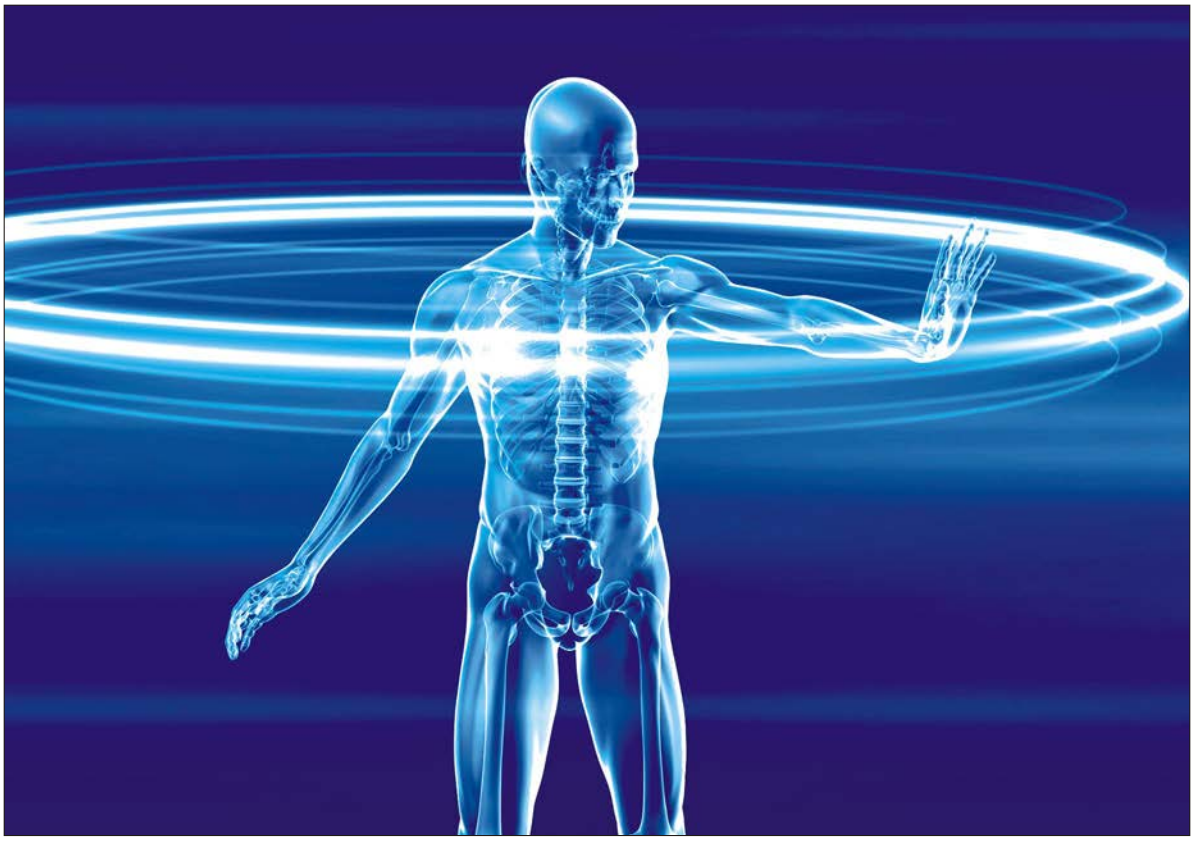

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Development of Gamma Irradiation Vaccine against *Mannheimia haemolytica*: A Preliminary Study

