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# Role of Liposome as an Adjuvanted Antigen Delivery System in Enterotoxaemia Vaccine in Rabbit

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**Abstract**

ENTEROTOXAEMIA disease in weaned rabbits has a significant impact on the rabbit industry. *Clostridium perfringens* type A caused an enterotoxaemia disease accompanied by severe bloat with brownish diarrhea and high mortalities. This study aims to compare the potency of a newly formulated enterotoxaemia vaccine and that of the presently available vaccine. Two types of enterotoxaemia vaccines were prepared from a toxigenic strain of *Clostridium perfringens* (CP) type A namely CP/liposome vaccine and CP/aluminum hydroxide gel vaccine. One received aluminum hydroxide vaccine in 2 doses (2 ml S/C with 3 weeks interval) and the other 2 groups, CP/liposome vaccine received liposome vaccine as one-shot dose (G2) and one as a control unvaccinated group (G3). Humoral immunity was quantified using indirect enzyme-linked immunosorbent assay (ELISA) and serum neutralization test. Out of two vaccines evaluated, CP/liposome vaccine produced higher protection till 11 months without any booster dose and no vaccinal reactions observed at injection site while the aluminum hydroxide gel vaccine give protection till 6 months. So, the study concluded the synergistic role of the prepared CP/liposome adjuvanted vaccine in eliciting a higher and persistent immune response and its superiority over the currently available vaccine.

**Keywords:** Aluminum hydroxide gel, *C. perfringens* type A, ELISA, liposome, Serum neutralization test.

## Introduction

*Clostridium perfringens* is located in the intestinal tract of living organisms as a normal inhabitant, until toxin released under stress conditions or dietary changes [1]. *Clostridium perfringens* was the main reason for enterotoxemic diseases occurrence in animals [2]. *Clostridium perfringens* type A was isolated from animals with severe diarrhea and healthy animals [3]. It is also associated with enteric diseases in animals and humans like foodborne diseases, diarrhea, and enteritis [4]. Investigations showed that rabbit outbreak in different farms associated with enterotoxaemia symptoms and Clostridium *perfringens* type A alpha toxin detected in cecal contents of dead rabbit with diarrhea [5]. A multi-drug-resistant *C. perfringens* strain was isolated by [6] from the fecal sample of a patient who was clinically suspected of gastrointestinal infection. Over the years, it was confirmed that vaccination is the most effective measure to control infectious diseases by enhancing both innate and adaptive immune responses [7]. Vaccine manufacturing progress is directed mainly to adjuvants that play an effective role in elaborating strong humoral and cellular immunity. Aluminum formulations widespread used as adjuvants in vaccine manufacturing of both human and veterinary vaccines [8]. Aluminum hydroxide adjuvants stored under unfavorable condition such as temperature, can cause adverse reactions [9]. Recent advances in vaccinology were the development of delivery systems such as polymeric nanoparticles and liposomes, which improve bioavailability and lower toxicity at site-specific delivery. Among the wide variety of the antigen- delivery systems, liposome in animals have been evaluated. Liposome are spherical vesicles with lipid bilayers [10]. Liposomes are proven as immunoadjuvants effective in eliciting high immune

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response against several antigens, biocompatible and easily to prepare [11].

The current study aimed to evaluate the role of liposome as an adjuvant and antigen delivery system in the prepared vaccine and compare it with the traditional vaccine prepared with aluminum hydroxide gel by determination of the potency of the prepared vaccines.

## Materials and Methods

*Ethical approval*

All work applied according to the committee of the Egyptian national research in animal ethical standards, (NLQO) and (VSVRI) ethical Committee.

*Bacterial cultures*

Highly toxigenic strain of *Clostridium perfringens* type A was isolated from dead weaned rabbit with severe post mortem lesions and identified morphologically, culturally and by serological tests in the Institute of (VSVRI) [12].

