Experimental studies on the efficacy of combined inactivated Infectious coryza and infectious bronchitis vaccine in chicken

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ABSTRACT

Two combined inactivated oil adjuvant vaccines against infectious coryza and infectious bronchitis diseases were prepared, one adjuvanted with mineral oil and the other with Montanide ISA-70. Evaluation of both vaccines were done by challenge test, haemagglutination inhibition test, ELISA test for *Avibacterium Paragallinarum***,** and ELISA test, indirect haemagglutination inhibition test and ciliostasis score for infectious Bronchitis virus (IBV)**.** The immune response for the antigenic component of both vaccines show high titre of antibodies, but Montanide ISA-70 adjuvanted vaccine was superior as shown in mean antibody titers against *Avibacterium paragallinarum* serovar A, B and C and infectious bronchitis. In addition, Montanide ISA-70 is lesser in viscosity that makes it easily injected, so, it is better to use for control of infectious coryza and infectious bronchitis diseases in chickens.

INTRODUCTION

The Egyptian poultry industry in recent years has observed an increasing incidence of respiratory and nephritis pathologies related to bacterial and viral diseases specialy infectious coryza and infectious Bronchitis virus (IBV) in vaccinated and non-vaccinated flocks that caused severe economic losses.

Infectious coryza is an acute respiratory disease of chickens caused by *Avibacterium paragallinarum*. The clinical signs of this disease include nasal discharge, facial swelling and lacrimation (Sakamoto et al., 2013). The economic impact of the disease attributes to increase culling rate in meat chicken and 10-40% reduction in egg production in laying and breeding hens causing severe economic losses in egg industry in many parts of the world (Blackall and Soriano, 2008). Infectious coryza remains a serious problem in the poultry industry in spite of the wide spread of different inactivated vaccines which are used to eradicate infectious coryza. Several outbreaks reported after using vaccines (Davis et al., 1976). There is no cross protection between serogroups (Rimler et al., 1977 and Kume et al., 1980).

Infectious bronchitis virus (IBV) is the causative agent of a highly contagious economically important disease of the chicken (King and Cavanagh, 1991). IBV belongs to the family Coronaviridae, genus Coronavirus and is considered a major cause of one of the most important respiratory disease in chickens of all ages which characterized by severe loss of production and egg quality in mature hens (Wit et al., 2010). Some strains cause nephritis in young birds and infectious bronchitis is occasionally reported to be associated with enteritis (Liu and Kong 2004; Susan et al., 2010). Vaccination programs against IBV rely on the use of vaccines containing virus antigenically similar to the virus present in the field. In Egypt, isolates related to Massachusetts, D128, D274, D08880, 4/91 have been isolated from different poultry farms (El-Kady 1989; Sultan et al., 2004 and Susan et al., 2010).

Controlling of these diseases by active immunization is of considerable importance as means for better control over the disease spread and ultimately

eradication of the infectious coryza and infectious bronchitis diseases in chickens with minimum cost. So, the objective of the present study was to prepare two combined inactivated infectious coryza and infectious bronchitis vaccines which containing two infectious bronchitis virus strains (H120 and CR88) and three *Avi. paragallinarum* serovar (A, B and C) one adjuvanted with mineral oil and the other with Montanide ISA-70, and evaluation of the immunizing and protective value of the two prepared vaccines.

MATERIAL AND METHODS

Avibactrium paragallinarum strains:

Standard *Av. paragallinarum* serovar A (W strain); serovar B (0222), and serovar C (Modesto strain) were supplied by the National Institute of Health (Ames, USA) and used in this study.

Infectious Bronchitis (IB) vaccinal strains:

a- H120 vaccinal strain

Attenuated vaccinal strain with titer of $10^{8.2}$ EID_{50}/ml was kindly obtained from IZO, S.P.a. Italy.

b- CR88 vaccinal strain

Attenuated vaccinal strain with titer of $10^{7.8}$ EID_{50}/ml was kindly obtained from Merial S.A.S, France.

Local nephropathogenic IBV virulent strain

It was isolated from vaccinated broiler chicken 24 day old at Dakahlia with a history of respiratory and renal signs. The isolates were matched for 96% with the isolated strain Egypt/F/03 strain with accession No. DQ. 487085 (NCBI). The isolate titre was 10^5 EID_{50}/ml

Adjuvants:

1. Montanide™ ISA-70: (Seppic-France) is used as W/O emulsion.
2. Mineral oil.

Imported Vaccines:

1- Imported inactivated infectious coryza aluminum hydroxide gel vaccine.
2- Inactivated combined IB+ND oil adjuvant vaccine.

