vaccinated with liquid toxoid of *C. perfringens* type A mixed with aluminium hydroxide gel (local vaccine) (vaccine No. 1)

Group (2): was vaccinated with lyophilized toxoid of *C. perfringens* type A (vaccine No. 2).

Group (3): was kept as a control unvaccinated.

All rabbits received subcutaneous injections of 3 ml of the prepared vaccines in two doses., 3 weeks apart, containing 60 MLD/ml (Elham *et al.*, 2014), except for the control negative group, which was left unvaccinated.

Blood samples were collected in the 1st week after the 2nd dose of vaccination from all vaccinated groups, then every 6 months till 24 months. The blood samples collected were left to clot and then spun in a centrifuge (10 minutes at 2500 rpm) for serum isolation. Serum samples from each rabbit were stored in sealed vials at -20°C until they were serologically tested for the presence of specific antibodies.

The potency evaluation of the prepared vaccines

1. Serum Neutralization Test (SNT)

Measuring of a test dose of alpha toxin of *C. perfringens* type A, followed by the measuring of the potency of unknown sera as detailed in the study by Gadalla *et al.* (1971).

2. ELISA

It was conducted following Buys *et al.*, (2020). The sera were considered positive when the absorbance values were as or more than the cut-off value (the cut value = double the mean of O.D “Optical Density” of negative sera).

Statistical analysis

Statistical analysis was performed using Microsoft Excel 2016. The data were obtained, expressed as a mean, and compared using a t-test. A p-value ≤ 0.05 was considered to indicate statistical significance.

RESULTS

Quality control of the prepared experimental vaccines

1. Sterility test:

There was no observed growth in the different media that were inoculated with the different prepared vaccines.

2 .Safety test :

By the end of the safety study, all rabbit cohorts remained in good health and survived without any adverse reactions noted, with no alterations in their consumption of water or food.

Evaluation of the potency of the vaccines

Tables (1 and 2) and figures (1 and 2) depicted antibody titers measured by serum neutralization test and ELISA in vaccinated rabbits with vaccine no. 1 and vaccine no. 2, showing vaccine no.1's protection remained protective up to 12 months while vaccine no. 2 remained protective up to 24 months with higher antibody titer than vaccine no. 1. A notable statistically significant difference (P ≤ 0.05) was observed in antibody levels by using serum neutralization test and/or ELISA among all vaccinated rabbit groups.

Table 1: Potency evaluation of prepared vaccines using Serum Neutralization Test (SNT) against *C. perfringens* type A

Month post vaccination	Types of vaccine	
	Liquid toxoid vaccine (local vaccine) (vaccine No.1)	Lyophilized toxoid vaccine (Vaccine No.2)
Pre vaccination	0	0
0 (1st week post booster)	12	20
6 months	9	18
12 months	5	15
18 months	0	10
24 months	0	6

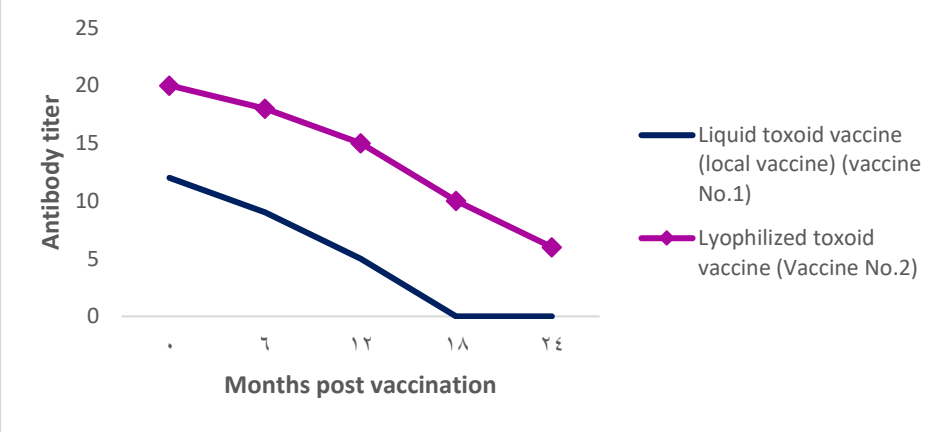

Fig. (1): Potency evaluation of prepared vaccines using Serum Neutralization Test (SNT) against *C. perfringens* type A

Table 2: Potency evaluation of prepared vaccines using ELISA Test for *C. perfringens* Type A

Months post vaccination	Types of vaccine	
	Liquid inactivated toxoid vaccine (local vaccine) (vaccine No.1)	Lyophilized inactivated toxoid vaccine (Vaccine No.2)
Pre vaccination	0.197	0.201
0 (1st week post booster)	1.478	1.765
6 months	1.109	1.589
12 months	0.616	1.324
18 months	0.287	0.883
24 months	0.211	0.53

