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Original Research Article

Development and Evaluation of Gamma Irradiated Fowl Cholera Mucosal Vaccine in Comparison with Commercial Fowl Cholera Vaccine

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Abstract

Fowl cholera is an avian bacterial disease caused by *Pasteurella multocid* which causes economic losses due to mortalities which may reach 100% and loss in body weight. The present work was designed to develop a novel improved local gamma-irradiated inactivated fowl cholera vaccine candidate, which stimulates vaccinated chickens mucosal immunity with different application routes to determine the most effective one. The antibody titers of vaccinated chickens measured by ELISA was found to be increased 2 weeks post vaccination until the third week, groups G3 and G3B which were vaccinated with the prepared irradiated FC vaccine via the intraocular route and they were the best as they recorded a titer of (399.528). On the fourth week, groups vaccinated by intranasal and oral route revealed positive immune status titers. The non-boosted group G4 was negative. Groups G3, G4B and G3B recorded (501.632, 517.274 and 665.037) respectively. On the fifth week, the antibody titers increased, but declined on the 6th week for groups G1, G2, G2B, G3 and G4 (257.746, 71.1541, 98.118, 395.339 and 230.759) but the titers increased for groups G1B, G3B, and G4B (483.433, 875.387 and 638.955). Complete protection was to the side of (0.3ml/dose) intraocular vaccinated-boosted group. While the commercial formalized vaccine was 90% for boosting group. Groups vaccinated-boosted by the gamma-irradiated intranasal (0.3 ml) vaccine recorded 80%. In conclusion, the study proved that gamma-irradiated I/O administrated vaccine is an effective protective vaccine. Montanide gel ISA 71 is a suitable adjuvant to enhance a protective immune response against FC.

Keywords: Fowl cholera, Gamma, Irradiation, Mucosal, Vaccine

INTRODUCTION

Fowl cholera (FC) is an avian bacterial disease caused by *Pasteurella multocida*. It causes economic losses due to mortalities which may reach 100% in addition to a loss in body weight (Glisson et al., 2008, Chrzastek et al., 2012). *Pasteurella multocida* is a Gram-negative non-motile, non-spore former, capsulated coccobacilli (Purushothaman et al., 2008). FC Vaccination is the most effective global strategy to protect the chickens from getting the infection (Kardos and Kiss, 2005). Inactivated formalized FC vaccines are used worldwide because of their great advantages to protect chickens against a homologous strain Pasteurella of pasteurella (OIE, 2012). Evaluation and quality control of the vaccine mainly depend on producing antibody titer post vaccination and result of challenge test. The most common *P. multocida* strain circulating in Egypt is serotype A1 and serotype A3

(Wafaa et al., 2018). The available commercial FC vaccine in Egypt is formalized oil adjuvant vaccine prepared from serotype A1 and 3 to be used 8-10 weeks of age with a booster dose at 16-17 weeks of age (Wafaa et al., 2020).

Despite the important role of the current available FC vaccine to control the disease burden, but it is still a big challenge in poultry production in Egypt (Fan et al., 2015). Owing to the available vaccine nature, it is expected to induce poor mucosal immunity due to its parenteral application. This explains the need for new vaccine formulation to achieve the desired immune response against such mucosal pathogen (Lycke, 2017).

The route of vaccination has a significant effect on the vaccine performance. Literally, parenteral vaccination induces low to no mucosal immunity which makes this route less effective immunity against mucosal pathogens (Miquel-Clopes et al., 2019). Additionally, administration of mucosal vaccines is easier than parenteral ones, so it is preferred in large scale chickens vaccination (Thanasarasakulpong et al., 2015). Moreover, the parenteral needs booster doses to overcome the low mucosal immunity with the produced humeral immunity.

