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One shot PLGA Nano vaccine against rabbits' enterotoxaemia

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Clostridium perfringens type A has a great impact in rabbit farms due to great loses and high mortalities specially among weaned rabbits, it causes severe diarrhea, bloat and enterotoxaemia. In the present work, *C. perfringens* alpha toxoid vaccine adjuvanted by either aluminum hydroxide gel or poly lactide co-Glycolic acid (PLGA) nanoparticles was prepared. It was revealed that enterotoxaemia and bloat vaccine adjuvanted by aluminum hydroxide gel in 2 doses protect rabbits for 6 months, while the vaccine adjuvanted by PLGA nanoparticles protect rabbits for 10 months after one shot of administration. Conclusion: It was found that PLGA vaccine gave prolonged immunity than the currently used aluminum hydroxide gel one.

Keywords: *C. perfringens*, poly-L-lactide-co-glycolidic acid (PLGA), nanoparticles.

INTRODUCTION

Enterotoxaemia in rabbits considered one of the most economically important and financially crippling enteric disease, causes the more commonly recognized fulminant infection which can result in outbreaks with mortality rates of up to 50% **(**McDevitt et al., 2006).

C. perfringens type A was isolated from caecum of rabbits died suddenly or shortly after severe diarrhea from different rabbit farms in Egypt (Diab et al., 2003).

Over years, it has become clear that vaccination is an effective and affordable measure to prevent infectious diseases, which is achieved by the activation of innate, nonspecific defenses and the subsequent development of adaptive immune responses to fight introducing pathogens (Look et al., 2009).

One of the endeavors of 21st century is delivery of vaccines and drugs through biocompatible and biodegradable polymer based nano or micro particles. PLGA is a nontoxic, FDA and European Medicines Agency (EMA) approved polymer, and widely used as a vehicle for drug and vaccine delivery (Lu et al., 2009).

Nanoparticles can offer significant advantages of high stability, high specificity, high carrying capacity, ability for controlled release, possibility to use in different routes of administration and the capability to deliver both hydrophilic and hydrophobic molecules (Pal et al., 2011).

The use of nanoparticles in vaccine formulations allows not only improved antigen stability and immunogenicity, but also targeted delivery and slow release (Zhaoa et al., 2014).

The use of biodegradable polymeric nanoparticles (PLGA) with entrapped antigens such as proteins, peptides, or DNA represents an exciting approach for controlling the release of vaccine antigens and optimizing the desired immune response via selective targeting of the antigen to antigen-presenting cells (APCs) (Akagi et al., 2012).

In this study, comparison between the currently used aluminum hydroxide gel enterotoxaemia vaccine with PLGA adjuvanted

one has been done to determine which one is able to induce more effective immunity in rabbits.

MATERIALS AND METHODS

Microorganism:

The identity tests used to identify the isolated organism were done according to Eyre (2009) for biochemical tests and Augustynowicz et al., (2002) for Polymerase Chain Reaction (PCR).

Toxoid preparation was done according to (Gadalla et al., 1974) *C. perfringens* type A was inoculated in cooked meat media and incubated anaerobically at 37 ° C for 24 hours, then sub cultured into primary peptone medium and incubated at 37 ° C for 4 hours then transferred to the toxin production medium which containing 1% sucrose and incubated at 37 ° C for 4 hours, pH was adjusted at 8 every one hour. Minimal Lethal Dose (MLD) in mice was determined. Toxin was converted to toxoid by addition of 0.5% formalin (v/v) and left for 7 days at 37°C till complete inactivation (Gadalla et al., 1969) then, 0.01% Merthiolate (v/v) was added as a preservative.

Encapsulation of toxoid with PLGA nanoparticles (Mukherjee *et al.,* 2008):

Briefly, encapsulation was done by dissolving PLGA obtained from Sigma® (500 mg) in Dichloromethane (DCM) in a beaker. Then a small amount of it was added to 2.5% of 2 g Poly vinyl alcohol (PVA) containing *C. perfringens* toxoid and was homogenized for 4 min. This mixture was added drop wise to 75 ml of 1.5% PVA in glass beaker and homogenized for 6 min. to produce w/o/w emulsion. The emulsion was then stirred on a magnetic stirrer overnight to evaporate DCM.