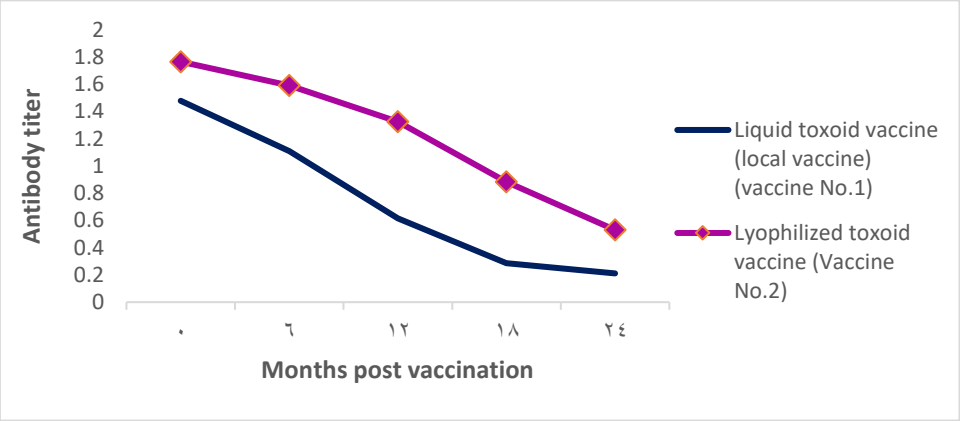

Fig. (2): Potency evaluation of prepared vaccines using ELISA Test against *C. perfringens* type A

DISCUSSION

Enterotoxemia in rabbits resulting from *C. perfringens* type A is regarded as a major enteric disease that leads to great losses and high mortality rates (up to 50%), particularly among weaned rabbits. An excellent manner to control the disease is through vaccination.

Vaccines, known as "weapons of mass protection," activate the immunity to build a robust, enduring shield against microbes (Cohen and Marshall, 2001; Curtiss, 2002).

Toxoid vaccines have been widely distributed for commercial use and have been extensively utilized in domesticated animals for many years; this may be due to using inactivated toxins in toxoid vaccines targeting the toxic activity created by the bacteria rather than targeting the bacteria itself. Also, there are many advantages of toxoid vaccines, as they are safer and cannot spread among non-immunized animals because the vaccine antigens are not undergoing active replication. Additionally, they are stable and durable because they are not easily affected by fluctuations in temperature, humidity, and light. (Baxter, 2007).

Inactivated rabbit enterotoxemia vaccine conjugated with aluminum hydroxide gel is widely available commercially.

It is always necessary to make a new modification in the preparation of the vaccine to achieve a high degree of safety and effectiveness.

The lyophilized vaccines are highly intriguing. Numerous vaccines are thermolabile, making it challenging to distribute and store them in countries with limited cold-chain management. The development of thermostable vaccines can

help in the distribution of vaccines in these areas and can additionally help in reducing waste of products that have been stored at temperatures above or below specified storage temperatures. Furthermore, lyophilized forms extend the storage duration of vaccines (Brandau *et al.,* 2003).

The objective of this work was to develop a new formula for inactivated rabbit bloat vaccine using the lyophilization technique that improves the keeping quality and commercialization of a vaccine. And also studying the effect of using saponin as an adjuvant in improving the immune response.

In this study, two vaccine formulae were prepared to determine which provides the greatest efficacy and lasts longer. The first one is the liquid-inactivated toxoid vaccine of *C. perfringens* type A adjuvanted with aluminium hydroxide gel (local vaccine) (vaccine no. 1), and the 2nd one is the lyophilized inactivated toxoid of C. perfringens type A (vaccine no.2), which were reconstituted at the vaccination time with saponin (1 mg/dose) as an adjuvant to enhance the humoral immune responses.

The sterility testing results of the prepared vaccines showed that they were free from any bacterial or fungal contamination on inoculated media, and this agreed with what is recommended by the WOAH (OIE) Terrestrial Manual 2023. It was also found that the safety of rabbits was even with a 2x field dose. These findings met the recommendations and requirements of safety of the WHO (OIE) Terrestrial Manual 2022.

The prepared vaccines were evaluated using serum neutralization and ELISA tests, and the results showed that the minimum protective antitoxic titer (4 IU/ml) of the serum from tested rabbits was achieved for *C. perfringens* type A (α toxin), according to the United States Department of Agriculture in 2015, from the 1st week post-vaccination and remained up to 12 months storage for liquid vaccine while remaining up to 24 months for lyophilized vaccine.