On the other hand, mucosal vaccines are known by its capability to elicit long-lived cellular immunity and humoral (Poolperm et al., 2018) which means there is a need to produce protective immunogenic mucosal vaccine against mucosal microorganisms. Commonly formalin is widely used to inactivate most of bacterial pathogens but it has many disadvantages as limited vaccine safety and less immunogenic due to modifications which may occur to the bacterial antigenic components making the vaccine less potent (Woodrow et al., 2012).

Recently, gamma rays were proved to be a potential tool for pathogens inactivation and sterilization in biological reagents manufacture and laboratories scale (Seo, 2015). Bacterial exposure to optimum amounts of gamma radiation is able to disrupt its genetic material and hinder the replication process. This makes the bacteria unable to make its usual infection, but it yet has some metabolically active residues, so the irradiated bacteria can easily find its predilection seat in its natural host and be effectively immunogenic (Fertey et al., 2020). The main advantage of using gamma radiation for vaccine production is its power to penetrate the biological matters effectively targeting the nucleic acids with least surface antigen protein damage. This makes gamma radiation preferable for developing safe vaccine simply (Bayer et al., 2018). Additionally, gamma-irradiated vaccines seem to be more durable (Syaifudin et al., 2011).

Moreover, addition of Montanide ISA71 to the prepared vaccine formulation as an adjuvant result in improved efficacy as Montanide ISA71 is known to help the vaccine to induce stronger, higher long lasting immune response when compared to ordinary oil adjuvants. Also Montanide ISA71 is able to stimulate both humoral and cellular immune response which in-turn makes the vaccine more potent (Ibrahim et al., 2017).

Accordingly, the present work was designed to develop a novel improved local gamma-irradiated inactivated fowl cholera vaccine candidate, which stimulates vaccinated chickens mucosal immunity with an easy application route, in addition to comparing it with the commercial vaccine.

MATERIAL AND METHODS

Bacterial Seed

A well-identified (phenotypic and molecular identification) *Pasteurella multocida* serotype A was kindly obtained from the Reference Strain Bank (RSB), Central Laboratory for Evaluation of Veterinary Biologics (CLEVB), Egypt.

Preparation of Fowl Cholera vaccine

Lyophilized *Pasteurella multocida* serotype A bacterial seed was diluted and homogenized by 2 ml of tryptose soya broth (TSB) then inoculated on tryptose soya agar with 10% horse serum with overnight incubation at 37°C to pick up a single colony into 2 ml of TSB containing 10% horse serum with 7 h incubation at 37°C. Later on, 0.5 ml was poured on 30 ml TSB of 10% horse serum with overnight incubation at 37°C (National Veterinary Institute/SOP, 2017). The obtained culture was adjusted to be 5.6×10^{9} CFU/ml. At this point, the culture was ready for vaccine preparation.

The culture was centrifuged at 4,000×g /min at 4°C for twenty minutes. The pelleted cell was washed by PBS and suspended equally with PBS to be subjected to 1 kGy gamma irradiation for one hour at 37°C (Dessalegn et al., 2021). Three samples were exposed to ^{60}Co source at variable gamma -ray doses of constant Gray. Irradiation was performed using gamma ray irradiator model CM-20 at the cyclotron facility of the Egyptian Atomic Energy Authority. The certified dose rate was 0.5 kGy/h. The irradiated culture was kept at 4°C with control non-irradiated one.

The prepared irradiated culture was adjuvanted with 20% Montanide ISA 71 gel (Seppic France) in a ratio of 29/71 as a final step of vaccine preparation, Montanide TM01 Gel Ready to Use Aqueous Polymeric Adjuvant for Veterinary Vaccines (2021).

Quality control of the irradiated prepared vaccine

It was performed according to Egyptian Standard Regulations for Evaluation of Veterinary Biologics (2017).

