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The protective efficacy of locally prepared combined inactivated *Mycoplasma gallisepticum* and *Pasteurella multocida* vaccine in chickens

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The present work was planned to study the immune response and protection of chickens vaccinated with locally prepared combined inactivated vaccine of *Mycoplasma gallisepticum* (*M. gallisepticum)* and *Pasteurella multocida* (*P. multocida)* adjuvanted with Montanide ISA70. The prepared vaccine was evaluated by measurement of nitric oxide in the supernatant of macrophage, Enzyme Linked Immuno sorbent assay (ELISA) and challenge tests. The results showed that combined inactivated vaccine of *M. gallisepticum* and *P. multocida* induced high and long duration of antibody response and significant protection against the challenge with virulent strain of *M. gallisepticum*.

Keywords: *M. gallisepticum*, *P. multocida*, vaccine, chicken, Montanide ISA70.

INTRODUCTION

Mycoplasma gallisepticum is a bacterial pathogen of poultry that is estimated to cause annual losses exceeding $780 million. The National Poultry Improvement Plan guidelines recommend regular surveillance and intervention strategies to contain *M. gallisepticum* infections and ensure mycoplasma-free avian stocks (Hennigan et al., 2012).

M. gallisepticum is a significant poultry pathogen involved in severe economic losses of the poultry industry due to a reduction in egg production, hatchability and downgrading of carcasses. Both horizontal and vertical disease transmission leads to rapid spreading of this pathogen in flocks. *M. gallisepticum* can cause severe chronic respiratory disease (CRD) when present in concert with other poultry pathogens including Newcastle disease virus, Infectious bronchitis virus and *E. coli* (Stipkovits et al., 2012). Infections with *Avibacterium paragallinarum* and *Pasteurella multocida (P. multocida)* should also be ruled out (OIE, 2012).

Control of pathogenic avian mycoplasmas can consist of one of three general approaches; Maintaining flocks free of infection, medication, or vaccination. Medication can be very useful in preventing clinical signs and lesions, as well as economic losses, but cannot be used to eliminate infection from a flock and is therefore not a satisfactory long-term solution. Vaccination against *M. gallisepticum* can be a useful long-term solution in situations where maintaining flocks free of infection is not feasible, especially on multi-age commercial egg production sites (Kleven, 2008).

Effective method to prevention of this infection is vaccination by inactivated vaccines (Ferguson-Noel et al., 2012). The major advantage of bacterins is their safety. Live attenuated vaccines may have residual pathogenicity or may revert to the status before attenuation (El Gazzar et al., 2011). Otherwise Ley (2008) stated that bacterins are considered to be of minimal value in the long-term control of *M. gallisepticum* infection in multiple-age commercial layer production sites. Also Faruque and Christensen (2007) concluded that inoculation of inactivated *M. gallisepticum* vaccine is not justified and is too expensive at farm levels.

P. multocida is a major animal pathogen that causes a range of diseases including fowl cholera. *P. multocida* infections result in considerable losses to layer and breeder flocks in poultry industries worldwide. *P. multocida* lipopolysaccharide (LPS) is a primary stimulator of the host immune response and a critical determinant of bacterin protective efficacy (Harper et al., 2016).

So the aim of this study was to study the potency of the locally prepared combined inactivated vaccine of *M. gallisepticum* and *P. multocida* adjuvanted with Montanide ISA70 against *M. gallisepticum*.

MATERIALS AND METHODS

Preparation of combined inactivated oil emulsion vaccine of *M. gallisepticum* and *P. multocida*:

Equal parts (V/V) of the inactivated broth of *M. gallisepticum* [field isolate of *M. gallisepticum* (Eis3-10) was kindly obtained from Mycoplasma Department, Animal Health Research Institute, Dokki, Giza, Egypt] and *P. multocida* strains (serotypes A and D were kindly obtained from Aerobic Bacterial Vaccines Department, Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo) were mixed using a magnetic stirrer. Aforementioned suspension was adjusted its concentration to contain $3x10^{10}$ colony forming unit (C.F.U.) per dose (5% packed cell volume) of *M. gallisepticum* according to Yoder (1979) and 3.25 x 10^{10} C.F.U./ml of each strain of *P. multocida* according to Mukkur et al., (1982). Equal amounts of aforementioned culture and Montanide ISA70 oil (SEPPIC, France) were mixed thoroughly in a ratio of 50/50 using a magnetic stirrer at approximately 300 rpm for 15 minutes (water-in-oil emulsions).

Evaluation and quality control of the vaccine:

The vaccine was tested for purity, sterility, safety and potency tests according to OIE (2012).