Evaluation of encapsulation efficacy:

It was determined by separating the *C. perfringens* type A toxoid encapsulated PLGA nanoparticles from the aqueous medium by centrifugation at 10,000 rpm for 30 min at 4°C. the amount of free antigen in the supernatant was determined by Sandwich ELISA. The result of ELISA reading is compared with that before encapsulation.

Characterization of protein-loaded nanoparticles:

Determination of morphology of PLGA nanoparticles was applied before being used as an adjuvant using transmission electron microscope (TEM). Measurement of the zeta potential has been done to allow predictions about the surface charge, particle size and the nature of material encapsulated within the nano capsules (Pangi et al., 2003).

In process control (OIE, 2015):

A- Sterility test:

It was carried out according to (British Veterinary pharmacopoeia, 2007).

The prepared Toxoid was inoculated into fluid Thioglycollate; nutrient agar; cooked meat medium, and Sabouraud dextrose agar and incubated at 37 ° C except last one incubated at room temperature. The media were observed for ten days.

B- Safety test:

Preliminary tests were made by injecting 0.5 ml of the toxoid alone and toxoid encapsulated into PLGA I/P into five mice (22 gm.). All mice survived without showing symptoms of the disease. Safety tests also were carried out by injection of 3 ml into five Guinea pigs I/M.

C- Keeping quality of prepared vaccine (Stability test):

The prepared vaccines were stored at 4◦C for time interval (3, 6 and 12 months) after preparation. Groups of Swiss albino mice were inoculated with the prepared vaccine. 14 days later, serum samples were collected and evaluated for Ab against *C. perfringens* type A by ELISA to determine the vaccine stability.

D- Potency test:

Vaccination Schedule

Thirty rabbits were screened for the absence of antibodies against alpha toxin, then assigned into three groups 10 in each one.

Group (1): It was vaccinated with vaccine # 1 *C. perfringens* type A toxoid adjuvanted with aluminum hydroxide gel, in 2 doses each dose of 2 ml S/C. with 21 days apart.

Group (2): It was vaccinated with vaccine # 2 *C. perfringens* type A toxoid adjuvanted with PLGA nanoparticles, in one dose of 2 ml S/C.

Group (3): It was kept as non-vaccinated control group.

Blood samples were collected 2 weeks after booster dose in the first group, 2 weeks after the only one dose in the second group as well as the control group, and then monthly till the antibody tier reached non -protective level.

Statistical Analysis

All statistical analyses were performed with Easy R (Saitama Medical Center, Jichi Medical University, Japan). One-way ANOVA followed by the Tukey's test was used to evaluate differences among three or more groups. Differences were considered to be significant for values of $p<0.05$.

Ethics Statement:

Care of laboratory and experimental animals were conducted in accordance with animal ethics guidelines and approved protocols of reference laboratory for veterinary quality control on poultry production (NLQO). It reviewed and supervised by the Ethical Committee of Veterinary Serum and Vaccine Research Institute (VSVRI).

RESULTS

Identity tests:

The identity tests confirmed that the isolated organism is *C. perfringens* type A.

Encapsulation efficacy:

The difference in optical density before and after encapsulation was calculated. The percent of free antigen was 20%. It means that the majority of antigen has been encapsulated.

Characterization of PLGA vaccine:

PLGA encapsulating *C. perfringens* type A toxoid was examined by high resolution transmission electron microscope (TEM). It appeared as spherical smooth particles with average size range from 130 to 200 nm as shown in Fig. (1).

Characterization of the PLGA nanoparticles with the Zeta-sizer showed that the surface charge of PLGA particles was -19.5 mV as shown in Fig. (2), and showed that 91.8% of PLGA nanoparticles were 134.9 nm as shown in Fig. (3).

In this work, comparing the results of Serum Neutralization test (SNT) in sera of rabbits of the three groups has been done as shown in Table (1&2); it was clear that the antibody titer of rabbits' sera vaccinated with aluminum hydroxide gel *C. perfringens* type A vaccine was equal to that vaccinated with PLGA *C. perfringens* type A one at the beginning then decreased gradually till reached the non-protected level after 7 months from the booster dose, while the titer of PLGA *C. perfringens* type A vaccine remained protective for more than 10 months without booster dose.