Table 1. Groups vaccination and challenge schedule

Group	Age of Vaccination and challenge		
	3 Weeks	6 Weeks	9 Weeks
G1	Prepared vaccine-I/N	-	-
G1B	Prepared vaccine-I/N	Booster	-
G1C	Prepared vaccine-I/N	-	Challenge
G1BC	Prepared vaccine-I/N	Booster	Challenge
G2	Prepared vaccine-oral	-	-
G2B	Prepared vaccine- oral	Booster	-
G2C	Prepared vaccine- oral	-	Challenge
G2BC	Prepared vaccine- oral	Booster	Challenge
G3	Prepared vaccine- I/O	-	-
G3B	Prepared vaccine- I/O	Booster	-
G3C	Prepared vaccine- I/O	-	Challenge
G3BC	Prepared vaccine- I/O	Booster	Challenge
G4	Commercial vaccine- S/C	-	-
G4B	Commercial vaccine- S/C	Booster	-
G4C	Commercial vaccine- S/C	-	Challenge
G4BC	Commercial vaccine- S/C	Booster	Challenge
G5	Control non vaccinated	Non	Non

To confirm that the Pasteurella was completely inactivated, blood agar plates were inoculated with the irradiated vaccine with 24 h of incubation at 37°C, the freedom from visible colonies after 3 consecutive passages meant complete bacterial inactivation (Completion of Inactivation Test).

The prepared Pasteurella vaccine was also tested for purity by culturing on tryptose agar to detect bacterial contamination after 24 h at 37 °C and also was plated on Sabouraud dextrose agar to assure the absence of fungal contamination after incubation at 25°C for 7 days. Concerning vaccine safety, ten specific pathogen free (SPF) chicks (3 weeks) were vaccinated with a double vaccinal dose of each vaccination route (intranasal "I/N", Oral, intraocular "I/O" and S/C) with the 14 day observation period.

Experimental design

Seventeen groups of SPF 3 week old chicks (30 per each) were obtained from the SPF Farm at Koom Osheem, Fayoum Province, Egypt and used for the experiment. The chicks were housed in Biosafety insulators' and divided according to the following design (Table 1). The groups vaccinated with the prepared vaccine received 0.3ml dose (with different routes) but the group vaccinated with the commercial one was injected S/C with 0.5 ml/dose according to manufacturer's recommendations.

Blood samples were collected every week for 6 weeks post vaccination for serum separation which was stored at -20°C for testing by ELISA.

Evaluation of protective efficacy of the prepared and commercial Fowl Cholera Vaccines by challenge test

Groups (1C, 1CB, 2C, 2CB, 3C, 3CB, 4C and 4CB) were challenged at the sixth week post vaccination (9 weeks of age) with 3.5×10^9 CFU/ml *P. multocida* intranasally (I/N) with 14 days of observation. Dead birds were examined for gross lesions of internal organs (lung, liver and spleen) and bacterial re-isolation from culled birds at the 14^{th} day post challenge. The protection percentages were recorded according to mortality percentage, gross lesions of internal organs of dead birds (congested edematous lung, petechial hemorrhage in the liver, intestinal hemorrhage, splenomegaly and peritonitis) and finally re-isolation of from culled birds (Bayer et al., 2018).

Measurement of vaccine antibody levels (humoral immune response) post vaccination

Serum samples from group G1 to G5 were tested for *Pasteurella multocida* titer by indirect ELISA kit (PMS-chick ver 0916 EN). The test was applied according to the manufacturer's instructions and S/P ratio was calculated as following equation:

$$S/P = \frac{OD_{Sample} - OD_{NC}}{OD_{PC} - OD_{NC}}$$

OD: Optical Density
NC: Negative Control
PC: Positive Control
An antibody titer of each sample was calculated by the following equation.
$\log_{10}$ titer = 1.09 x $\log_{10}$ (S/P) + 3.360
Titer = Antilog $\log_{10}$ titer

