Composing of an oil adjuvant containing BCG as an immunomodulating for rabbit clostridial enterotoxaemia and bloat vaccine

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1. Abstract

Clostridium perfringens (*C. perfringens*) is one of the most important pathogen that is widely distributed in all over the world and responsible for many diseases among life stock. *C. perfringens*is responsible for toxin production in the intestinal tract of rabbits and that was considered the main cause of enteritis and enterotoxaemia causing high mortality especially at the early weaned rabbits, which lead to high economic losses in rabbits industries. So, this study was aimed to evaluate the ability of oil adjuvant containing sonicated BCG vaccine to increase the immune response for rabbit clostridial enterotoxaemia and bloat vaccine. The study was conducted on twenty New Zealand rabbits divided in to two groups each of 10,Group A(control group)was vaccinated with alpha toxoid adjuvanted with aluminum hydroxide gel at two doses with 21 days apart inoculated subcutaneously, Group B was vaccinated with BCG sonicated oil adjuvant vaccine of alpha toxoid of *C. perfringens* type A, with 2 doses subcutaneously with 21 days. Rabbits in group (B) which vaccinated with BCG sonicated oil adjuvant vaccine of alpha toxoid of *C. perfringens* type A, their serum contain antibody titer of 8 IU/ml(7.1 IU-8.0 IU/ml) greater than that of control group (A) vaccinated with aluminum hydroxide gel adjuvant vaccine (W=0 , p=0.0001503). From this study, we can concluded that the vaccination of rabbits with oil adjuvant containing sonicated BCG vaccine is effective to increase the immune response against rabbit clostridial enterotoxaemia and bloat vaccine.

Keywords: BCG, Clostridia, Vaccine, Tuberculin test, Clostridial vaccine

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2. Introduction

Recently, rabbits' industry has acquired significant attention worldwide to increase the meat resources, particularly in low-income countries. Moreover, rabbits' meat is healthier, of lower cholesterol and fat, than other livestock meats, plus their valuable for production **[1]**. Clostridia are spore-forming bacteria that exist as inhabitants in intestinal tract of animals and under stress condition retract their pathogenicity, producing harmful toxins and causing serious diseases. *Clostridium perfringens* (*C. perfringens*) type A is the main infectious etiology of acute and sub-acute enterotoxaemia and bloat amongst rabbits, accompanied by severe diarrhea, high mortalities (27-50%) especially at 5 to 7 weeks of age and significant economic losses **[1], [2], [3].**

Antibiotic treatment of such infections is often ineffective and prophylactic vaccination with the suitable toxoid formulation was proved satisfactory for its control **[4], [5]**. In Egypt, a toxoid vaccine was produced from locally isolated toxigenic strains of *C. perfringens* type A and has been in use for disease control in many rabbit farms **[6].**

Antibody response to an antigen is improved when they are injected as water-in-oil (w/o) emulsion that primes the host for a transient onset of delayed hypersensitivity, with extensive leucocytes infiltration. Inclusion of tubercle bacilli in such a formula induces true long-lasting delayed hypersensitivity with qualitative changes in the immune response, generating surplus of immunoglobulin classes **[7], [8].**

Bacillus Calmette-Guerin (BCG), an attenuated strain of *Mycobacterium bovis* (*M. bovis*), has been developed as a vaccine against human tuberculosis by Calmette and Guerin, since 1908 at Pasteure's Institute, France. BCG or its derivatives can potentiate cell-mediated immunity and antibody synthesis in a variety of systems. Previously, investigators attributed the BCG-induced effects to stimulation of the reticuloendothelial system (RES) and augmentation of the host immune system **[9], [10], [11].** BCG was validated as a non-specific immunostimulant against a variety of pathogens including bacteria, viruses, fungi and protozoa **[12], [13], [14].** Moreover, BCG has been shown to generate a tangible heterologous immunological memory, termed trained immunity, enabling the primed immune cells to escalate a strong response to similar and dissimilar pathogens. Such a trained immunity is characterized by immune system cells' metabolic, transcriptomic, epigenetic, and functional modifications, leading to useful events, such as an increased production of cytokines **[15].** BCG has also been shown to increase adaptive antibody response to concurrent or subsequent vaccinations **[16] [17].**Recently, it has been hypothesized that BCG vaccination might be a potential preventive measure against COVID -19 **[14].**