The results obtained from titration of rabbits' sera vaccinated with aluminum hydroxide gel *C. perfringens* type A vaccine as shown in Table (1) revealed that the mean antibody titer in sera of vaccinated rabbits was 4.1 IU/ml in respective to the dose of 2 ml and after 2 weeks after booster dose, while, the mean antibody titer in sera of vaccinated rabbits with PLGA *C*. *perfringens*.

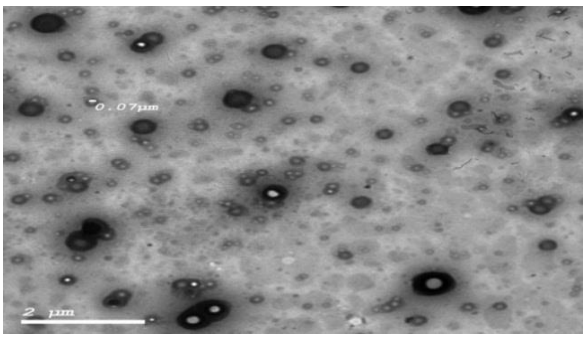

Figure.(1): TEM micrograph showing the PLGA nanoparticles.

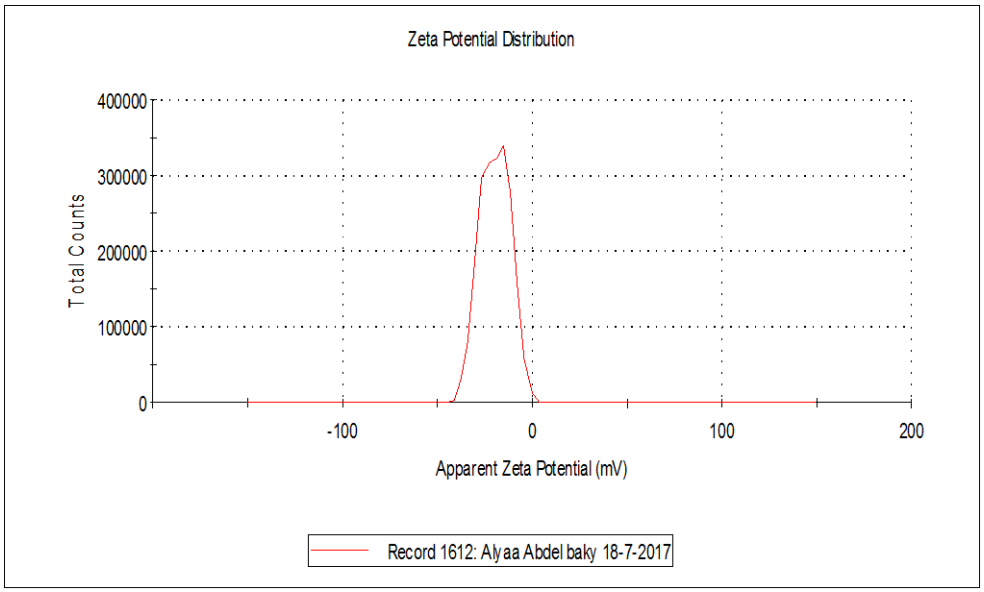

Figure.(2): Surface charge on PLGA nanoparticles using Zeta-sizer was -19.5 mV.