Table 2. Humoral immune responses against *P. multocida* using ELISA to determine *P. multocida* antibody titer in vaccinated chickens

groups	Weeks post vaccination					
	1st	2nd	3rd	4th	5th	6th
G1	80.062	100.396	260.211	275.044	322.437	257.746
G1B	80.062	100.396	260.211	322.437	372.955	483.433
G2	4.889	47.162	75.597	111.847	139.714	71.154
G2B	4.889	47.162	75.597	77.827	196.789	98.118
G3	144.406	199.200	399.528	501.632	588.135	395.339
G3B	144.406	199.200	399.528	665.037	735.581	875.387
G4	137.373	163.298	250.362	322.437	360.211	230.759
G4B	137.373	163.298	250.362	517.274	548.675	638.955
G5	2.155	2.150	2.100	2.135	2.156	2.170

Test interpretation

S/P ≤ 0.2 or ELISA antibody titer ≤ 396 immune status was negative
S/P > 0.2 or ELISA antibody titer > 396 immune status was positive

Ethical statement

The work was ethically approved by (CLEVB) Central Laboratory for Evaluation of Veterinary Biologics, Egypt.

Competing interests

All authors declare there is no competing interests.

RESULTS

The commercial and prepared vaccines were confirmed for their freedom from contamination and safe to be used.

Measurement of vaccines antibody levels (humoral immune response) post Fowl Cholera vaccination

Antibody titers of all collected serum samples were measured by an indirect ELISA and recorded in Table (2). The levels of antibody titers of vaccinated chickens were found to be gradually increased 2 weeks post first vaccination for both the prepared gamma-irradiated vaccine (different routes) and commercial vaccine. All groups recorded a high increase high at the third week pvaccination, but but still the immune status was considered negative except groups G3 and G3B which were vaccinated with the prepared irradiated FC vaccine via the intraocular route (I/O) as they recorded a titer of (399.528).

On the fourth week, neither groups vaccinated by intranasal route nor the groups vaccinated by oral route revealed positive immune status titers. Also the non-boosted group G5 was still negative. On the other hand, groups G3, G3B and G4B were the best where they recorded (501.632, 665.037 and 517.274) respectively.

On the fifth week, the antibody titers followed its increase nearly by the same pattern and started to decline on the sixth week for groups G1, G2, G2B, G3 and G4 (257.746, 71.154, 98.118, 395.339 +ve and 230.759) but the titers increased for groups G1B, G3B, and G4B (483.433, 875.387 and 638.955) as shown in Table (2) and Figure (1).

Protective Efficacy of prepared and commercial Fowl Cholera Vaccines post challenge

Protection rates of chicken groups vaccinated with prepared Fowl Cholera Vaccine and the commercial formalized one at 3rd weeks post challenge were recorded in Table (3). Completely protected chickens from fowl cholera infection were to the side of (0.3ml/dose) intraocular vaccinated-boosted group (G3BC). While the protection percentage of the chickens vaccinated and boosted the commercial formalin-inactivated vaccine was 90% (G4BC), but in those vaccinated-boosted by the gamma-irradiated intranasal (0.3 ml) vaccine was 80% (G1BC).

Post challenge Pasteurella re-isolation and macroscopic gross lesions

The dead chickens along the experiment had the most fowl cholera characteristic lesions such as congested lung and liver, splenomegaly, and peritonitis (Figure 2). These findings were supported by bacterial re-isolation of *P. multocida* which revealed recovery of *P. multocida* from all dead birds' samples.