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ABSTRACT

The study aimed to use the several advantages of nuclear techniques for developing irradiation vaccine against *Mannheimia haemolytica* using different gamma radiation doses for vaccines preparation and different inoculation doses of irradiation vaccine. The *M. haemolytica* was exposed to different doses of gamma radiation. The dose rate was considered the optimum irradiating dose that was Lethal to *M. haemolytica* cells and selected for optimal gamma irradiation vaccine. Experimental animals were divided into four groups. The experimental groups injected twice with three weeks interval for tested vaccines. The first group (G^1) inoculated with 4×10^9 bacterial cells/dose from optimum irradiation vaccine. The second group (G^2) inoculated with 2×10^9 bacterial cells/dose from optimum irradiation vaccine. The third group (G^3) inoculated with 4×10^9 bacterial cells/dose from high irradiation vaccine. The fourth group (C) injected (S/C) with 2 mL sterile PBS and was kept as a control group. Vaccination challenge with wild *M. haemolytica* life organism (0.5 mL of 3.6×10^{10} mL^{-1}) was two doses for all experimental animals. ELISA was used to evaluate the efficiency of vaccines. The antibodies production are evaluated using Optical Density (OD) value as an indication of the efficiency of vaccine against *M. haemolytica*. The results revealed that after the second vaccination dose, the OD value of G^2 showed a significant difference compared to G^1 and G^3 groups and it was non-significant between G^1 and G^3 groups. Comparative analysis of control and the different doses of gamma irradiation vaccines showed that after the second vaccination dose, the mean of OD value of the G^2 was a significant different while it was non-significant in the $G^1$and G^3 compared to the control group. After vaccination challenge, the mean of OD value of G^2 was with high significant different compared to all of vaccinated and control groups.

Key words: Vaccine, gamma radiation, *M. haemolytica*, ELISA, respiratory disease

INTRODUCTION

Mannheimia haemolytica (*M. haemolytica*) is the principal bacterial pathogen of respiratory disease, causing considerable economic losses in cattle, sheep and goats. Moreover, it is responsible for mastitis in ewes and camels and abortion in cattle (Blackall *et al*., 2002; Christensen *et al*., 2003; Dewani *et al*., 2002; Odugbo *et al*., 2004; George *et al*., 2008).

In Egypt, the Mannheimia disease reported as the major cause of death in several farms of ostrich (Fatma and Hala, 2008). Kaoud *et al*. (2010) isolated *M. haemolytica* from pneumonic sheep, goat, cattle and buffalo (14.10, 11.80, 3.60 and 3.90%, respectively). They also isolated the microorganism from healthy animals with a relatively high number. Zaher *et al*. (2014) recorded the frequent association between Bovine Respiratory Disease Complex (BVD) and *M. haemolytica* in Egyptian cattle, sheep and goat.

Vaccination is arguably the most effective defense ever deployed to fight disease. Vaccination strategies have saved billions of animals and people from death, sickness and hardship. Progress has been made towards the development of vaccines against causative pathogens showing protection and lasting immunity.

Vaccine development is an activity that focuses on a variety of technological initiatives and applied research which enhance and promote improved systems and practices for vaccine safety. Gamma irradiation is a technically destroyed the DNA of pathogen, making the microorganism unable to replicate so it cannot establish an infection but some residual metabolic activity may survive, so, the irradiated microorganism can still find its natural target in the host (Datta *et al*., 2006). Gamma irradiation is widely used by many researchers to inactivate parasite for the preparation of vaccines, instead of traditional heat or chemical methods of inactivation. It has the advantage of a longer storage life than live, attenuated and killed, inactivated vaccines (Syaifudin *et al*., 2011).

The objective of this study was to develop gamma irradiation vaccine against *M. haemolytica* using different gamma radiation doses (optimum and high radiation) for vaccines preparation and different inoculation doses of irradiation vaccine.

MATERIALS AND METHODS

Samples collection: Samples from both healthy and pneumonic lungs were obtained from Basateen automated Slaughterhouse (Cairo-Egypt) of freshly slaughtered animals. The samples were cultured overnight at 37°C in Erlenmeyer flasks containing 200 mL of brain/heart infusion broth.

Bacterial isolation and identification: Based on morphology under microscopy, suspected colonies were cultured on (Oxoid) Trypton soya agar with 10 g L^{-1} NaCl and 10 mL sheep blood selective medium for *Mannheimia haemolytica* and on MacConkey. The plates were incubated aerobically and anaerobically at 37°C for 24-72 h, followed by purification through sub-culturing. The isolates were subjected to further identification using Gram staining and biochemical reactions (MacFaddin, 2000).