Considering these beneficial nonspecific effects of BCG, rabbits as a sensitive experimental animals, and *C. perfringens* type A as a heterologous toxigenic, spore-forming pathogen, this endeavor was designed to achieve two main objectives. First, to construct a typical oil adjuvant for veterinary vaccines that confer features of being potent, stable, non-irritant, easy injected and inexpensive, without tuberculin test interfering. Second, to explore the efficacy of sonicated BCG particulates to augment the protective immune response induced by the local rabbit enterotoxaemia and bloat vaccine as a right model.

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3. Materials and Methods

3.1. Ethical approval

All procedures performed according to Egyptian ethical standards of the national research committee, approved from Institutional Animal Care and Use Committee (IACUC), Cairo University, under number 1221 /2013. All activities involving the use of vertebrate animals were reviewed and approved prior to their initiation.

3.2. Bacterial strain.

A local vicinal strain of *C. perfringens* type A, was kindly supplied by El-Sergany and El- Sawy **[18],** was used in this study for vaccine preparationsat the Anaerobic Bacterial Vaccines Research Department, Veterinary Sera and Vaccines Research Institute (VSVRI), Abbassia, Cairo.

3.3. Experimental Animals.

3.3.1. Mice

Ninety five Albino Swiss mice were obtained from Laboratory Animal Breeding Farm at VSVRI and were used to study the safety of the prepared vaccines, minimal lethal dose (MLD) of *C. perfringens* alpha toxin, and toxin neutralization test (TNT).

3.3.2. Guinea pigs.

Six albino Guinea pigs, weighing about 300-400 grams each, were used for performing tuberculin skin test. The animals were inoculated with either gel or oil emulsion toxoid vaccine.

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3.3.3. Rabbits.

Twenty New Zealand Rabbits (n = 20) weighing about 2.5-3kg each, were used for evaluation of the prepared vaccines and Ten rabbits were used for safety test.

Before vaccination, all rabbits were proved to be negative to bacterial components of the prepared vaccines and were examined for internal and external parasites. They were kept in a hygienic animal facility at the VSVRI during the period of experiment.

3.4. Vaccines preparation.

3.4.1. Rabbit enterotoxaemia and bloat (REtB) gel toxoid vaccine:

Briefly, the lyophilized *C. perfringens* type A vaccine seed was rehydrated into Robertson's cooked meat medium (Oxoid) and grown under anaerobic condition using Gas pack, at 37 ºC for 24 hours **[19].** Grown culture was transferred into the toxin production medium for high toxin yield and re-incubated at 37 ºC for 4 hours **[20].** The *C. perfringens* type A alpha toxin (α-toxin) was separated and concentrated from bacterial culture then, its minimum lethal dose was determined **[21].** The α-toxin was inactivated by adding formalin37% (sigma-Aldrich, UK) as 1% (v/v) and incubated for about 7 days at 37ºC, until complete inactivation. The produced inactivated α-toxin, namely α-toxoid, was adjusted to contain 40 MLD/ vaccine dose. The sterile aluminum hydroxide gel® (Suprex, Copenhagen, Denmark; 2%) was then added to α-toxoid as 20 % (v/v) and thoroughly stirred until complete homogenization according to **OIE [22]**.

3.4.2. Freeze dried BCG vaccine[23]

It was prepared and obtained from Bacterial Diagnostics Researches Department, VSVRI. To prepare the BCG sonicated antigenic particulates, the reconstituted BCG fluid (containing 10^9cfu/ml) was sonicated on ice for 15 min at intensity of 80% and 5 rounds of 3 min each.

3.4.3. Rabbit enterotoxaemia and bloat-BCG (REtB-BCG) oil emulsion toxoid vaccine (El-Ayouby et al.[12]):

In brief, the α-toxoid was emulsified in white paraffin oil at a ratio of 30:70. The surfactant blend, made of span and tween-80 as 1:1 (v/v), was added with stirring until the hydrophilic-lipophilic blance of 7 was achieved, then stirred thoroughly with BCG sonicated antigenic particulates to complete the REtB-BCG oil emulsion toxoid vaccine formula.