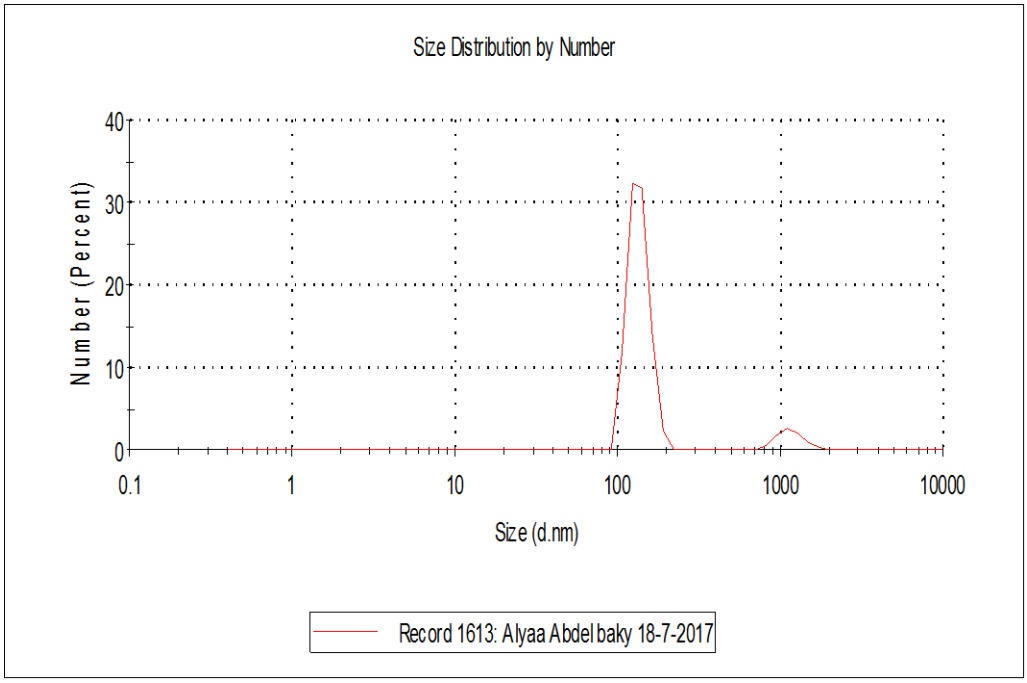

Figure.(3): The average particle size by Zeta-sizer showed that 91.8% of PLGA nanoparticles were 134.9 nm.

Table (1): Antibody titer in sera of rabbits vaccinated with aluminum hydroxide gel *C. perfringens* type A vaccine (#1) measured by SNT:

	Period elapse from 1st dose of vaccinated rabbit with aluminum hydroxide gel vaccine							
	5 weeks (2 weeks after booster dose)	2 months	3 months	4 months	5 months	6 months	7 months	8 months
Antibody titer in sera of vaccinated rabbits by IU	4	1	1	1	1	0.5	0.2	0
	3	3	1	1	1	0.5	0.1	0
	5	2	2	2	2	0.5	0.1	0
	3	3	2	2	2	1	0.5	0.1
	4	3	1	1	1	0.5	0.1	0
	3	2	1	1	1	0.5	0.1	0
	5	3	2	2	2	1	0.3	0.1
	6	3	2	2	2	1	0.3	0.1
	4	3	2	2	2	1	0.2	0.1
	4	2	1	1	1	0.5	0.1	0.1
Mean	4.1±0.99	2.5±0.7	1.5±0.52	1.5±0.52	1.5±0.52	0.7±0.36	0.2±0.4	0.05±0.21
control	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**

perfringens type A vaccine after single dose administration was 4.3 IU/ml as shown in Table (2). So, this dose covers the permissible requirement allowed for production of vaccine.

An observed increase in Ab titer in sera of vaccinated rabbits with aluminum hydroxide gel vaccine after 2 weeks after booster dose (5 weeks from 1st dose), then steady decrease of Ab titer from 2 months till 7 months post vaccination has been occurred, but it still able to protect rabbits from enterotoxaemia. At the 8 month post vaccination the Ab titer reached to 0.05 IU which is unable to protect rabbits.

DISCUSSION

Vaccination occurs through the administration of antigens in order to elicit an adaptive antigen-specific immune response and confer long-term protection against subsequent exposure to the antigen (Leleux and Roy, 2013).

In this study, development of *C. perfringens* type A vaccine was done by using well-tolerated polymers (PLGA) as they are injectable, non-irritant, biodegradable and sustain the release of the antigen in a pulsatile pattern to induce protective immunity by a single administration of vaccine delivery system. So, they give long-lasting immunity and control the release of the antigen (Abd El-Bakey, 2014).

The development of more efficient and safe adjuvant/vaccine delivery systems, requiring only a single administration to obtain high and long lasting immune responses, is of primary importance. They used PLGA, PLA and Chitosan polymeric systems as adjuvants for hepatitis B vaccines that are easy to deliver and elicit a long-lasting immune response (Sivakumara et al., 2010).

In particular, a method for regulating the size of polymeric nanoparticles is essential for effective vaccine delivery, and to elicit a specific immune response (Akagi et al., 2012).