Molecular identification: Bacterial genome was extracted using Wizard genomic DNA isolation kit (#A1120, Promega Corporation, USA). The 16S rRNA gene sequencing was used for molecular identification of *M. haemolytica* sample according to James (2010). PCR amplification of 16S rRNA gene was carried out using forward 8F primer "5' AGA GTT TGA TCC TGG CTC AG" and reverse U1492R primer "5' GGT TAC CTT GTT ACG ACT T", PCR green master mix (Promega Corporation, USA) and 0.2 μg of purified bacterial DNA. Thermal cycle of the reaction was that the pre-denaturation at 95°C for seven minutes one cycle, followed by 35 cycles (Denaturation at 95°C for 1 min, annealing at 50°C for 1 min, extension at 72°C for 1 min) and finalized at 72°C for 7 min

one cycle. The PCR product was loaded in 1.5% gel agarose for electrophoresis separation and molecular weight calculation using molecular weight standard ladder (100-bp DNA ladder, Promega Corporation, USA).

Vaccines preparation: A single colony of *M. haemolytica* was inoculated into 5 mL tryptone soya broth. Inoculated flask was incubated at 37°C for 18-24 h in shaking incubator. *Mannheimia haemolytica* was exposed to different doses of gamma radiation ranged from 2-20 kGy. The process was achieved (under cooling) by using Co_{60} source (Russian facility, Model Issledovatel). Bactericidal activity of different radiation doses was assessed by cultivation on soya tryptone agar media, the optimum irradiating dose was the lowest amount of radiation that was lethal to *M. haemolytica* cells (Aquino *et al*., 2005; Abo-State *et al*., 2010). A complete abolishing of *M. haemolytica* was obtained in media exposed to 20 kGy.

Animals: White New Zealand rabbits, four weeks old were used in present experimental studies. The animals were obtained from Animal Production Research Institut's New Zealand rabbit farm. The rabbits were barrier-bred, unvaccinated and free of a variety of pathogens. Animals were allowed a one-week period of acclimatization following their arrival at the vivarium. The animals were individually housed in stainless steel cages with slatted bottoms but did not contain bedding. The rabbits were allowed *ad libitum* access to fresh tap water by water bottles and were fed a balanced commercial feed.

Bacterial infection challenge: *Mannheimia haemolytica* organism was grown confluent on dextrose starch agar plates overnight at 37°C. The cells were harvested in 0.01 M phosphate-buffered saline, centrifuged, washed twice with phosphate-buffered saline and diluted to 3.6×10^{10} cells/mL^{-1}. All groups were inoculated subcutaneously with the challenge organisms at dose 0.5 mL per rabbit, the challenge dose was according to Lu and Pakes (1981).

Experimental design: Experimental study divided into two experiments: (1) Comparative study between different doses of optimum gamma irradiation vaccine and high gamma irradiation vaccines. (2) Vaccination challenges for all experimental animals to test the efficiency of different irradiation and inoculation doses against the infection with wild *M. haemolytica*.

The animals were classified into four groups and subjected to treatment as follows:

- Group one (G^1) : Vaccinated subcutaneously (S/C) with two doses of optimum gamma irradiated *M. haemolytica* at 4×10^9 bacterial cells/dose
- Group two G^2 : Vaccinated subcutaneously (S/C) with two doses of optimum gamma irradiated *M. haemolytica* at 2×10^9 bacterial cells/dose
- Group three (G^3) : Vaccinated subcutaneously (S/C) with two doses of gamma irradiated high dose at 4×10^9 bacterial cells/dose
- Group four (C) : Injected (S/C) with 2 mL sterile PBS and was kept as a control group

For all experimental animals, the second dose was given after three weeks from the first dose. Vaccination challenge with live *M. haemolytica* (0.5 mL of 3.6×10^{10} mL^{-1}) was twice for all experimental animals. The first dose was three weeks after the second dose of vaccination. The second dose of challenge was given after one week of first challenge (0.5 mL of 3.6×10^{10} mL^{-1}).

Samples collection for vaccine evaluation: Blood samples were collected at the beginning of every week after first dose of vaccination till one week after the second dose of challenge. The collected samples were centrifuged at 4500×g for 10 min at 4°C. Plasma samples were transferred to 1.5 mL tubes and frozen at -20°C until used.