3.5. Quality control of the prepared vaccines.

3.5.1. Sterility testing:

All in process steps of vaccines' preparations, concerning bacterial culturing, inactivation and the final products, were subjected to in process testing for sterility as was described by OIE [22] to confirm that prepared vaccines were free from any contaminant.

3.5.2. Safety of prepared vaccines [22]

3.5.2.1. Safety in mice:

Twenty seven adult albino mice were used to test safety of both prepared vaccines. The mice were divided into three groups each of 9 mice. The first and second groups were inoculated intraperitoneally (ip) with 0.2 ml/mice with the prepared **REtB gel and REtB-BCG** oil emulsion toxoid vaccines, respectively. Whereas the third one was inoculated with physiological saline using the same dose and route and kept as a control group.

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3.5.2.2. Safety in Rabbits **[18]**

Ten rabbits were assigned into two groups, five each for safety testing of **REtB gel and** REtB-BCG oil emulsion toxoid vaccines, separately. Each rabbit in both groups was inoculated intradermal (Id) with the vaccine double dose (4 ml) and observed up to 21 days afterwards for development of any clinical abnormalities.

3.5.2.3. Potency in Rabbits **[18]:**

Susceptible rabbits (n = 20) were randomly assigned into 2 groups (A) and (B) each of ten rabbits for testing potency of both vaccines, independently. Eachof **REtB gel and** REtB-BCG oil emulsion toxoid vaccines was inoculated per a group by the same route (S/C) and dose (2 ml / rabbit). Three weeks later, all vaccinated rabbits in the 2 groups received a similar booster vaccination.

All rabbits were bled and sera were collected on the day of initial vaccination (Zero day, as controls); on the day of booster vaccinations (3 weeks post vaccination, PV); two weeks post booster vaccination (PBV)**.**Sera were complete inactivated at 56°C for 30 min and stored at -20°C to be examined for antitoxin titers.

3.6. Tuberculin skin test **[22]:**

Six albinos, tuberculin negative Guinea pigs were divided into two groups each of three guinea pigs. The first group (Group A) was injected s/c with 2 ml/animal of REtB-BCG oil emulsion toxoid vaccine. The second group (Group B) was injected intradermally with 0.1 ml of freeze dried BCG vaccine only for sensitization. After 4 weeks, all guinea pigs in both groups were tested for skin reactivity by inoculating 5 units/ animal intradermally of mammalian PPD tuberculin (VSVRI, Egypt) as instructed, in a pre-shaved abdominal area. The tested Guinea pigs were observed daily for skin reactivity at the inoculation site.

3.7. Toxin neutralization test (TNT).

It was done **[24], [25]** to verify the α-toxin-antitoxin titers against *C. perfringens* type A in sera of vaccinated rabbits. Briefly, Lethal dose dose of *C. perfringens* type A α-toxin was determined (one Lethal dose of toxin is defined as the smallest amount of toxin that can be combined with 0.5 unit of the standard antitoxin and cause death when injected into mice). Serum samples were two-fold serially diluted and an equal volume of α-toxin dose was added to each serum dilution, then the mixture was incubated at 37°C for 1 hr, allowing neutralization. Three mice were injected I/V with 0.2 ml from each serum/toxin dilution mixture and observed for 24 hours. The reciprocal of the highest dilution of serum that caused death of all mice divided by 2 was regarding as the antitoxin titer which was expressed as IU/ ml. Aα-antitoxin titer of < 1 IU /ml was regarded as negative and non-protective. The diagnostic standard α-toxin of *C. perfringens* type A (Dartford tuning and diagnostic services, UK) was used as a positive control in the TNT for rabbits' humoral serum conversion to the prepared vaccines.

Statistical test for descriptive analysis and statistical data analysis of the two groups and the difference between them was done by using R program version (1.4.1717), Wilcoxon sign rank test and the results were considered significant at $p<0.05$.