Embryonated chicken eggs

Specific pathogen free (SPF) eggs (9-11 day old) were obtained from SPF Egg Production Farm, Koum Oshiem, Fayoum, Egypt, and used for propagation, titration and assurance of complete inactivation of IB virus strains.

Chickens:

Three hundred SPF chickens used in this study were obtained from Koum Oshiem, Fayoum, Egypt. Chickens were divided into six groups (50 chickens per each).

IB virus propagation, titration and inactivation:

Each of two IB viruses strains (H120 and CR88) was propagated separately in 10 day old SPF embryonated chicken eggs according to the method of Cunningham (1973). The titers of obtained virus strains were $10^{8.2}$, $10^{7.8}$ EID_{50}/ml respectively. IB virus strains were inactivated using formalin (0.1%) and tested for complete inactivation by two successive blind passages in SPF eggs.

Avibacterium paragallinarum bactrin preparation:

It was prepared according to the method described by Blackall et al., (1992).

Inactivated combined Infectious coryza and infectious bronchitis vaccine preparation:

Two vaccines were prepared; the first was mineral oil combined IBV and *Av. paragallinarum* vaccine and the second was Montanide ISA 70 combined IBV and *Av. paragallinarum* vaccine. Vaccines were prepared according to **Otsuki and Iritani (1974).** The vaccines were confirmed to be stable at 4˚C. Stability was defined as the maintenance of a homogenous emulsion and instability was defined as the separation of the emulsion into oil and water phases.

Experimental Design:

Three hundred SPF chickens were divided into six groups (50 chickens per each) as follows:

Group (1): Vaccinated with mineral oil combined *Av. paragallinarum* and IBV vaccine.

Group (2): Vaccinated with Montanide ISA 70 combined *Av. paragallinarum* and IBV vaccine.
Group (3): Vaccinated with Imported infectious coryza aluminum hydroxide gel vaccine.
Group (4): Vaccinated with imported inactivated combined IB + ND vaccine.
Group (5): Kept as control positive group.
Group (6): Kept as control negative group.

Evaluation of the two prepared combined inactivated Infectious coryza and infectious bronchitis vaccines:

1. Sterility tests:

They were done according to (OIE, 2013).

2. Safety test:

They were done according to (OIE, 2013).

3. Potency tests:

A- ***Avibacterium Paragallinarum*** **bacterin:**

Protection rates of two prepared mineral oil and Montanide ISA 70 vaccine to *Av. paragallinarum* serovar A, B, and C were carried out by challenge test. For evaluation of immune response in chickens, serum samples were collected weekly from vaccinated chicken and tested by haemagglutination inhibition test and ELISA test.

- **Haemagglutination-inhibition test (HI):**

The test was performed for estimation of HI antibodies against *Av. paragallinarum* serovar A, B and C according to Sakamoto et al., (2013). The HI antibody titer was expressed as the reciprocal of the highest dilution of the sample that completely inhibited haemagglutination.

- **Enzyme linked immune sorbent assay (ELISA):**

Antibody titer specific for *Av. paragallinarum* serovar A, B and C was measured by ELISA according to Sakamoto et al., (2012). Optical density (OD) was measured at 490 nm by using a micro plate reader (DYANA Tech., USA). The absorbance was expressed as ELISA titer, the cut-off value of the ELISA was determined as double the optical density of an average mean of negative control.

- **Challenge test:**

It was done according to Blackall et al. (1992). The immunity of vaccinated and unvaccinated chickens to *Av. paragallinarum* was tested by challenge with 2 $x10^7$ CFU/chicken of *Av. paragallinarum* serovar A, B, and C separately. Strains were administrated intra nasal four weeks post vaccination. Chicken were examined for clinical respiratory signs and swelling of the face (signs of infectious coryza)**.** A protected chicken was defined as a chicken that exhibited no clinical signs during the observation period.

B- Infectious bronchitis virus (IBV)

Protection rates of two prepared mineral oil and montanide ISA 70 vaccine to IBV were carried out by challenge test. For evaluation of immune response of chickens, serum samples were collected weekly from vaccinated chicken and tested by ELISA test, indirect Haemagglutination inhibition test and ciliostasis score.

- **Enzyme linked immune sorbent assay (ELISA)**

It was carried out for estimation of antibodies against IBV vaccines according to kit manufacture (Biochek).