Evaluation of vaccine efficiency using Enzyme Linked Immuno-Sorbent Assay (ELISA): The antibody production was evaluated using Optical Density (OD) value as an indication of the efficiency of vaccine against *M. haemolytica*. Plasma samples were assayed for anti-bodies against *M. hemolytica* by ELISA. The polystyrene microtiter wells were coated with sonicated antigen (The bacterial cells were diluted in the bicarbonate buffer (pH 9.6) at an absorbance of 1.0 measured spectrophoto-metrically at 450 nm). The suspension was sonicated for 15 min at 35% power using a cell disrupter with a microtip-probe.), 100 μL of 1:10 diluted antigen in carbonate-bicarbonate buffer (pH 9.6) were added to each well of a 96 flat bottom. The plate was then incubated at 4°C overnight. The plates were washed three times with PBS (pH 7.4) containing 0.5% (v/v) Tween 20 and then incubated for 30 min at 37°C with 1% (w/v) bovine serum albumin (Sigma Chemical, St Louis, MO). Immediately before samples were tested, wells were washed three times with PBS-Tween 20. Based on preliminary assays, plasma samples were diluted 1:5 in PBS and incubated in duplicate PTE-coated wells and uncoated wells (to control for non-specific absorption) for 1 h. Then, the wells washed with PBS-Tween 20, 100 μL of the diluted Rabbit IgG-heavy and light chain antibody conjugated horseradish peroxidase (HRP) (Bethyl laboratories. Inc, USA Cat No. A120-101P) (1:10,000) were added to all wells and incubated at 37°C for 1 h. The 100 μL of the substrate 3, 3, 5, 5, -tetramethyl benzidine (TMB) (Bethyl laboratories. Inc,, USA Cat. No. E102) solution was added and kept for 15 min at 37°C. A color reaction was developed with the wells. The reaction was stopped by the addition of 25 μL of sulphuric acid (95-97%) per well. The plates were read at 405 nm spectrophoto-metrically using the ELISA reader (bio Tek ELX800, Using soft wear Gen5 2.00).

Statistical analysis: The results of OD values were analyzed using the arithmetic mean, standard deviation and variance ANOVA, Posthoc multiple comparisons tests according to Pipkin (1984).

RESULTS

Identification of *M. haemolytica*: According to MacFaddin's methods (MacFaddin, 2000), the results proved that the isolated microorganism from collected samples identified as *M. haemolytica* was gram-negative rods, did not produce indole, grew in MacConkey's agar, non-motile, catalase positive, oxidase positive, attacks sugars fermentatively like lactose, non-motile and heamolysis. The PCR amplified product of *M. haemolytica* 16S rRNA gene was 1.5 Kbp. BLAST analysis of *M. haemolytica* 16S rRNA gene sequence indicated that the isolated *M. haemolytica* sequence showed identity to *Mannheimia haemolytica* D174 complete genome in the region of 16S ribosomal DNA sequence (NCBI Sequence ID: gb|CP006574.1|). This result confirmed that the isolated microorganism from the study samples was *Mannheimia haemolytica*.

Detection of different doses of gamma- radiation on the survival of *M. haemolytica*: The D10 value was 2.5 kGy and the sub-lethal dose was found to be 18 kGy. In present experiment, a

complete abolishing of *M. haemolytica* was obtained in media exposed to 20 kGy. This dose rate was considered as the optimum irradiating dose that was lethal to *M. haemolytica* cells and selected for optimal gamma irradiation vaccine. The *M. haemolytica* exposed to 25 kGy was used for high gamma irradiation vaccine.

Evaluation the results between the control and the different gamma vaccines inoculation groups: Comparative study between control and vaccinated groups was illustrated in Table 1 and Fig. 1-4. The vaccinated G^1 and G^2 groups showed significant difference at the three weeks of the first vaccinated dose compared to the control group while the OD value of G^3 group showed significant difference only at the first week compared to the control group. After the second vaccination dose, the OD value of the G^2 and G^3 groups showed a significant difference at the first week while it was non-significant in the G^1 group compared to the control group. At the second week, the OD value of G^1 group was significantly different while it was non-significant difference in G^2 and G^3 groups compared to the control group. At the third week, the OD value of the G^2 and G^3 groups showed a significant different while it was non-significant difference in the G^1 group compared to the control group.