*Toxoid vaccine preparation*

The isolated strain rehydrated in cooked meat medium under an anaerobic condition at 37 °C then examined on blood agar at 37 °C for 24 hours then firstly inoculated on peptone media for 4 hours then transferred to the production media with 1% sucrose for 4-5 hours and adjust pH at 8 at 37°C to enhance optimum toxin production then determination of minimal Lethal Dose in mice [13] .Toxin inactivated by 0.5% formalin (v/v) addition at least 7days then adding merthiolate (v/v) (0.01%) the day after formalin addition as preservative agent. Toxoid then filtered by millipore filter (0.22um/cross) for separation and concentration.

*Formulation of aluminum hydroxide gel vaccine*

Respectively after toxoid preparation, adjuvant was added about 20%aluminum hydroxide gel and 80% toxoid [14]. Homogenization by magnetic stirring then vaccine examined for safety and sterility.

*Formulation of liposome vaccine*

Liposome Kit according to Sigma-Aldrich a Lipid mixtures is a lyophilized powder contains: cholesterol, L-α-phosphatidylcholine, and stearyl amine. This lipid mixture may be used to prepare cationic (positively charged) liposomes for incorporation of materials into cells.

To prepare liposomes, add 1 ml of an aqueous solution containing the solute to be encapsulated into the vial at the desired temperature (>4 °C) and mix well by vertexing for 30 seconds. A homogenous milky suspension should result. Added DMSO or ethanol, transfer 1–10 µl of this solution into the vial then add 0.2 ml of aqueous medium. Vortex well at room temperature. Agitate for 30 minutes. The organic solvent should be<1% of the final needed, it can be removed by gel filtration or dialysis. Average particle size was 100 nm.

*In control process test* [15]

Sterility test of both vaccines carried out by inoculating the prepared toxoid in fluid thioglycolate, nutrient agar, cooked meat media and sabouraud dextrose agar tubes then ten days observation for no bacterial or fungal growth. Vaccine's safety done by injecting five mice (22g) I/P by 0.5 ml of the prepared vaccine ~~o~~r five guinea pigs I/M by 3ml then any death or symptoms appearance registered. For stability testing, both vaccines kept at 4 ºC for 14 days before vaccination.

*Vaccination Schedule and Vaccine potency determination*

Thirty bosket rabbits assigned into three groups were confirmed that all rabbits free from *C. perfringens* alpha. Group (1) CP/ aluminum hydroxide gel vaccinated group: rabbits vaccinated with aluminum hydroxide gel, in 2 doses each dose of 2 ml S/C. with 3 weak interval, Group (2) CP/ liposome vaccine group: rabbits vaccinated with liposome vaccine in one dose 2ml S/C and Group (3): unvaccinated control group. Serum samples were collected monthly from all groups and then immune response determination by seurm neutralization test (SNT) and ELISA.

*Challenge test*

According to [16] ten randomly chosen rabbits from three groups, the first group was challenged by s/c injection with 0.1 ml of the virulent alpha toxin strain 2 weak after second dose and the second group was challenged after 35 days then all observed and results recorded after 15 days post-challenge according to [17].

*Statistical analysis*

One-way ANOVA (Tukey's test) to evaluate three or more groups' differences values with significant value $p < 0.05$.

## Results

*Characterization test*

The procured strain of *C. perfringens* type A showed typical morphological, cultural and toxigenic properties. The toxin titer was 100 MLD/ml.

*Sterility, safety and stability testing of vaccines*

All media inoculated are free from any growth, all mice vaccinated with the prepared vaccine very safe and vaccines are very stable in $4^0$C.

*Determination of immune response by ELISA and SNT in rabbit's sera.*

Standard alpha antitoxin was used for determination antibodies titer by SNT and ELISA. The results of mean antibody titers for both vaccines determined were illustrated in Tables (1 and 2) and Figures (1 and 2). Group (G1) vaccine adjuvanted with aluminum hydroxide gel vaccine was 3.09 IU by ELISA and 3.1 IU by SNT with 80% protection while group (G2) vaccine adjuvanted by liposome was 4.0 IU by ELISA and 3.7 IU by SNT with 100% protection. It is showed that the gradual rise of antibodies titer for both vaccines at the beginning of vaccination then gradually declined till 8 months from the booster dose at aluminum hydroxide gel (0.50 IU) vaccine and reached unprotected level (less than 0.5 IU), while liposome vaccine with one shot dose remained protective for more than 10 months (0.64 IU). In all vaccinated rabbits a strong antibody response against alpha toxoid was evaluated and the results detected it 6 months after immunization. So, the prepared vaccine dose showed a production-permissible requirement. Interestingly, the liposome formulation is safer compared to aluminum hydroxide gel vaccine and has a potent and prolonged immunity. Also, these results agreed with challenge test in Table (1).