This indicates that the efficiency of the prepared vaccine by the lyophilization method did not have a negative impact on the antigenicity and immunogenicity of the vaccine. These results are consistent with those of Atwa *et al.* (2020), who mentioned that the lyophilized inactivated RVF vaccine is highly immunogenic and cost-effective compared to the aluminium hydroxide-inactivated RVF vaccine.

It was also found that there is a significant difference ($P \leq 0.05$) between the two vaccines, as the lyophilized inactivated toxoid of the *C. perfringens* type A vaccine gave a higher antitoxic titer than the liquid form of the inactivated toxoid of *C. perfringens* type A when measured by SNT, and this is due to the use of saponin as an adjuvant and these findings were documented by Oda *et al.* (2000) and Rajput *et al.* (2007), who reported that the use of saponin as an adjuvant has the ability to stimulate not only a cell-mediated immune response but also encourage antibody production and also has the important advantage that only a low dose is needed for the activity of the adjuvant.

Using saponin (1 mg/dose) resulted in high and protective titers from the 1st week post-vaccination, as shown by this outcome, which aligns with Ojiako *et al.* (2019), who found that saponin adjuvant usage was more effective than other ones, as it can boost antibody production. Furthermore, Maha *et al.* (2020) discovered that utilizing saponin in a vaccine for bovine viral diarrhoea virus (BVDV) in mice was able to enhance both cellular and humoral immune responses, resulting in increased levels of total specific IgG against BVDV in all immunized groups that received saponin-adjuvanted vaccines. The results of the ELISA test also confirmed the results of the SNT.

CONCLUSION

Lyophilized inactivated rabbit bloat vaccine could be provided as a safe, potent vaccine, suggesting that it has a long shelf life. Lyophilized inactivated toxoid of the *C. perfringens* type A vaccine gave a higher antibody titer than the liquid form of the local vaccine. Also, using saponin as an adjuvant gave high and protective titers with recommendations to track the immune response of prepared vaccines.

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كفاءة اللقاح المثبط المجفد لمرض التسمم المعوي الأرنبي

الهام فضل السرجاني ، حامد عادل الحلو ، ياسر أحمد عبد الله ، مروه مصطفي أحمد ، نورا محمد خلف ، علياء عبد الباقي

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تسبب الكلوستريديم بيرفرينجنز نوع أ مرض التسمم المعوي في الارانب والذي يعتبر من أهم الأمراض المعوية من الناحية الاقتصادية. ويتسبب في خسائر ونفوق كبير (يصل إلي ٥٠٪) خاصة في الأرانب المفطومة. التطعيم هو وسيلة مهمة وأفضل للسيطرة على المرض. تم تطبيق هذه الدراسة لتحضير وتقييم لقاح النفاخ الارنبي المثبط المحضر من كلوستريديم بيرفرينجنز نوع أ في صورة مجفدة بالتجميد وتم اختباره وقت التطعيم باستخدام مادة الصابونين والتي تعمل كمحفز مناعي. تم اختبار تقييم الاستجابة المناعية (باستخدام اختباري سيرم المصل المتعادل (SNT) واختبار الأليزا وجودة وصلاحية اللقاح المحضر في الأرانب كل ٦ أشهر حتى ٢٤ شهرًا عند تخزينه عند درجة حرارة ٤ درجة مئوية ومقارنته باللقاح المحلي المثبط المحضر من الكلوستريديم بيرفرينجنز نوع أ المحضر من جل هيدروكسيد الألومنيوم. أظهرت النتائج أن جميع الأرانب المحصنة لديها أجسام مضادة وقائية في الأسبوع الأول بعد التطعيم ضد الكلوستريديم بيرفرينجنز نوع أ. وقد تحقق الحد الأدنى من الاجسام المضادة والذي كان ٤ وحدة دولية / مل لسم الالفا المفرز من الكلوستريديم بيرفرينجنز نوع أ في مصل الأرانب. كما وجد أن اللقاح المجفد بالتجميد المحضر من الكلوستريديم بيرفرينجنز نوع أ قد أعطى اجسام مضادة مناعية أعلى من اللقاح المحلي والذي استمر ذو جودة وصلاحية حتى ٢٤ شهرا، بالمقارنة باللقاح المحلي الذي استمر حتى ١٢ شهرا فقط. لقد تم توضيح أن استخدام طريقة التجفيد لا يؤثر فقط على فعالية اللقاح ولكن أيضًا يزيد من مدة حفظ اللقاح ويقلل من الوقت المستغرق أثناء نقل اللقاح. كما أن استخدام الصابونين كمحفز مناعي يساعد بكفاءة في تحفيز الاستجابة المناعية.