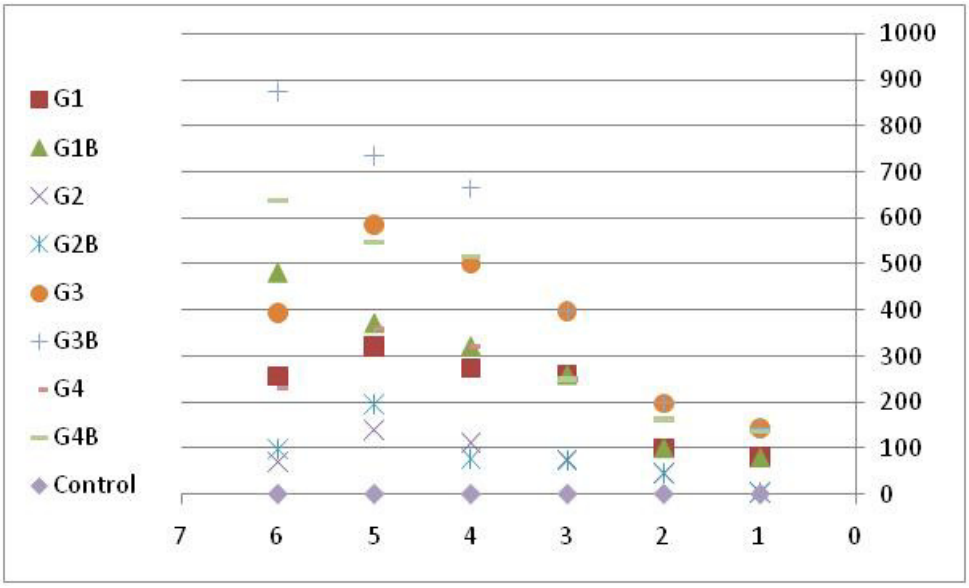

Figure 1. Six weeks Post vaccinal *P. multocida* antibody titers using ELISA test

Table 3. Protection percentages of vaccinated chickens post *P. multocida* challenge

Groups	Mortality (n=10)	Protection percent
G1C	6	40
G1BC	2	80
G2C	8	20
G2BC	8	20
G3C	4	60
G3BC	0	100
G4C	4	60
G4BC	1	90
G5	10 (within 5 days)	0

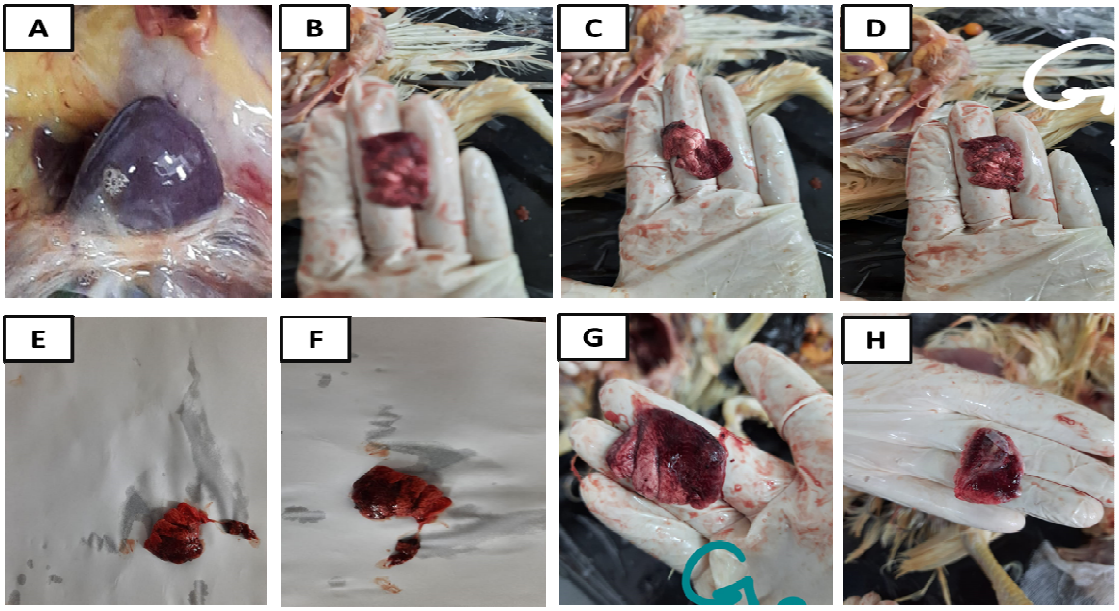

Figure (2) : *Pasteurella multocida* gross lesions post-challenge
(A) Edematous spleen (B, C, D, E, F, G and H) Congested lung from different challenge groups

DISCUSSION

Fowl cholera is a disease which is caused by *P. multocida* infection. It is a contagious worldwide avian disease affecting poultry industry (Wubet et al., 2019). Vaccination is the best control method to protect poultry flocks from getting infection. It also provides a long term protection (Harper and Boyce 2017).