## Discussion

Rabbit faces a very great economic losses diseases like enterotoxaemia [18] which responsible for sudden death and increase the rate of mortalities was investigated by [19]. *Clostridium perfringens* was classified into seven types according to the type of toxin released. Toxigenic isolates were typed by [20] and 68.2% of the isolates produced toxins. *C. perfringens* type A is the main reason of severe losses of rabbit's farms, especially in weaned rabbits as a result of introducing new food so cause increase the fermentation rate in intestine, which enhance the production of alpha toxin and cause severe loss in young rabbit accompanied with brownish diarrhea with offensive odor and bloat. *C. perfringens* α toxin is secreted in all types of toxins and is closely related to necrotic enteritis in poultry [21]. Vaccination is an important way to ensure the health of animals. The vaccine efficacy depends mainly on the virulency of antigen and adjuvants to elicit a significant immune response. Adjuvant is a substance that enhances immune responses against a vaccine [9]. Its role is to increase the weak antigens' immunogenicity, prolonged immune response; cell mediated immunity (CMI) enhancement and mucosal immunity induction [22]. Many adjuvants are available now in vaccine production as Aluminum hydroxide gel, liposome and other recently discovered adjuvants.

Aluminum hydroxide is the widely commonly used adjuvant. Although aluminum hydroxide adjuvanted vaccines are beneficial, sometimes they may cause adverse reactions [9]. Liposomes are bilayer lipid or phospholipid vesicles consist of amphiphilic lipids and phospholipid molecules. Liposomes improve the encapsulation and delivery to target cells by enhancing stability and efficacy. Over few decades, liposomes and lipid nanoparticles developed subunit vaccines against infectious diseases, including TB [23]. Liposomes are spherical lipid structures with an aqueous core. Phospholipids are the main constituents of the shell protect the aqueous core. They are amphiphilic with hydrophobic tail composed of two fatty acids. The liposomes' structure made it act as carriers for antigens [24]. The liposomes adjuvant action is their ability to involved in APCs, antigen exposure, immunostimulatory and acts as delivery antigens. Flexibility is one of the most important liposomes is that it allows the combination of molecules with liposome dispersion [25].

This study decided to make a comparison between two different adjuvants like liposome and aluminum hydroxide gel by using serological tests. The prepared vaccines quality control was conducted according to [26] and Egyptian Standards Regulations for Evaluation of Veterinary Biologics (2017). We not have any evidence of local side effects among rabbits vaccinated with the prepared toxoid. In vaccinated rabbits, the antibodies production against alpha toxin was monitored by ELISA and SNT.

It is cleared that rabbits vaccinated with the prepared CP/liposome vaccine with one shot dose showed a stronger immunity than that those vaccinated with CP/aluminum hydroxide gel. The

mean antibody titers of liposome vaccine groups increased after vaccination and then declined gradually till they reached the non-protective level after 11 months, but the antibody titers of rabbits vaccinated with aluminum hydroxide gel vaccine group reached 6 month .The results agreed with[27] The use of liposome as an adjuvant and a vaccine carrier was effective in enhancing strong and prolonged immune response in comparison with aluminum hydroxide gel and this agreed with the results of the challenge test and the results of [25] concluded that liposome was safer compared to the live B. abortus S19 vaccine at 15 days post challenge and [28] found that administration of liposomes-entrapped enhances the immune system and gave protection.[29] proved that the New castle disease liposome vaccine elicited a significantly high immune response and did not have any toxic effects. It is concluded from this study that vaccines prepared from liposome with one shot dose enhance a strong immunity.

## Conclusion

This study concluded that adjuvant choice is crucial in a vaccine manufacture to be safe and effective. The adjuvant selected must give the same immune response in all batches. The results showed clearly that the high immunogenicity of liposome vaccine was very high, prolonged duration immunity and safe with 90% protection with no report of post vaccinal reaction.