Experimental design

Sixty, 4 weeks old specific pathogen free (SPF) chickens (were obtained from Kom Osheem farm in Fayoum, Egypt) were divided into four groups (15 chickens for each group), the 1st group was vaccinated with *M. gallisepticum* vaccine, the 2nd group was vaccinated with combined vaccine of *M. gallisepticum* and *P. multocida*, the 3rd group was vaccinated with imported inactivated *M. gallisepticum* vaccine and the 4th group was kept unvaccinated as a control group. The vaccinated chickens were received vaccines in a dose of 0.5 ml in 2 doses with 1 month interval. Blood samples were collected at 3th, 7th and 15th days after second vaccination and after challenge for the determination of the cellular immunity by measurement of nitric oxide (NO) in the supernatant of macrophage according to Rajaraman et al., (1998) and Municio et al., (2013). Also serum samples were collected every 2 weeks till 25 weeks of age for the determination of the humoral immune response of the vaccinated chickens by Enzyme Linked Immuno sorbent assay (ELISA) technique (*M. gallisepticum* antibody test kit; Proflok®, Synbiotics® Corporation, No. 96-6533). At the same time the vaccine was evaluated by challenge test (at 11 weeks of age) against the challenge with the virulent strain of ***M. gallisepticum*** (Eis3-10 strain) according to Whithear (1996).

RESULTS AND DISCUSSION

For many years, the control of *M. gallisepticum* in most of the world has been based on the maintenance of breeding stock that is free of *M. gallisepticum* and on biosecurity (Ley, 2008). However, *M. gallisepticum* vaccines may be employed in situations where this approach is not feasible such as endemically infected multi-age facilities and areas of dense poultry populations (Kleven, 2008). While *M. gallisepticum* bacterins reduced the severity of lesions and egg production losses but did not completely prevent *M. gallisepticum* colonization of the chicken respiratory tract upon challenge (OIE, 2012).

M. gallisepticum is further complicated with other poultry pathogens causing avian influenza, New Castle disease, infectious bronchitis, fowl cholera, coryza and *E. coli* (Liu et al., 2001). So this study was conducted for preparation of locally prepared combined inactivated vaccine of *M. gallisepticum* and *P. multocida* adjuvanted with Montanide ISA70, evaluation of it and comparison of its efficacy with the imported *M. gallisepticum* vaccine.

Table (1): Concentration of NO in the supernatant of macrophage:

Interval times of blood collection	Types of vaccines			
	M. gallisepticum	**Combined vaccine**	**Imported vaccine**	**Control**
Pre vaccination	10.9	15.4	15.1	8.12
Post 2nd vaccination				
At 3rd day	19.7	25.2	24.1	11.06
At 7th day	45.2	53.9	46.3	16.3
At 15th day	29.4	47.4	43.9	14.0
Challenge				
At 3rd day	23.3	47.8	41.5	11.7
At 7th day	78.3	102.6	94.1	15.2
At 15th day	44.7	74.6	62.7	10.8
Overall means	35.9	52.4	46.8	12.4

NO**:** nitric oxide

Cellular immune response of chickens that vaccinated with different *M. gallisepticum* vaccines was evaluated by estimation of NO concentration in the supernatant of macrophage (Table 1). Group of chickens vaccinated with combined *M. gallisepticum* and *P. multocida* vaccine showed a significant increase of overall mean of concentration of NO in supernatant of macrophage. These data were in the same manner with Obukhovska et al., (2015) who concluded that the level of macrophages in chickens increased rapidly during the first 10 days after the second injection of inactivated *M. gallisepticum* vaccines adjuvanted with Mantanide ISA 70. It was shown that inoculation of inactivated vaccines against avian mycoplasmosis in chickens promoted stimulation for primary link of cellular immunity (macrophage).

These data were explained by Zhang et al., (2013) who stated that the capsule is a major virulence factor of *P. multocida* serotype A: 3 strain. Also Harper et al., (2013) reported that *P. multocida* is a Gram-negative pathogen and the causative agent of fowl cholera and the major outer membrane component LPS is both an important virulence factor and a major immunogen.

Nascimento et al., (2005) stated that genus *Mycoplasma* has ability to stimulate macrophages, monocytes, T-helper cells and NK cells, results in the production of substances, such as tumor necrosing factor (TNF-α), interleukin (IL-1, 2, 6) and interferon (α, β, γ). Moreover Majumder (2014) explained that *M. gallisepticum* cytadheres to the tracheal epithelium and mediates infiltration of macrophages, heterophils and lymphocytes to the tracheal submucosa.