4. Results

4.1. Results of Toxin Neutralization Test(TNT):

As shown in table (1) and figure (1), Group (A) control group showing median antibody titer 4.0 IU/ml (inter-quartile range 4.0 IU/ml- 4.5 IU/ml). While, the Group (B) oil emulsion vaccine showing median antibody 8.0 IU/ml (inter- quantile range (7.1 IU/ml -8.0 IU/ml).We found that group (B) of rabbit where vaccinated with oil emulsion vaccine showed antibody titer

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than that of control group vaccinated with aluminum hydroxide gel vaccine (W=0, *p*=0.0001503).

4.2. Results of tuberculin skin test in albino Guinea pigs sensitized with human mycobacteria:

As demonstrated in figure(2), the Group (A) that injected with emulsified clostridial toxin combined with sonicated BCG vaccine, was negative for tuberculin skin test. While, in the group (B) which was injected with freeze dried BCG vaccine only shown severe skin reaction as shown in figure (3).

6. Discussion

Enteric diseases were responsible for high morbidity rate characterized by poor food conversion rate, growth depression or high mortality rate due to sudden death especially in young rabbits. The enterotoxaemia means toxemia of intestinal origin occur when the toxin produced in intestinal tract then absorbed in bloodstream. The main causes of enterotoxaemia in rabbits is *Clostridium perfringens* which is Gram positive, spore forming, rod shape and anaerobic bacterium **[26], [3]**. The enterotoxaemia due to *C. perfringens* type A mainly affecting rabbits at early weaning time, it causes high economic losses in rabbit farms. So the prevention of enteric disease by vaccination considered the main target in the rabbits industry **[27]**. BCG is a live attenuated vaccine obtained from *Mycobacterium bovis* isolated strain. Also, the vaccination with BCG has nonspecific protective effects against a number of diseases, non-mycobacterial infections and some types of malignancies **[28], [29], [14].** So, in order to enhance the immunogenic effect of the prepared vaccine, the study was performed to evaluate the ability of oil adjuvant containing sonicated BCG vaccine to increase the immune response for rabbit clostridial enterotoxaemia and bloat vaccine. Results in table (1) and figure (1) illustrated that the that group (B) of rabbit which vaccinated with BCG sonicated Oil adjuvant vaccine of Alpha toxoid of *C. perfringens* type A, their serum showed antibody titer 8 IU/ml (7.1 IU-8.0 IU/ml) greater than that of control group (A) vaccinated with aluminum hydroxide gel adjuvant vaccine (W=0 , *p*=0.0001503). These findings agree with Leake et al. **[30]** who showed that the BCG vaccine very effective and efficient vaccine against Caseous lymphadenitis of sheep. While**,** Ikeda et al. [31] observed that the subcutaneous inoculation of muramyl dipeptide (MDP), which is one of mycobacterial cell wall components gave protection against *Vaccinia* virus and *Herpes simplex* virus infection in mice. Also, El-Ayouby et al. **[12]** found that the vaccination of guinea pigs mixed vaccine (Rev.1 + BCG vaccine) give 90% protection against *Brucella melitensis*. Moreover, Stewart-Tull **[29]** indicated that the killed γ– irradiated BCG induced long term innate and adaptive immune responses. As demonstrated in figure(2), the Group (A) that injected with emulsified clostridial toxin combined with sonicated BCG vaccine, was negative for tuberculin skin test. While, in the group (B) which was injected with freeze dried BCG vaccine only shown sever skin reaction as shown in figure (3). The possible explanation for this phenomenon is that the important antigens released by live bacilli or exposed on their surface, which are not present in dead tubercle cells **[32].**

Online ISSN: 2537-1045
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7. Conclusions

From the above mentioned results, it could be concluded that the vaccination of rabbits with oil adjuvant containing sonicated BCG vaccine is effective to increase the immune response against rabbit clostridial enterotoxaemia and bloat vaccine.

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Table (1): Results of Toxin Neutralization Test (TNT) among the vaccinated groups.