*Declarations*

*Ethical consideration*

All work has been applied according to the international biological animal welfare instructions, and according to ethical guidelines at the Cairo Veterinary Medicine University and VSVRI ethical Committee.

*Competing interests*

Authors declared that this study have no any conflict of interests.

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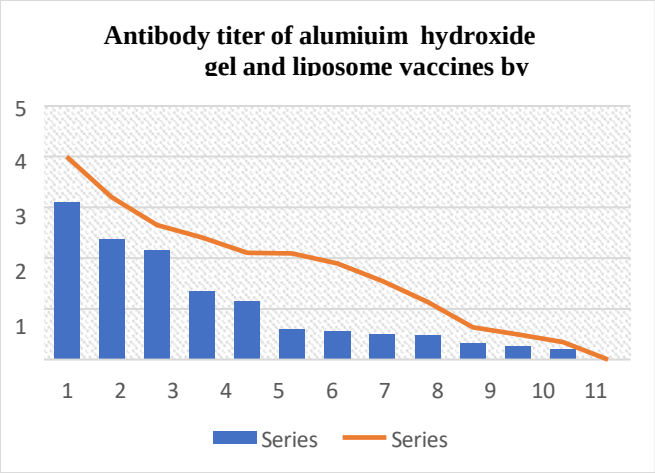
*Authors Contributions*

El Jakee J., Yasser Abdulla and Heba N.Dief designed experiments, wrote and reviewed the manuscript. Riham Wahied applied the manuscript experiments, performed and written. reviewed and accepted for the final version by all authors.

**TABLE 1. The immune response of CP/aluminum hydroxide gel and CP/liposome vaccines in rabbits' sera by ELISA**

| Gps | Mean antibody titer in serum of rabbits vaccinated with aluminum hydroxide gel and liposome by ELISA | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1M | 2M | 3M | 4M | 5M | 6M | 7M | 8M | 9M | 10M | 11M | 12M |
| G1 | 3.09 ±0.62 | 2.37 ±0.68 | 2.15 ±0.67 | 1.35 ±0.62 | 1.16 ±0.43 | 0.60 ±0.14 | 0.57 ±0.09 | 0.50 ±0.094 | 0.48 ±0.074 | 0.32 ±0.21 | 0.26 ±0.22 | 0.21 ±0.21 |
| G2 | 4.0 ±0.79 | 3.2 ±0.53 | 2.65 ±0.74 | 2.41 ±0.81 | 2.11 ±0.75 | 2.09 ±0.6 | 1.89 ±0.64 | 1.54 ±0.45 | 1.14 ±0.36 | 0.64 ±0.22 | 0.50 ±0.10 | 0.34 ±0.22 |
| G3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

ELISA: enzyme linked immunosorbent assay. G1: aluminum hydroxide gel vaccine, G2: liposome vaccine, G3: control unvaccinated groups and CP: *C. perfringens.*

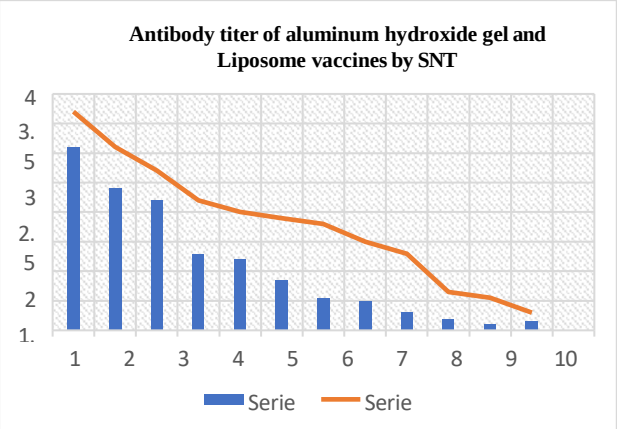



Bar: aluminum hydroxide gel vaccine (blue), Line: liposome vaccine (orange).