The zeta potential of a nanoparticle is commonly used to characterize the surface charge property of nanoparticles (Couvreur et al., 2002). Nanoparticles with a zeta potential above (+/-) 30 mV have been shown to be stable in suspension, as the surface charge prevents aggregation of the particles. The zeta potential can also be used to determine whether a charged active material is encapsulated within the centre of the nano capsule or adsorbed onto the surface (Mohanraj and Chen, 2006).

By characterization of the PLGA nanoparticles with the Zeta-sizer, the surface charge of PLGA particles was -19.5 mV as shown in Fig. (2). It means that the antigen is encapsulated within the center of the nano capssule and stable in suspension with no aggregation of the particles.

Because cells are negatively charged, cationic particles induce immune cells uptake more efficiently than anionic particles, owing to electrostatic attraction to the negatively charged APC membranes (Skwarczynski and Toth, 2014).

There is a compromise between a small size and maximum stability of nanoparticles (Redhead et al., 2001).

Desai et al., (1997) found that 100 nm nanoparticles had a 2.5 fold greater uptake than 1 µm micro particles, and 6 fold greater uptakes than 10 µm micro particles.

This is in agreement with the present results, as the size of 91.8% of the nanoparticles was about 134.9 nm. So, it has a lower degradation rate that can keep the antigen for longer period with greater uptake rate.

Table (2): Antibody titer in sera of rabbits vaccinated with PLGA *C. perfringens* type A vaccine (#2) measured by SNT:

	Period elapse from 1st dose of rabbit vaccinated with Enterotoxaemia Nano Vaccine											
	2 weeks after one dose	1 months	2 months	3 months	4 months	5 months	6 months	7 months	8 months	9 months	10 months	11 months
Antibody titer in sera of vaccinated rabbits by IU	4	2	2	2	1	1	1	0.5	0.5	0.5	0.5	0
	3	3	3	3	1	1	1	1	0.5	0.5	0.5	0
	5	3	2	2	2	2	1	0.5	0.5	0.5	0.5	0
	4	3	2	2	2	1.5	1	1	1	0.5	0.5	0
	4	3	3	1	1	1	1	0.5	1	0.5	0.5	0
	4	3	2	3	2	1	1	1	1	0.5	0.5	0
	5	3	2	2	2	2	2	2	1	0.5	0.5	0
	6	3	2	2	2	2	1	1	1	0.5	0.5	0
	4	3	2	2	2	2	1	1	0.5	0.5	0.5	0
	4	4	2	2	2	1	1	1	1	0.5	0.5	0
Mean	4.3±0.82	3±0.47	2.1±0.56	2.1±0.56	1.6±0.51	1.5±0.52	1±0.4	0.95± 0.47	0.8± 0.15	0.5± 0.14	0.5± 0.1	0
control	0	0	0	0	0	0	0	0	0	0	0	0

Table (3): Antibody titer in sera of rabbits vaccinated with *C. perfringens* type A vaccine adjuvanated with aluminum hydroxide gel measured by ELISA:

	Period elapsed from 1st dose of vaccinated rabbit with aluminum hydroxide gel vaccine							
	5 weeks (2 weeks after booster)	2 months	3 months	4 months	5 months	6 months	7 months	8 months
Antibody titer in sera of vaccinated rabbits by IU	4.15	1.96	1.77	1.59	1.33	0.67	0.53	0
	3.72	1.95	1.54	1.43	1.21	0.66	0.49	0
	5.25	3.05	2.65	2.45	2.29	0.57	0.33	0
	3.41	3.15	2.33	2.22	2.09	0.78	0.56	0.1
	4.5	3.47	1.98	1.65	1.43	0.88	0.69	0
	3.59	2.5	1.65	1.55	1.39	0.66	0.49	0
	5.5	3.7	2.34	2.21	2.1	1.01	0.83	0.1
	6.211	2.92	2.78	2.65	2.44	0.78	0.63	0.1
	4.51	3.63	2.46	2.33	2.19	0.99	0.67	0.1
	4.64	2.34	1.77	1.66	1.34	0.67	0.45	0.1
Mean	4.54± 0.8	2.86± 0.7	2.2± 0.52	1.97± 0.52	1.78± 0.52	0.76± 0.36	0.56± 0.4	0.05± 0.21
control	0	0	0	0	0	0	0	0

Table (4): Antibody titer in sera of rabbits vaccinated with *C. perfringens* type A vaccine adjuvanated with PLGA nanoparticles measured by ELISA.