The current study aimed to develop a gamma irradiated mucosal vaccine against FC. Mucosal vaccines known to be suitable for mass vaccination with less stress effect when compare it to ordinary parenteral routes. Economically, it needs low labor requirements and short time (Thanasarasakulpong et al., 2015).

Choosing of gamma irradiation as an alternative method for inactivation of *P. multocida* is based on its penetrability into the bacteria leading to a large inactivation volume with short period. Bacterial inactivation by gamma radiation mainly depends on damaging the bacterial nucleic acid which was reported previously to be the primary target of gamma rays without damaging the epitopes required for protective immune response production (Sahar et al., 2015, Jwa et al., 2018, Fertey et al., 2020, Dollery et al., 2021).

From the study results, the application of prepared vaccine by the intraocular and intranasal routes could induce higher antibody response than the commercial vaccine as the gamma radiation was able to inactivate *P. multocida* successfully without affecting the bacterial antigenic structure to give a strong fast immune response. On the other hand, the commercial formalized vaccine just induced the methylene bridges formation of amino acids causing cross liking of protein and antigenic effect (Thavarajah et al., 2012).

Inactivation by gamma-radiation could induce serum IgG in chickens (Varinrak et al., 2017). The study finding indicates that the serum antibody titers increased 3 weeks post-vaccination, but clear titer shooting was reported at 5^{th} and 6^{th} weeks post-vaccination. This finding came in agreement with that recorded by Tuasikal et al. (2012), Poolperm et al. (2018) and Dessalegn et al. (2021) who mentioned that intranasal and intraocular gamma-irradiated vaccines induced higher immune response and protective efficacy against many intracellular and extracellular bacteria. Also Mahmoud et al. (2016) recorded that the irradiated developed vaccines strongly induced immune responses which confirmed the powerful effect of these vaccines.

Also the high levels of *P. multocida* antibody may be due to preserved high immunogenic activity of antigenic protein even post radiation treatment. However, commercial formalin inactivation vaccine could make cross-linking of different amino acid residues leading to low immunogenic response (Babb et al., 2016).

Moreover, Montanide gel ISA 71 was selected as an adjuvant known to improve the vaccine efficacy. Montanide gel ISA 71 composed very stable sodium polyacrylate particles (Damiana et al., 2019) which depot slow release effect due to property of polymer adsorption so it can improve the elicit of innate immune response of the vaccine. Accordingly, systemic and mucosal immune responses are enhanced significantly with a good and safe performance (Jafari et al., 2017). Based on the study results, using Montanide gel 71 for vaccine preparation resulted in better immunogenic protective effect when compared to commercial formalin-inactivated vaccine.

Regarding the efficacy, intraocular vaccination of the gamma-irradiated vaccine resulted in full protection of vaccinated group when compared by the intranasal and oral routes, immunization via oral and intranasal route resulted in lower efficacy. Because of production of local IgA in the mucosal airways, as it is the natural infection route of *P. multocida* (Jwa et al., 2018).

CONCLUSION

In conclusion, the study proved that gamma-irradiated I/O administrated vaccine is an effective protective vaccine. Montanide gel ISA 71 is a suitable adjuvant to enhance a protective immune response against FC.

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Supervision: Mounir Elsafty.
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Visualization: Marwa Fathy, Reem Soliman.
Writing – original draft: Hala Mahmoud, Heba Soliman .
Writing-review and editing: Mounir Elsafty, Hala Mahmoud, Marwa Fathy, Reem Soliman, Maha Elshazly.

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