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Comparative study between formalin-killed vaccine and developed gamma irradiation vaccine against *Mannheimia haemolytica* in rabbits

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Abstract: *Mannheimia haemolytica* is responsible for considerable economic losses to sheep, goats, and cattle and other livestock industries in Egypt. This study aimed to evaluate the effectiveness of a newly developed gamma irradiation vaccine against *Mannheimia haemolytica* in comparison to a formalin-killed vaccine. Three groups of rabbits were used in this study. Group 1 animals were inoculated with 4×10^9 bacterial cells per dose of the formalin-killed vaccine. Group 2 was inoculated with 2×10^9 bacterial cells per dose of gamma-irradiated vaccine. Group 3 (control group) was injected with 2 mL of sterile PBS. The vaccines were injected subcutaneously into experimental animals twice with 3-week intervals between inoculations. Three weeks after the second vaccination dose, the animals in all groups were infected with *M. haemolytica* twice with 1-week intervals between inoculations. Blood samples were collected weekly after the first vaccination until one week after the second *M. haemolytica* infection challenge. ELISA results revealed that the gamma irradiation vaccine developed in this study provided protective effects that reached high levels at the time of challenge. Furthermore, the second dose of gamma irradiation vaccine could act as a booster dose resulting in increased antibody production.

Key words: Mannheimiosis, vaccine, gamma radiation, formalin-killed, ELISA

1. Introduction

Vaccination is aimed at inducing active immunity in an individual so that subsequent contact with a pathogen following natural infection induces a strong protective immune response. Even though no vaccine is entirely safe or completely effective, their use is strongly supported by their benefit-to-risk ratio (1). Conventional vaccines fall into one of three types: live, attenuated vaccines; killed, inactivated vaccines; and toxoids. Live vaccines are prepared from organisms that have no virulence in the target animal. They are prepared from naturally occurring (or induced) mutated organisms, or culture passages to reduce their pathogenicity. Killed vaccines are prepared from highly immunogenic strains of organisms that are treated with chemicals that do not interfere significantly with the conformation of their surface proteins, and toxoid vaccines are based on antigenically altered toxins that are secreted by the pathogen and produce the clinical symptoms associated with the disease. In this case, the vaccine does not prevent infection but protects against the effects of the toxins produced by the pathogen (2,3).

Many studies reported several disadvantages of these types of vaccines. Live vaccines cannot be given safely to immunosuppressed individuals and administration of live attenuated vaccines to individuals with impaired immune function can cause serious illness or death in the vaccine recipient. The attenuated vaccine may also revert to its virulent form and cause disease (1,3,4).

Vaccine development focuses on a variety of technological initiatives and applied research, which enhances and promotes improved systems and practices for vaccine safety. Among the practical applications of radiobiological techniques that may be of considerable interest for public health is the use of ionizing radiation in the preparation of vaccines. Vaccines developed by irradiation have been tested and reported as strong inducers for cellular and humoral immune response that make this type of vaccine highly effective (5–7).

The objective of this study was to evaluate the effectiveness of a gamma irradiation vaccine compared to a formalin-killed vaccine against *Mannheimia haemolytica* in three groups of experimental animals and two challenges.

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2. Materials and methods

2.1. Sample collection and bacterial isolation

Samples from both healthy and pneumonic lungs were obtained from freshly slaughtered sheep of the Basateen automated slaughter house (Cairo, Egypt). The samples were collected in separate plastic bags, which were labeled and kept cooled in an ice-chest until being transported to the laboratory. The samples were cultured overnight at 37 °C in Erlenmeyer flasks containing 200 mL of brain/heart infusion broth (gelatin peptone 10 g, sodium chloride 5 g, beef/heart infusion 10 g, disodium phosphate 2.5 g, calf brain infusion 7.5 g, dextrose 2 g, and distilled water up to 1 L; final pH 7.4 ± 0.2 at 25 °C, autoclaved).

Based on morphology under a microscopy, suspected colonies were cultured on Tryptone Soya Agar (Oxoid) with 10 g/L NaCl and 10 mL of sheep blood selective medium for *M. haemolytica* and on MacConkey. The plates were incubated aerobically and anaerobically at 37 °C for 24–72 h, followed by purification through subculturing. The isolates were subjected to further identification using Gram staining and biochemical reactions (8).