Evaluating the results of the different gamma vaccines inoculation: The results of comparative analysis between the three vaccines revealed that the OD value of the three groups

Table 1: OD values in all experimental groups

	D^1 (First dose)				D^2 (Second dose)			
Vaccine treatment/time/dose	W^1	W^2	W^3	Mean	W^1	W^2	W^3	Mean
G^1	1.468	1.306	1.241	1.338	1.145	1.286	1.241	1.220
G^2	1.497	1.417	1.007	1.307	1.426	1.263	1.316	1.340
G^3	1.446	1.267	1.121	1.278	1.299	1.188	1.050	1.179
C	1.061	1.196	1.125	1.127	1.152	1.168	1.168	1.163
LSD dose *time1	0.108608							
LSD type *time	0.133017							

[1]Only LSD values for significant interactions are shown

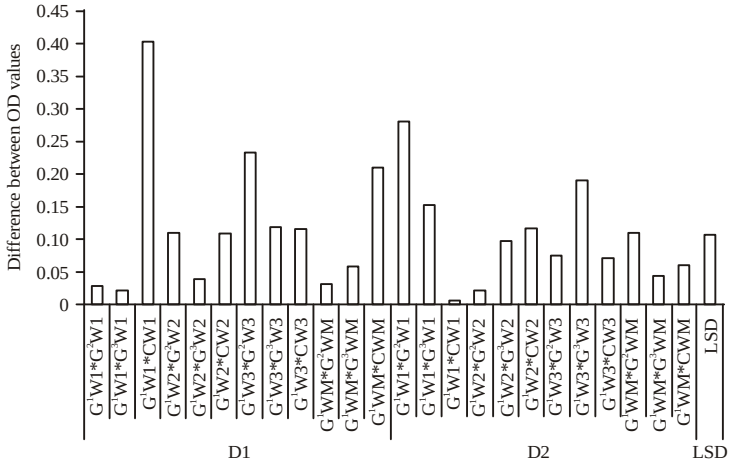

Fig. 1: Output of the OD values of the G^1 group deducted from the G^2, G^3 and C groups compared to the LSD

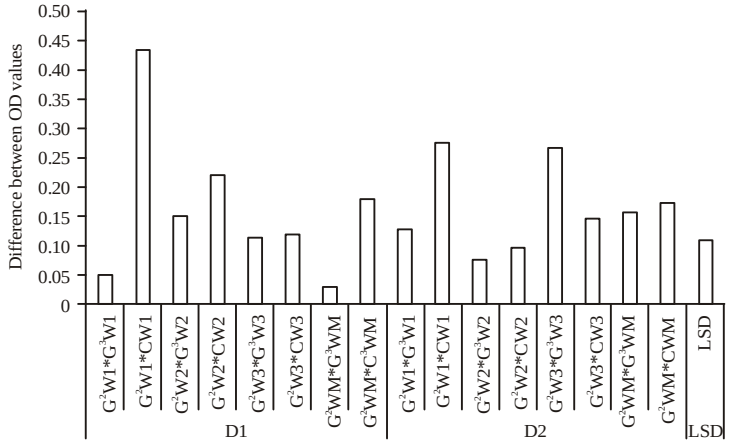

Fig. 2: Output of the OD values of G^2 group deducted from the G^3 and C groups compared to the LSD

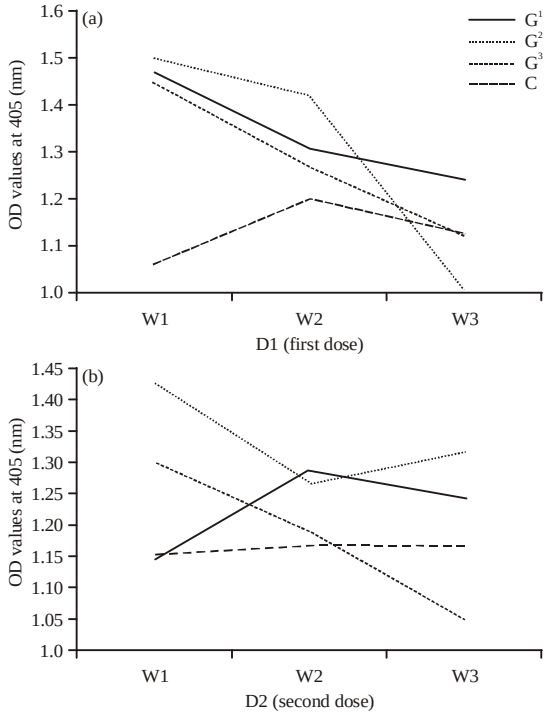

Fig. 3(a-b): OD values for G^1, G^2, G^3 and C groups after the (a) 1st and (b) 2nd vaccination dose