	Period elapsed from 1st dose of rabbit vaccinated with Enterotoxaemia Nano Vaccine											
	2 W after one dose	1 M	2 M	3 M	4 M	5 M	6 M	7 M	8 M	9 M	10 M	11M
Antibody titer in sera of vaccinated rabbits by IU	4.99	2.89	2.11	1.78	1.66	1.39	1.21	1.18	0.86	0.77	0.66	0.2
	4.23	3.67	2.78	2.29	1.95	1.44	1.23	0.965	0.87	0.75	0.62	0.17
	5.78	4.01	3.01	2.69	2.11	1.78	1.45	1.3	1.09	0.98	0.79	0.2
	4.89	3.89	3.24	2.77	2.01	1.89	1.38	1.26	1.13	0.94	0.78	0.15
	5.23	4.11	3.26	2.59	1.94	1.29	1.01	0.87	0.67	0.55	0.56	0.09
	4.99	3.78	2.99	1.99	1.45	1.11	0.9	0.76	0.66	0.6	0.53	0.1
	5.34	4.78	2.89	2.09	1.85	1.26	1.09	0.99	0.79	0.65	0.54	0.12
	6.57	5.34	3.28	2.24	1.85	1.24	1.14	0.99	0.79	0.69	0.55	0.13
	4.76	4.35	3.33	2.34	2.09	1.79	1.44	1.24	1.1	0.98	0.78	0.19
	4.89	4.23	2.46	1.96	1.48	1.19	1.08	0.89	0.69	0.59	0.45	0.1
Mean	5.16±0.8	4.1±0.47	2.93±0.5	2.27±0.33	1.83±0.23	1.43±0.28	1.19±0.18	1.04±0.18	0.86±0.18	0.75±0.16	0.62±0.12	0.14±0.04
control	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**

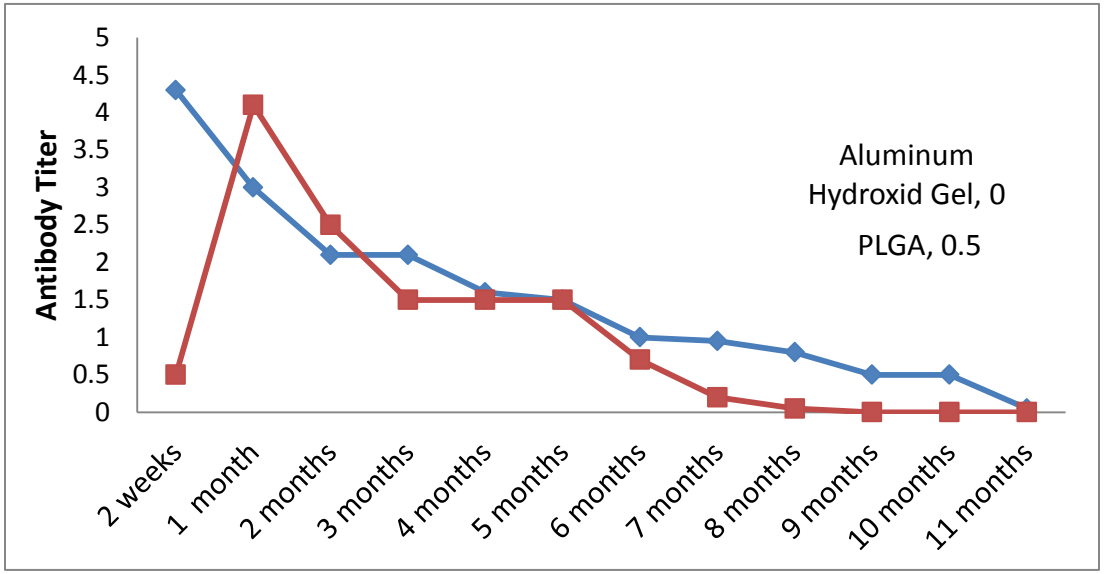

Figure. (4): Antibody titer of *C. perfringens* type A PLGA vaccine compared with aluminum hydroxide gel one measured by SNT.