2.2. Molecular identification of *M. haemolytica*

Bacterial genomic DNA was extracted from colonies on a plate using a Wizard genomic DNA isolation kit (#A1120, Promega Corporation, USA). 16S rRNA gene sequencing was used for molecular identification of *M. haemolytica* (9). PCR amplification of the 16S rRNA gene was carried out using forward primer 8F 5' AGA GTT TGA TCC TGG CTC AG and reverse primer U1492R 5' GGT TAC CTT GTT ACG ACT T, PCR green master mix (Promega Corporation), and 0.2 µg of purified bacterial DNA per reaction. PCR thermal cycling conditions consisted of initial denaturation at 95 °C for 7 min for one cycle followed by 35 cycles of denaturation at 95 °C for 1 min, annealing at 50 °C for 1 min, and extension at 72 °C for 1 min. A final extension step was performed at 72 °C for 7 min (one cycle). PCR products were electrophoresed on 1.5% agarose gel together with a 100-bp DNA ladder (Promega Corporation) for molecular weight estimation.

2.3. Preparation of vaccines

2.3.1. Formalin-killed vaccine

The vaccine was prepared according to Selim et al. (10) and Ruzauskas (11). A single colony of *M. haemolytica* was inoculated into 5 mL of Tryptone Soya Broth and incubated at 37 °C for 18–24 h in a shaking incubator. After incubation, the broth culture was centrifuged at 6000 × *g* at 4 °C for 15 min. The supernatant was discarded and the pellet of cells was washed twice with acetone and thereafter twice with diethyl ether. The pellet was then resuspended in 1% formalin saline. Tween 80 was added to the suspension at a final concentration of 3%–4%. This procedure was repeated three times with saline solution and the inactivated bacterial cells were resuspended to a final concentration of 4×10^9 cells/mL.

2.3.2. Gamma irradiation vaccine

A single colony of *M. haemolytica* was inoculated into 5 mL of Tryptone Soya Broth and incubated at 37 °C for 18–24 h in a shaking incubator. *M. haemolytica* was exposed to different doses of gamma radiation ranging from 2 to 20 kGy. The process was achieved (under cooling) by using a Co60 source (Russian Facility, Model Issledovatel). Bactericidal activity of different radiation doses was assessed by subcultivation of *M. haemolytica* cells on Soya Tryptone Agar medium after irradiation. The optimum was the lowest amount of radiation that was lethal to *M. haemolytica* cells (12,13).

2.4. Animals

Four-week-old white New Zealand rabbits (Animal Production Research Institute, New Zealand) were used in experimental infection studies. The rabbits were barrier-bred, unvaccinated, and free of a variety of pathogens. Animals were allowed a 1-week period of acclimatization following their arrival at the vivarium. The animals were individually housed in stainless steel cages and slatted bottoms 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.

2.5. Bacterial infection challenge

M. haemolytica was grown confluent on dextrose starch agar plates overnight at 37 °C. The cells were harvested in 0.01 M phosphate-buffered saline (PBS), centrifuged, washed twice in PBS, and diluted to a final concentration of 3.6×10^{10}/mL. The animals in all groups were inoculated subcutaneously with the challenge organism at a dose of 0.5 mL per rabbit, the challenge dose according to Lu and Pakes (14).

2.6. Experimental design

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

Group 1 (formalin-killed vaccine, FKV) was vaccinated subcutaneously with two doses of formalin-killed vaccine with *M. haemolytica* at 4×10^9 bacterial cells/dose. The second dose was given 3 weeks after the first dose.

Group 2 (gamma irradiation vaccine, GIV) was vaccinated subcutaneously with gamma-irradiated *M. haemolytica* (2×10^9 bacterial cells/dose). The second dose was given 3 weeks after the first dose.

Group 3 (control group, CG) was injected subcutaneously with 2 mL of sterile PBS and was kept as the negative control group.

2.7. *M. haemolytica* infection challenge

Challenge with wild *M. haemolytica* (0.5 mL of 3.6×10^{10}/mL) was done twice for all experimental animals. The first infection was 3 weeks after the second dose of vaccination.

The second infection was given 1 week after the first infection (0.5 mL of 3.6×10^{10}/mL).

2.8. Sample collection for vaccine evaluations

Blood samples were collected at the beginning of every week after the first dose of vaccination until 1 week after the second infection. 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.