of vaccinated animals was non-significant at first week of first vaccination dose. At the second week the OD value between G^1 and G^3 was non-significant (Table 1 and Fig. 1) while the OD value of G^2 group showed significant difference compared to the G^3 group (Table 1 and Fig. 2). At third week, a significant difference was observed among all vaccinated groups, where the OD value of G^1 was 1.241 compared to 1.007 and 1.121 for G^2 and G^3, respectively. After second vaccination dose, the OD value of G^2 group at first week showed a significant difference compared to the G^1 and G^3

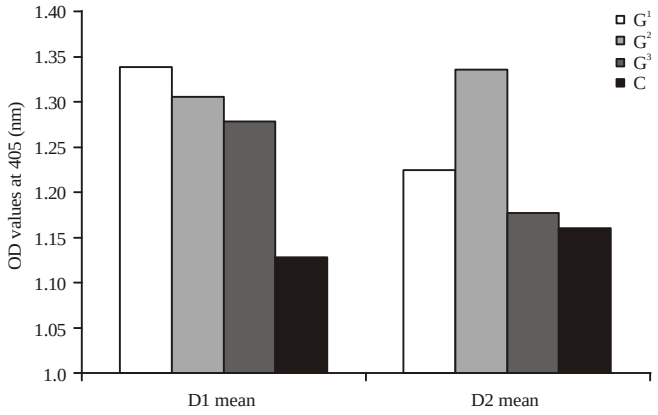

Fig. 4: Mean of OD values after first and second vaccination doses for the G^1, G^2, G^3 and C groups

groups. At the second week, the OD value showed non-significant between all of the vaccinated groups. The OD value at the third week was significantly different between G^1 and G^3 as well as between G^2 and G^3 while it was non-significant between G^1 and G^2 groups.

Evaluation the results between different doses of optimum gamma irradiation vaccine: After the first vaccination dose, the results of OD values between the two doses of optimum gamma irradiation vaccine showed that the difference observed non-significant at first week while it was with significant differences at the second and third weeks. After the second vaccination dose, the G^2 vaccinated group at first week observed with significant difference in the OD value compared to the OD value of G^1 group while it was non-significant at second and third weeks (Table 1).

Evaluation the results of the total OD mean values after first and second inoculations between all experimental groups: Comparative analysis of the total OD mean value in control and different doses of optimum gamma irradiation and high gamma irradiation vaccines after first vaccination dose revealed that a significant difference was observed between control and vaccinated groups. After the second vaccination dose, the total mean of OD value of the G^2 showed a significant difference while the total mean of OD value of G^1 and G^3 vaccinated groups was non-significant compared to the control group.

The total OD mean values between the three vaccinated groups after first vaccination dose observed non-significance. After the second vaccination dose, the total OD mean value of G^2 group showed a significant difference compared to G^1 and G^3 groups and it was non-significant between $G^1$and G^3 groups (Table 1 and Fig. 4).

Vaccination challenge: The mean values of OD in various challenge treatments illustrate in Table 2. The mean value of OD in G^1, G^2 and G^3 groups were significantly different compared to the control group after the first and second *M. haemolytica* challenge treatments. The vaccinated animals by optimum irradiation vaccine at 2×10^9 dose observed with a highest OD value after first and second challenges compared to inoculation animals with optimum irradiation vaccine at 4×10^9 dose and high gamma irradiation vaccine at 4×10^9 dose. The OD value between G^1 and G^3 at the second *M. haemolytica* challenge treatment was non-significance. The results of the total OD mean values between experimental groups after challenge treatments were shown in Table 3. The total OD mean value of G^2 group showed highly significant difference compared to G^1, G^3 and control groups.