- **Indirect Haemagglutination-inhibition test (HI):**

Trypsinization of IB antigen

Working solution of reagent grade trypsin (Sigma chemical company St. Louis, Mo, USA) was prepared containing 2% trypsin in PBS with pH adjusted at 7.2. Allantoic fluid from inoculated embryos was collected 72 hrs post inoculation (PI) and treated directly by mixing 0.25 ml of allantoic fluid with 50µl from the working solution (Mahmood et al. 2004).

Trypsin-induced Haemagglutiontion assay:

Trypsin-induced Haemagglutination assay was performed for all samples by gently mixing 50 µl trypsin-treated allantoic fluid with 50 µl of 2% solution of

the chicken RBCs in a micro titration plate (**Olsen et al., 2003**).

- **Ciliostasis test**

Assessment of ciliostasis test according to Cubillos et al. (1991); Cavanagh et al. (1997).

At day 7 post challenge, 5 chicks from each group were euthanizes and the trachea was removed immediately then rinsed with physiological saline at 37°C. Small transverse sections of the trachea were cut by scalpel then examined for ciliary activity, briefly each of 10 explants (3 from top, 4 from middle and 3 from bottom) prepared from each trachea was examined by low-power microscopy and the ciliary activity for each explants was scored as follow: 0=100% cilia beating, 1=75% of cilia beating, 2=50% of cilia beating, 3=25% of cilia beating and 4=complete ciliostasis. This gave a maximum possible ciliostasis score for a trachea of 40 score if there was complete ciliostasis (Cubillos et al., 1991; Cavanagh et al., 1997). A protection score was calculated by the formula:

$$\frac{\text{Mean ciliostasis score for vaccinated challenge group}}{\text{Mean ciliostasis score for corresponding infected controls}} \times 100$$

RESULTS

Inoculated chickens with double dose of combined inactivated Avibacterium paragallinarum and IB prepared vaccines either mineral oil adjuvenated vaccine or Montanide vaccine revealed no clinical signs of coryza during the 14 days of observation so the prepared vaccines were safe. Also, the present study was found that the prepared vaccine was sterile and no evidence of any bacterial (aerobic or anaerobic contaminants) or fungal growth has been detected even after prolonged incubation

Regarding the humoral antibody responses HI and ELISA tests were conducted and antibody titers were calculated as shown in table (1, 2, 3 and 4).

The results illustrated in tables (1,2) indicated that combined vaccine adjuvanated with Montanide ISA 70 resulted in a higher humoral immune response as detected by the HI inhibition test and ELISA test when compared to the combined vaccine adjuvanated with mineral oil. These results were supported by the results of the challenge test as shown in table (3, 4).

Table (1): Mean antibody titer of *Avibacterium paragallinarum* serovar A, B and C using haemagglutination inhibition test

Types of vaccines		Pre vaccination	HI Antibody titer (week post vaccination)					1 WPC
			1	2	3	4	5	
Mineral oil	A	0	2.63	10.55	36.75	24.25	17.14	13.92
	B	0	2.29	7.46	29.85	22.62	13.92	9.84
	C	0	3.24	9.18	34.29	25.99	14.92	11.31
Montanide ISA 70	A	0	2.82	14.92	42.22	42.22	25.99	18.37
	B	0	2.63	9.84	34.29	36.75	22.62	13.92
	C	0	3.03	10.55	39.39	45.25	27.85	21.11

Table (2): Geometric mean titre (log_2) of the two prepared vaccines against IB virus using indirect haemagglutination inhibition

Test groups	Types of vaccines	Mean antibody titer (week post vaccination)					1 WPC
		1	2	3	4	5	
1	Mineral oil adjuvant	5	5.4	6.4	7.6	7.8	6
2	Montanide ISA 70	5.4	5.8	7	8.2	8.6	6.8
3	Non vaccinated challenged	-	-	-	-	-	3.8
4	-ve control	-	-	-	-	-	0

WPC: Week post challenge

Table (3): Optical Density of antibody titer against Avibacterium paragallinarum serovar A, B and C using ELISA

Types of vaccines		Pre vaccination	Optical density (week post vaccination)					1 WPC
			1	2	3	4	5	
Mineral oil	A	0.02	0.093	0.345	0.974	0.728	0.496	0.353
	B	0.303	0.402	0.566	1.213	1.047	0.803	0.641
	C	0.294	0.424	0.639	1.547	1.291	0.846	0.716
Montanide ISA 70	A	0.017	0.1	0.405	1.11	1.233	0.712	0.553
	B	0.315	0.415	0.641	1.276	1.495	1.247	0.999
	C	0.301	0.416	0.689	1.731	1.943	1.417	0.81