2.9. Evaluation of vaccine efficiency using enzyme linked immune-sorbent assay (ELISA)

The antibody production was evaluated using optical density (OD) as an indication of the efficiency of the tested vaccines against *M. haemolytica* to generate an immune response. Plasma samples were assayed for antibodies against *M. haemolytica* by ELISA. The polystyrene microtiter wells were coated with sonicated antigen, which was prepared as follows: the bacterial cells were diluted in bicarbonate buffer (pH 9.6) at an absorbance of 1.0 measured spectrophotometrically at 450 nm. Thereafter, the suspension was sonicated for 15 min at 35% power using a cell disrupter with a microtip-probe and diluted (1:10) in carbonate-bicarbonate buffer (pH 9.6). Finally, 100 µL of the diluted bacterial antigen solution was added to each well of a 96-well (flat-bottom) ELISA plate. The plate was then incubated at 4 °C overnight. The plates were washed 3 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 Co., St. Louis, MO, USA). 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 nonspecific absorption) for 1 h. Thereafter, the wells were washed with PBS-Tween 20, 100 µL of the diluted rabbit IgG heavy and light chain antibody conjugated horseradish peroxidase (Bethyl Laboratories Inc., USA; Cat. No. A120-101P) (1:10,000) was added to all wells, and wells were incubated at 37 °C for 1 h. Next, 100 µL of substrate 3,3,5,5-tetramethylbenzidine solution (Bethyl Laboratories; Cat. No. E102) was added and kept for 15 min at 37 °C, after which a color change was observed in the wells. The reaction was stopped by the addition of 25 µL of sulfuric acid (95%–97%) per well. The plates were read at 405 nm spectrophotometrically using an ELISA reader (BioTek ELX800, with software Gen5 2.00).

2.10. Statistical analysis

The results of OD values were analyzed using the arithmetic mean, the standard deviation, and ANOVA and post hoc multiple comparison tests according to Pipkin (15).

3. Results

3.1. Identification of *M. haemolytica*

The PCR-amplified product of the *M. haemolytica* 16S rRNA gene was visualized at 1.5 kbp. BLAST analysis of the *M. haemolytica* 16S rRNA gene sequence indicated that the isolated *M. haemolytica* sequence showed similar identity to the *Mannheimia haemolytica* D174 complete genome in the region of the 16S ribosomal DNA sequence (NCBI Sequence ID: gb|CP006574.1|). This result confirmed that the isolated microorganism in this study's samples was *M. haemolytica*.

3.2. Evaluation of the results between control and vaccinated groups

The OD values of the GIV group showed significant difference at 3 weeks after the first vaccination dose, while the OD values of the FKV group showed significant difference at the first and second weeks and nonsignificant difference at the third week compared to the control group. After the second vaccination dose, the OD value of the FKV group showed a significant difference at the first week while it was nonsignificant at the second and third weeks compared to the control group. The OD value of the GIV group showed nonsignificant difference only at the second week of the second vaccination compared to the control group. The mean OD value of the two vaccines showed a significant difference after the first and second vaccinations compared to the mean OD value of the control group (Figure).

3.3. Evaluation of the results between the FKV and GIV inoculation groups

The results of OD values of the FKV and GIV groups are shown in Table 1. The differences between OD values in the 3 weeks after the first vaccination dose varied between the two vaccines. At the first and second weeks, the OD values of the FKV and GIV groups had a nonsignificant difference, while at the third week the OD values between the FKV and GIV groups was significantly different. The estimated OD values at the second vaccination dose between the GIV and FKV groups were significantly different at the first and third weeks (1.426, 1.316 and 1.265, 1.129, respectively), while they were nonsignificant at the second week (1.263 and 1.263, respectively). The mean of OD values between the two vaccinated groups after the first vaccination dose was nonsignificant, while the mean of OD values of the second vaccination dose showed a significant difference between the GIV and FKV groups (Figure).

3.4. Challenge results

The mean values of OD in various challenge treatments are illustrated in Table 2. The mean value of OD in the FKV and GIV groups was significantly different compared to the control group after the first and second *M. haemolytica*

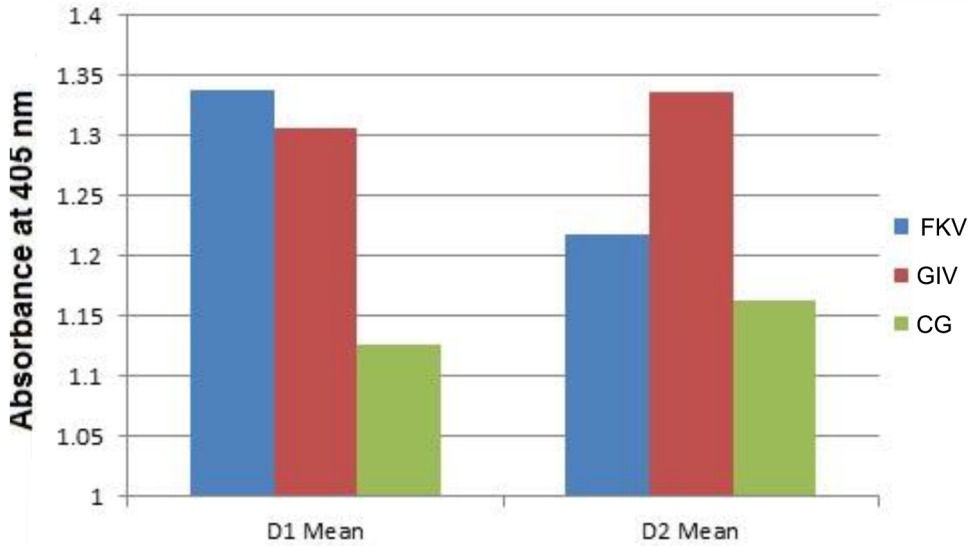

Figure. The mean of OD values at the first and second vaccination doses in FKV, GIV, and CG groups.