No. Animal	Group(A)	Group(B)
1	4	8
2	4.5	8
3	5	9
4	4	7
5	3	7.5
6	4	7
7	4	8
8	4.5	9
9	4	8
10	5	6
Median	- 4.2	7.8

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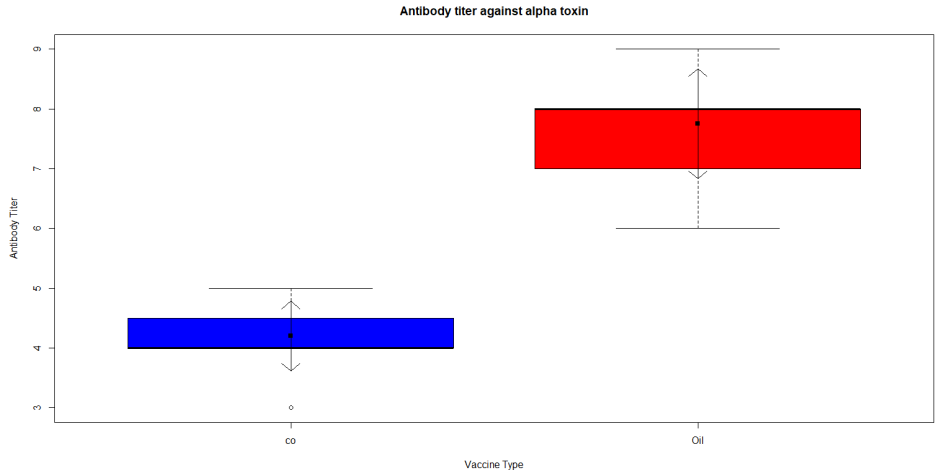

Figure (1): Antibody titer against alpha toxin.

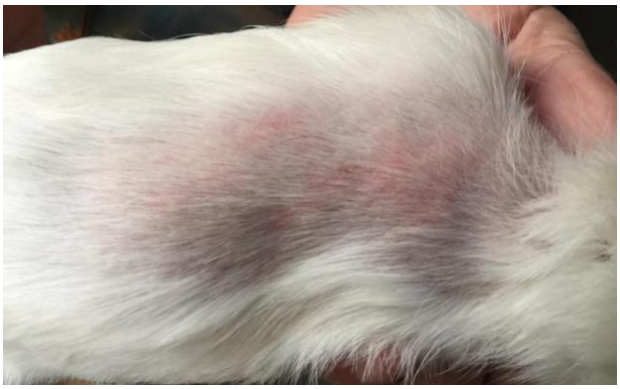

Figure (2): Negative tuberculin skin reaction in guinea pig that was injected with clostridial toxin combined with sonicated BCG vaccine.

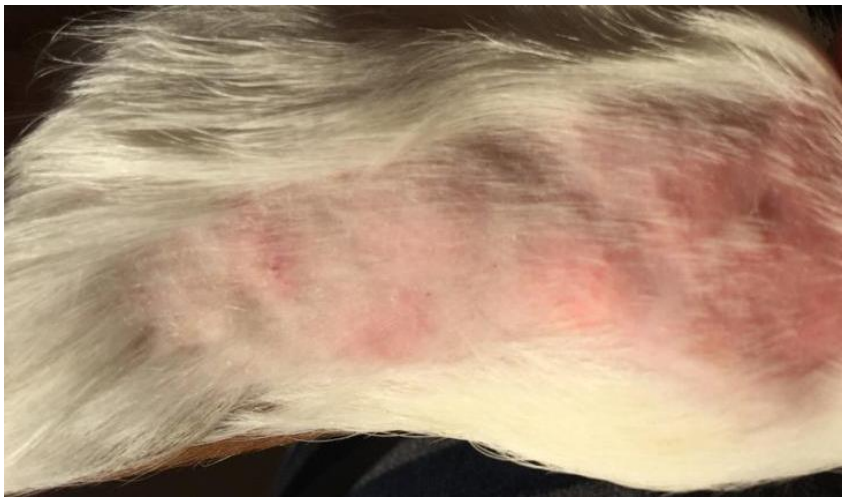

Figure (3): Positive tuberculin skin reaction in guinea pig that was injected with freeze dried BCG vaccine only

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