Cut off A: 0.030 Cut off B: 0.333 Cut off C: 0.325

Table (4): ELISA antibody titer against IB virus of the two prepared vaccines

Test group	Type	Mean antibody titer week post vaccination					1 WPC
		1	2	3	4	5	
1	Mineral oil adjuvant	1987	3789	4281	4552	4782	3778
2	Montanide ISA 70	2769	3916	5382	6734	6874	4024
3	Non vaccinated challenged	-	-	-	-	-	1961
4	Non vaccinated non challenged	-	-	-	-	-	634

WPC: Week post challenge

Concerning the protection efficacy of the local prepared of combined inactivated Infectious coryza and infectious bronchitis vaccine, the data in table (5), showed that there was generally good cross-protection among all serovars.

Ciliostatic scores as shown in table (6), the ciliostasis protection was 75% and 70% in montanide vaccine and the adjvant vaccine respectively.

Table (5) Results of challenge test of chicken vaccinated with mineral oil and Montanide ISA 70 combined vaccines against *Avibacterium paragallinarum* serovar A, B and C

Vaccine type	Strain used in challenge	No. of chicken	No. of protected chicken	No. of chicken have clinical signs	Protection rates %
Mineral oil	**A**	10	8	2	80%
	B	10	7	3	70%
	C	10	8	2	80%
Montanoide ISA 70	**A**	10	9	1	90%
	B	10	9	1	90%
	C	10	8	2	80%
Control Un-vaccinated	**A**	10	0	10	0 %
	B	10	0	10	0 %
	C	10	0	10	0 %

Table (6): Ciliostasis score in chicks examined 6 days after challenge with IBV local isolate strain

Test group	Type	Ciliostasis score	Mean	Protection %
1	Oil Adjuvant	1-2	12	70%
2	Montanoide ISA 70	0-1	9.8	75%
3	Non vaccinated challenge	4 complete ciliostasis	-	0%
4	Non vaccinated Non challenge	100% cilia beating	-	100%

The results in Tables (7 and 8) showed that the average antibody titers at 4th week post vaccination of the group vaccinated with Montanide ISA 70 adjuvant vaccine were higher than those of the group vaccinated with mineral oil adjuvant vaccine and group vaccinated with imported vaccine either for *Avibacterium paragallinarum* or IB viru

Table (7): Comparison between the Optical Density of ELISA antibody titer of the groups vaccinated with mineral oil, ISA 70 oil vaccines and imported infectious coryza alum hydroxide gel vaccine using *Avibacterium paragallinarum* serotype A, B and C

		Mean ELISA antibody titers		
		Type of *Avibacterium paragallinarum* vaccines		
4th week PV	Type of antigen	Mineral oil adjuvant	Montanide ISA 70	Imported infectious coryza aluminum hydroxide gel vaccine
	A	0.728	1.233	0.813
	B	1.047	1.495	0.027
	C	1.291	1.943	1.458

Table (8): Comparison between the mean ELISA antibody titer of the groups vaccinated with mineral oil, ISA-70 oil vaccines and imported IB vaccine using virulent IB virus

Mean ELISA antibody titers	Type of IB vaccines		
	Mineral oil adjuvant	Montanide ISA 70	Imported combined IB +ND oil adjuvant vaccine
4th week PV	4552	6734	3762

DISCUSSION

Vaccine needs a potent adjuvant to induce protection. The reactogenic properties of the inactivated bacteria and virus used as antigen in the present vaccines require a safe adjuvant. The acceptable balance between efficacy and safety is obtained through the use of a specific adjuvant formulation. An effective combination of a relevant vaccine strain and suitable adjuvant are critical for ensuring that the vaccine provides optimal protection. (Dungu *et al*., 2009).

Mineral oils are generally considered non-toxic and have a long history of use in food, cosmetics, medicine, and other products (Yoshiki Kuroda et al., 2003). However, it is also well described that mineral oils have significant inflammatory or immunological effects following their injection, ingestion, or inhalation.