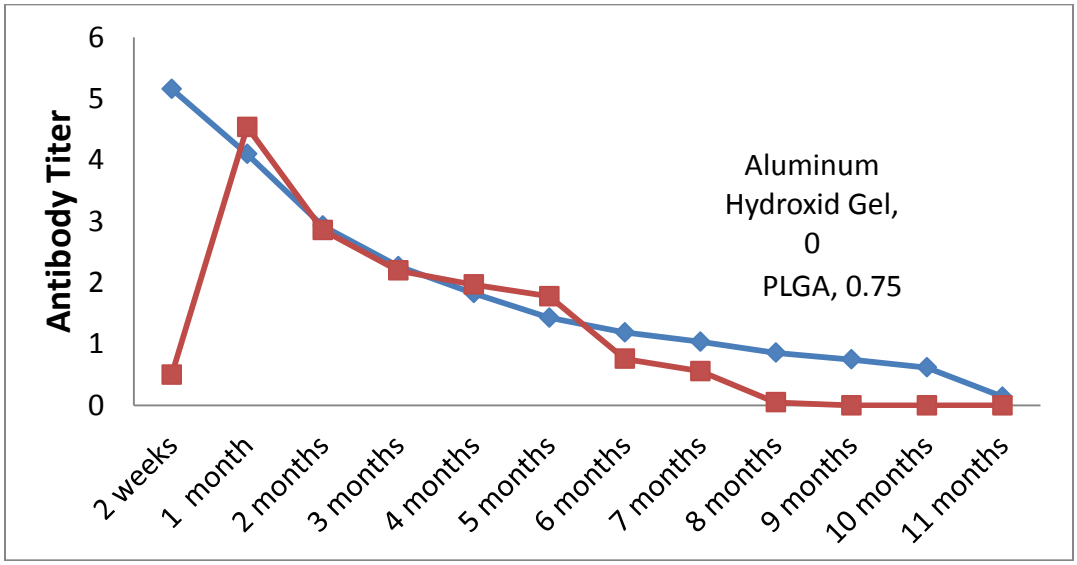

Figure.(5): Antibody titer of *C. perfringens* type A PLGA vaccine compared with aluminum hydroxide gel one measured by ELIS

Dealing with the results of ELISA; it was calculated by linear regression equation using standard curve and antibody titer was calculated according to equation:

Y=a+bX

Where a: slope = 0.196, b: intercept = 0.0811, Y =optical density, and X =calculated antibody titer.

the first reading in Table (1): (2 weeks after booster dose of vaccine #1) was higher in antibody titer than the first reading in Table (2) (2 weeks after one dose administration of vaccine # 2), but this increase no longer continued. After 6 months the titer starts to decline gradually in group (1) but still constant for longer period in group (2).

Hiremath et al., study **2016** demonstrated how influenza H1N1 conserved peptides cocktail entrapped in biodegradable nanoparticles in pigs for the first time and delivered intranasally as mist. They found that it induced epitope specific T cell response, despite not boosting the antibody response.

An increase in Ab titer in sera of vaccinated rabbits after 2 weeks from vaccination with *C. perfringens* type A PLGA one shot nano vaccine was observed, then significant decrease of Ab titer from 1 month to 4 months where $P < 0.05$ but the mean Ab titer was sufficient to protect rabbits from enterotoxaemia where the range of Ab titer 1.83 to 4.1 IU/ ml. After 5 months post vaccination there was steady decrease in Ab titer with no significant difference where $P > 0.05$ from 6 months to 8 months (mean titer 1.19 to 0.86) then significant decrease reached to 0.62 IU at 10 months where this titer still able to protect rabbit from infection, but there is reduction in titer at 11 months where 0.14 IU unable to protect rabbit and must be revaccinated.

So, there was significant difference of antibody titers of *C. perfringens* type A vaccine adjuvanted with aluminum hydroxide gel and that of PLGA one, in which there was prolonged period of persistence of the antibody titer for 10 months in PLGA vaccine compared with 6 months only in aluminum hydroxide gel one, as shown in Fig. (4) and (5).

CONCLUSION

From the previous work, it was found that PLGA vaccine gave prolonged immunity than the currently used aluminum hydroxide gel one that protect rabbits against enterotoxaemia and bloat.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

M. A. and H. Designed the experiment, wrote and reviewed the manuscript. H. E. and A. A. performed the experiments and also wrote the manuscript. A. A. designed and performed the expereiments and also wrote the manuscript
All authors read and approved the final version.

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