The humoral immune response of the vaccinated chickens with different *M. gallisepticum* vaccines was evaluated by ELISA as illustrated in Table (2) noticed that a significant increase of the overall mean of the antibody titers against *M. gallisepticum* by ELISA test was in the group of chickens vaccinated with combined *M. gallisepticum* and *P. multocida* vaccine. These data agreed with Gondal et al., (2013) and Bekele (2015) who reported that the formaldehyde inactivated Montanide ISA70 based *M. gallisepticum* vaccine induced protective level of anti *M. gallisepticum* antibodies in chickens. Also Sarfaraz et al., (2017) reported that oil based combined *M. gallisepticum* and avian influenza (H9N2) vaccine adjuvanted with Montanide ISA-70 induced effective antibody response in the vaccinated birds measured by ELISA and haema gglutination inhibition (HI) tests.

These data were explained by Harper et al., (2012) who reported that the capsule and LPS of *P. multocida* constitute the major components of the bacterial cell surface. They play key roles in a range of interactions between the bacteria and the hosts they colonize or infect. Both polysaccharides are involved in the avoidance of host innate immune mechanisms, such as resistance to phagocytosis, complement-mediated killing, and the bactericidal activity of antimicrobial peptides; they are therefore essential for virulence. In addition, LPS is a major antigen in the stimulation of adaptive immune responses to infection.

Potency of the vaccines were evaluated by the challenge test against *M. gallisepticum* (Eis3-10 strain) in chickens vaccinated with different *M. gallisepticum* vaccines was illustrated in Table (3) showed that the protection percentage (P %) against the challenge with *M. gallisepticum* was 93% for combined *M. gallisepticum* and *P. multocida* vaccine.

Table (2): Level of antibody titers against *M. gallisepticum* by ELISA:

Interval times of blood collection	Types of vaccines			
	M. gallisepticum	**Combined vaccine**	**Imported vaccine**	**Control**
Pre vaccination	0	0	0	0
1st vaccination				
2 weeks post 1st vaccination	157	360	241	0
Booster vaccination (at 4 weeks post 1st vaccination)				
6 weeks post 1st vaccination	729	996	965	0
Challenge (at 7 weeks post 1st vaccination)				
9 weeks post 1st vaccination	1039	1902	1636	0
11 weeks post 1st vaccination	2423	4166	3665	0
13 weeks post 1st vaccination	2541	4958	3927	0
15 weeks post 1st vaccination	2106	3551	3229	0
17 weeks post 1st vaccination	1624	2768	2199	0
19 weeks post 1st vaccination	1010	1860	1487	0
21 weeks post 1st vaccination	743	969	892	0
Overall means	1237	2153	1824	0

Table (3): Challenge test against *M. gallisepticum* (Eis3-10 strain):

Types of vaccines	*M. gallisepticum*	Combined vaccine	Imported vaccine	Control
Total no. of chickens	15	15	15	15
No. of chickens showing respiratory signs	3	1	2	15
Protection%	80	93	87	0

These data were in the same manner with those of Bekele (2015) who concluded that the formaldehyde inactivated Montanide ISA70 based *M. gallisepticum* vaccine induced 100 % protection against *M. gallisepticum*. All chickens did not show clinical signs or post mortem changes after challenge test. Also Ferguson-Noel et al., (2012) found that the *M. gallisepticum* bacterin was protective and resulted in significant differences in air sac lesions, tracheal lesions, and ovarian regression compared to the non-vaccinated controls.

Moreover Shafay (1995) concluded that the locally prepared combined inactivated vaccine of *M. gallisepticum* and *P. multocida* gave acceptable protection level in comparison with the monovalent *M. gallisepticum* vaccine in vaccinated chickens. Also Gadallah (2015) reported that the locally prepared inactivated combined *M. gallisepticum* and *E. coli* vaccine induced protection against the chronic respiratory disease and elicited the humoral immune response in broiler chickens.

These data were explained by Gong et al., (2013) who stated that two outer membrane proteins (OmpH and OmpA) are the major immunogenic antigens of avian *P. multocida*, which play an important role in inducing immune responses that confer resistance against infections. Moreover Boyle and Finlay (2003) found that the outer membrane proteins promote adherence to host cell surfaces and are therefore likely to be involved in *P. multocida* virulence. Also Noor mohammadi (2007) found that lipoproteins (LPs) reside on the surfaces of the cell wall-less mycoplasmas and are important factors in pathogenesis.

CONCLUSION

So it could be concluded that the locally prepared combined inactivated *M. gallisepticum* and *P. multocida* vaccine induced a considerable immunity in chickens as it gave early, high and long duration of antibody response. Also it was efficient and safe in protection of chickens against *M. gallisepticum* and *P. multocida* infections. Depending on this study, it could be suggested to use this combined vaccine for control of *M. gallisepticum* in poultry industry.

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CONFLICT OF INTEREST

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

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AUTHOR CONTRIBUTIONS

FF performed the experiments and wrote the manuscript. El-JJ, WAA and EM designed the experiments and reviewed the manuscript. MM and FF designed the experiments and prepared the vaccine. All authors read and approved the final version.

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