Table 2: OD values in all experimental groups after vaccination challenge

Challenged vaccine type/challenge dose	G^1	G^2	G^3	C
CD^1	1.349	1.633	1.228	0.870
CD^2	1.451	1.571	1.377	1.276
L.S.D 5%	0.102061154			

Table 3: Overall mean of OD values ELIS for antibody of various studied vaccines

Vaccine type	Mean values of ELISA (OD)
G^1	1.28100^{ab}
G^2	1.32108^{a}
G^3	1.22850^{b}
C	1.14492^{c}

Different superscript letters in a column shows that results are significant

DISCUSSION

Although, *Pasteurella* has been described from a long time ago by Louis Pasteur but still remains a ubiquitous organism with a worldwide distribution which causes several serious diseases in domestic animals and milder infections in humans. Its species are microbiologically characterized as gram-negative, non-motile, facultative anaerobes (not requiring oxygen) that have a fermentative type of metabolism (Odugbo *et al*., 2004; Oladele *et al*., 1999; Martino, 2000).

ELISA was used to evaluate the efficiency of a newly developed gamma irradiation vaccines. The antibodies production were evaluated using Optical Density (OD) value as an indication of the efficiency of vaccine against *M. haemolytica*. Comparative analysis of the results obtained from different doses of optimum gamma irradiation vaccine, high gamma irradiation vaccine compared to the C group revealed that the overall mean of OD values of vaccinated G^1, G^2 and G^3 groups showed significant difference after first vaccinated dose compared to the C group. After the second dose, the overall mean of OD value of vaccinated G^2 group was significant different while it was non-significant in the G^1 and G^3 groups compared to the C group (Table 1, Fig. 1-3). These results suggested that the antibodies were highly produced after first dose compared to second dose of vaccines in G^1 and G^3 groups due to the memorial cells were initiated after first dose and produced significant amount of antibodies compared to the C group. The results are in agreement with Datta *et al*. (2006). After the second dose of vaccination, the antibodies of G^1 and G^3 groups increased compared to the C group but this increases showed less than that observed after first dose. These results are in agreement with Sun (2009). Regarding to the results of the G^2 vaccinated group, the same significant amount of antibodies were produced after the second dose of vaccine inoculation as well as after first inoculation. The results indicated that the booster dose (second inoculation dose) of optimal gamma vaccine inoculation (2×10^9 bacterial cells/dose) stimulated the antibodies production and kept the animals with highly immune defense.

The results of comparative analysis between the three vaccines revealed that the overall mean of OD values of vaccinated groups after first vaccination dose were non-significant between G^1 and G^2 as well as between G^1 and G^3 groups while G^2 showed significant difference compared to the G^3 group. After the second vaccination dose, the overall mean of OD value of G^2 group was significantly compared to G^1 and G^3 groups and the difference between G^1 and G^3 groups was non-significant. The results suggested that the second dose of G^2 vaccine could act as booster dose resulting in the increase in the amount of antibodies production while this advantage does not exist in G^1 and G^3 vaccines. These results confirmed by vaccine challenge experiment, whereas the G^2 group recorded highly significant amount of antibodies detected by ELISA assay compared to G^1 and G^3 groups (Table 2 and 3).

The experimental results proved that the gamma irradiation vaccine at inoculation dose 2×10^9bacterial cells/dose was a significant vaccine which could provide a highly significant amount

of antibodies that reached a high level at the time of challenge. The results are in agreement with the study applied by Eberl *et al*. (2001). He recorded the protective effect of irradiation gamma vaccine against *Schistosoma mansoni* and proved that the irradiation vaccine induced high immune response illustrated in humoral and cellular immune response. The same observation was reported by Datta *et al*. (2006) against *Listeria monocytogenes*, Syaifudin *et al*. (2011) against malaria and Dabral *et al*. (2014) against *Brucella*. Although, that none of these studies evaluated the efficiency of different loses of gamma irradiation vaccines on immunity response. The present applied work is the first study evaluating the efficiency of different gamma radiation vaccines, however, we consider these results as a preliminary study which need further studies.

CONCLUSION

The irradiation vaccine keep the structural features of the bacterial pathogen without destroying the natural antigens, therefore, a strong immune response is induced in the vaccinated host. Our results proved that: (1) The optimal gamma vaccine inoculation at 2×10^9 bacterial cells/dose provided protective effects against Mannheimia disease that reached high levels at the time of challenge and (2) The second inoculation dose of optimal gamma vaccine inoculation (2×10^9 bacterial cells/dose) could act as booster dose resulting in the increase in the amount of antibodies production while this advantage does not exist in optimal gamma vaccine (4×10^9 bacterial cells/dose) and high gamma vaccine (4×10^9 bacterial cells/dose).

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