Montanide™ ISA adjuvants are based on a homogenous ready to use mix of purified mineral oils and refined oleic esters of anhydrous mannitol of vegetable origin (Dungu et al., 2009). Emulsifying properties of specific surfactants can only be obtained through strict synthesis parameters, and have a direct impact on the vaccine safety and efficacy (Stone, 1988). The least severe vaccination reactions were seen in the chickens which received the Montanide™ ISA formulation, which can be attributed to the fact that this was the only formulation in which the continuous phase is aqueous (water- in-oil-in-water), therefore causing the least inflammatory local reaction as compared to water in oil emulsions (Jansen et al., 2006).

Recently, several authors as (Abd El- Hady et al., 2002 and Kamal et al., 2004) noted that using of oil adjuvant of Montanide series (Seppic- France) as Montanide ISA 25, 70, 206 and IMS 1113 resulted in an improvement of immune response and those new oil formulations have favorable characteristics of low viscosity, lower reactivity and high potency. Other Montanide adjuvants are primarily prophylactic adjuvants that utilize a variety of mineral and metabolizable oils and are in various phases of human and veterinary trials (Yoshiki Kuroda et al., 2003).

The results illustrated in tables (1,2) indicated that combined vaccine adjuvanated with Montanide ISA 70 resulted in a higher humoral immune response as detected by the HI inhibition test and ELISA test when compared to the combined vaccine adjuvanated with mineral oil. These results were supported by the results of the challenge test as shown in table (3, 4).

The result obtained in this study concerning the use of another type of oil adjuvant rather than mineral oil, it was clear that infectious coryza vaccine adjuvanated with Montanide ISA 70 yielded earlier and higher humoral immune responses as measured by HI test as shown in table (1) in chicken sera when

compared with the vaccine adjuvanated with mineral oil.

These finding were also noticed when Abd-Elhady et al. (2002) also mentioned that Newcastle Disease vaccine adjuvanated with Montanide ISA 70 oil adjuvant yielded earlier antibody titer at 2 weeks post-vaccination and peaked at 4 weeks, in addition to its superior stability performance. This was due to the new immunostimulant listed as GRAS substances included in the Montanide oil and which elicited both humoral and cellular immune response.

The HI results expressed as $\log_2$ of the reciprocal of the geometric mean 0.(GMT) as shown in table (2),the bivalent vaccine of IBV (H120 and CR88) strains induced high titers to classical M41 antigen (7.8 $\log_2$) and(8.6) in oil adjuvant and Montanide ISA70 vaccines respectively, these findings were also obtained by (Finney et al., 1990)

The results of Kamal et al. (2004) coincided with the results of this study as they found that, infectious coryza vaccine adjuvanated with Montanide ISA 206, 25 or IMS 1113 revealed a higher immune response in the chicken superior to those adjuvanated with liquid paraffin as an oil adjuvant.

ELISA test revealed an excellent antibody titers against IB vaccines reached (6874) in Montanide vaccine which was higher than the oil adjuvant vaccine (4782). These results were supported by those found by Ladman et al. (2002).

Coryza vaccine should contain three serotypes to obtain a broad protection against all serotypes. Within Kume serogroup A, there was generally good cross-protection among all serovars; however, within Kume serogroup C, there was evidence of a reduced level of cross-protection between some of serovars (Soriano et al., 2004).

Ciliostatic scores as shown in table (6) in chickens were better in montanide vaccine than the adjvant vaccine (75 % and 70) ciliostatic protection respectively (Cavanagh et al., 1997).

The kidneys of each bird were examined macroscopically for evidence of the damage attributable to IB infection at 7 and 10 days post challenge (d.p.c) were all normal in vaccinated challenged groups wherever in non vaccinated challenged group two birds at 7 d.p.c had mild kidney lesion and at 10 d.p.c the kidney of three birds examined showed sever lesions typical of IBV infection.

These present study confirm the results of (Ladman et al., 2002) who said that inactivated IBV vaccination stimulates increased local respiratory as well as systemic immunity upon challenge with nephrotropic strain of IBV, also the vaccination of live vaccine alone did not protect trachea against IBV strains.

Because of Massachusetts serotype alone were not effective against challenge with different strains (Cooke et al., 2001), also (Cooke et al., 1999 and Lambrechts et al., 1993 and 1994) mentioned that the combined vaccination programs provided excellent protection against other nephrotropic strain.

The results in table (7) confirm that the imported infectious coryza vaccine contain serogroup A and serogroup C only so there is no induction of ELISA antibody titer against serogroup B.

The present study highlights the importance of a suitable vaccine formulation that generates protection throughout the productive life of chickens and the need for incorporation of dominant strains in the vaccine.

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