**Fig. 1. Mean antibody titer of aluminum hydroxide gel and liposome vaccines measured by ELISA**

**TABLE 2. The immune response of CP/aluminum hydroxide gel and CP/liposome vaccines in rabbits' sera by SNT.**

| Gps | Mean antibody titer in serum of rabbits vaccinated with aluminum hydroxide gel and liposome by SNT | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1M | 2M | 3M | 4M | 5M | 6M | 7M | 8M | 9M | 10M | 11M | 12M |
| G1 | 3.1 ±0.54 | 2.4 ±0.80 | 2.2 ±0.74 | 1.3 ±0.64 | 1.2 ±0.40 | 0.85 ±0.24 | 0.55 ±0.16 | 0.50 ±0.15 | 0.30 ±0.24 | 0.20 ±0.24 | 0.10 ±0.20 | 0.15 ±0.22 |
| G2 | 3.7 ±0.90 | 3.1 ±0.46 | 2.7 ±0.64 | 2.2 ±0.87 | 2.0 ±0.77 | 1.9 ±0.83 | 1.8 ±0.74 | 1.5 ±0.67 | 1.3 ±0.45 | 0.65 ±0.22 | 0.55 ±0.15 | 0.30 ±0.24 |
| G3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |




SNT: serum neutralization test. G1: aluminum hydroxide gel vaccine, G2: liposome vaccine, G3: control unvaccinated groups and CP: *C. perfringens.*

Bar: CP/aluminum hydroxide gel vaccine (blue), Line: CP/liposome vaccine (orange).

**Fig. 2. Mean antibody titer of CP/aluminum hydroxide gel and CP/liposome vaccines measured by SNT.**

**TABLE 3. Challenge test results for evaluation of the prepared vaccines.**

| Groups | No. of rabbits tested | No of mortality/total no of rabbits | Protection % |
|---|---|---|---|
| G1 | 10 | 2\10 | 80 % |
| G2 | 10 | No mortality | 100 % |
| G3 | 10 | 8\10 | 20 % |

G1: CP/aluminum hydroxide gel vaccine, G2: CP/liposome vaccine and G3: control group (unvaccinated).

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## دور الليبوسوم كنظام توصيل مستضد مساعد في لقاح التسمم المعوي في الأرانب

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**الملخص**

مرض التسمم المعوي في الأرانب المفطومة له تأثير كبير في صناعة الأرانب. هذا المرض الناجم عن الكلوستريديم بيرفرينجنز من النوع A مصحوبا بانتفاخ شديد مع الإسهال البني وارتفاع معدلات الوفيات. الهدف من هذه الدراسة هو مقارنة فعالية لقاح التسمم المعوي باضافة الليبوزوم كمحفز مناعىواللقاح المتاح حاليا. تم تحضير نوعين من لقاحات التسيم المعوي من سلالة الكلوستريديم بيرفرينجنز من النوع A وهما لقاح الليبوسومولقاح هلام هيدروكسيد الألومنيوم. تلقت مجموعة واحدة من الأرانب لقاح هيدروكسيد الألومنيوم على جرعتين (2 مل S / C بفاصل 3 أسابيع) والمجموعات الأخرى ، تلقت واحدة لقاح الليبوسوم كجرعة واحدة والأخرى كمجموعة ضابطة. تم قياس المناعة الخلطية باستخدام مقايسة الممتز المناعي غير المباشر المرتبط بالإنزيم (ELISA) واختبار تحييد المصل (SNT). من بين لقاحين تم تقييمهما ، أنتج لقاح الكلوستريديم بيرفرينجنز باضافة الليبوسوم كمحفز مناعى حماية أعلى حتى 11 اشهر دون أي جرعة معززة ولم يتم ملاحظة أي تفاعلات لقاحية في موقع الحقن بينما كان لقاح الكلوستريديم بيرفرينجنز باضافة هيدروكسيد الألومنيوم كمحفز مناعى اظهر حماية حتى 6 أشهر. لذلك ، خلصت الدراسة إلى الدور التآزري للقاح المساعد الليبوسوم في استنباط استجابة مناعية أعلى ومستمرة وتفوق لقاح الليبوسوم على اللقاحات المتاحة الاخرى.

**الكلمات الدالة:** هلام هيدروكسيد الألومنيوم، C. perfringens من النوع أ، ELISA، الليبوزوم، اختبار تحييد المصل.