Table 1. The OD values in all experimental groups.

Vaccine treatment/time/dose	D1 (first dose)				D2 (second dose)			
	W1	W2	W3	Mean	W1	W2	W3	Mean
FKV	1.423	1.406	1.185	1.338	1.265	1.263	1.129	1.219
GIV	1.497	1.417	1.007	1.307	1.426	1.263	1.316	1.34
CG	1.061	1.196	1.125	1.127	1.152	1.168	1.168	1.163
LSD dose × time[1]	0.108608							
LSD type × time	0.133017							

[1]Only LSD values for significant interactions are shown.

Table 2. The OD values in all experimental groups after challenge.

Challenged vaccine type/challenge dose	FKV	GIV	CG
CD1	1.409	1.633	0.870
CD2	1.468	1.571	1.276
LSD 5%	0.102061154		

infections. The mean values of OD in the two challenges showed that the OD of the GIV group was highly significant compared to the FKV group.

4. Discussion

Pneumonic mannheimiosis caused by *M. haemolytica* is one of the major problems in sheep, goats, and cattle. It is responsible for considerable economic losses in these animals and other livestock industries in many parts of the world (16). In Egypt, *M. haemolytica* is found in the upper respiratory tract of healthy animals as well as in diseased animals. *M. haemolytica* was also recorded with frequent association with the bovine respiratory disease complex, causing severe pneumonic damage in Egyptian cattle, sheep, and goats (16,17). A vaccine that has the potential to provide protection against *M. haemolytica* from a local strain is needed in order to combat and control the disease in Egyptian livestock. Therefore, this study aimed

to investigate the effectiveness of two different candidate vaccines against *M. haemolytica*.

Evaluation of the results obtained for the ELISA assay between gamma-irradiated vaccine and the conventional formalin-killed vaccine revealed that after the first vaccination dose the ELISA assay of the two vaccines was nonsignificant at the first and second weeks. At the third week the OD value of the FKV and GIV groups was significantly different (Table 1). The results suggested that animals injected with the gamma-irradiated vaccine were able to avoid the vaccination's side effects and their bodies maintained their normal states, which could support the animal body's functions to perform its full production process.

After the second vaccination dose, a significant difference was observed between the GIV and FKV groups at the first and third weeks (1.426, 1.316 and 1.26, 1.129, respectively), while it was nonsignificant at the second week (1.26 and 1.2, respectively) (Table 1). The same observation was reported by Confer et al. (18) and Sun (19). They recorded a decrease of specific antibody against whole-cell *M. haemolytica* antigens in vaccinated animals at day 14 after vaccination and an increase at day 21, but the average response still remained higher than that of control animals.

Regarding the results of the GIV group, after the second dose of vaccine inoculation the amount of antibodies was the same as after the first inoculation. This indicated that the second dose of gamma-irradiated vaccine could act as a booster dose stimulating antibody production and thus facilitate a higher immune response upon exposure to *M. haemolytica*. This advantage does not exist with the formalin-killed vaccine. The results of the second dose inoculation related to antibody production are in agreement with previous studies on irradiation vaccines against *Schistosoma mansoni*, *Listeria monocytogenes*, malaria, and *Brucella* (5,6,20,21).

The strength of vaccine immunization has been evaluated with wild *M. haemolytica* infection. The experiment was carried out by infecting rabbits with *M. haemolytica* 3 weeks after the second vaccination dose twice with a 1-week interval. Each challenge dose was 0.5 mL of 3.6×10^{10}/mL. The present study applied the experiment using rabbits as an model animal, which are naturally not susceptible to infection by *M. haemolytica*. This gave the chance to compare the effectiveness of vaccine treatment on immunity of treated and nontreated animals after challenge (22). The results proved that the OD of the GIV group was highly significant compared to the control and FKV groups (Table 2). This result suggests that the gamma-irradiated vaccine could provide high immunity against mannheimiosis.

The present study indicates that the newly developed gamma irradiation vaccine provided protective effects against *M. haemolytica* infection in rabbits that reached high levels at the time of challenge. The second dose of gamma irradiation vaccine could act as a booster dose resulting in an increase in the amount of antibody production.

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