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TRIAL FOR PREPARATION OF COMBINED OIL INACTIVATED VACCINE AGAINST NEWCASTLE AND FOWL CHOLERA DISEASES BY USING MONTANIDE ISA206 OIL FOR IMPROVING THE IMMUNE RESPONSE IN CHICKENS

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SUMMARY

One hundred and fifty chickens were divided into 3 equal groups (50 birds each). Groups one and two were vaccinated with combined inactivated fowl cholera (FC) and Newcastle (ND) vaccines using ISA206 and white oil (paraffin oil) as adjuvants respectively while group three was kept as non-vaccinated control group. The results of haemagglutinating antibody titres against NDV revealed that ISA206 gave earlier and higher immune response than white oil till 8 weeks post vaccination (WPV) but from 16 till 20 WPV it was noticed that paraffin oil was protective than ISA206. On the other hand, results of indirect haemagglutinating (IHA) and ELISA tests indicated that the same above results till the 6 WPV for FC by using ISA206, meanwhile paraffin oil recorded higher antibody titre from 8 WPV till the end of experiment and these results were confirmed by protection tests against virulent NDV and virulent FC.

INTRODUCTION

Fowl Cholera (FC) and Newcastle diseases (ND) are the most two important diseases affecting chickens, turkeys in large of expanding Egyptian poultry breeding industry (Sheble, 1960). Such two diseases are also act as the biggest depressing factors on profit margin (Allan et al., 1973). Vaccination has played a major role in the control of infectious diseases in veterinary and human medicine.

The inactivated oil vaccine against FC was locally produced and still represent safe and effective means for controlling FC in chickens. Furthermore, early inactivated vaccines of NDV using aluminum hydroxide adjuvants but the development of oil emulsion vaccines proved a major ad-

vancement.

ND inactivated vaccines had been produced many years ago. The combination of ND and FC production have the advantage of providing protection against more than one disease at the same time thus reducing vaccination expenses and number of vaccination per farm as well as saving time and costs (Nadia et al., 1993).

Mineral oil emulsions has been widely used to produce a prolonged, slow release of solutes from the aqueous phase. They have been used with antigens to potentiate the antibody response, also to prolong their action (Cox and Coulter, 1997). Tween 80 is known to cause a reduction in viscosity, interfacial tension and an increase in the coagulation rate of the aqueous globules of water in oil emulsion (Chiejina and Sewell, 1974).

Montanoide ISA206 is incomplete specific adjuvants type of mineral oil based adjuvant from a complex water in oil emulsion characterized by highly efficient fluidly with low viscosity and easily reproducible (Hala et al., 2002 and Abd El-Wanis, et al., 2003).

In a trial to improve the combined vaccines against FC and ND, the present work was designed to use two different adjuvants for preparing this experimental vaccine. The first adjuvant type of mineral oil of low viscosity Montanoide ISA206 incomparison with white oil (paraffin oil) for potentiating antibody response in chicken for preventing ND and FC diseases.

MATERIAL AND METHODS

1- Chickens:

One hundred and fifty, one day old, chickens were used in the present study. They were obtained from commercial poultry farm and reared under strict hygienic measures till six weeks old. Random serum samples were tested for maternal antibodies against NDV and Pasteurella multocida. They were used for evaluation of the experimentally prepared vaccines using immunization and bioassay procedures.

2- Strains:

a- Vaccinal strain of NDV (LaSota strain):

It was locally prepared in Veterinary Serum and Vaccine Research Institute (VSVRI), Abbasia, Cairo. It had an infectivity titre of $10^{10.5}$ EID_{50}/ ml before inactivation. The vaccine was kept at -20°C till used.

b- Virulent strain of NDV:

It is a field local isolate, Velogenic Viscerotropic Newcastle Disease Virus (VVNDV), obtained from Newcastle Disease Department, Veterinary Serum and Vaccine Research Institute (VSVRI), Abbasia, Cairo. Its infectivity titre is 10^6 EID_{50} / dose (Reda and Sheble, 1976).

c- Pasteurella multocida strains:

Four serovars (5:A, 8:A, 9:A & D:2) of P. multoc-

ida were used for preparation of the experimental vaccines and antigens, used for serological assay (Indirect haemagglutination and ELISA tests).

3- Adjuvants:

a- Montanoide ISA206:

It was obtained from Seppoc-Paris, France. It is a mineral oil based adjuvant from complex water in oil emulsion and mixed with the vaccine according to manufacturer instructions.

b- Whilerex 309, white oil quality, FDA /A/ USP with span 80 as a classical emulsifier:

It was prepared in Aerobic bacterial vaccines department, Veterinary Serum and Vaccine Research Institute (VSVRI), Abassia, Cairo.

4. Preparation of vaccines:

a. Preparation of inactivated NDV vaccine:

Preparation and titration of NDV was done in specific pathogen free (SPF) 10-day-old embryonated chicken eggs according to Allan et al., (1973). Its titre was 10^{12} EID_{50}/ml. Inactivation and testing of complete inactivation was done according to Madkour (1995).

b. Preparation of inactivated Pasteurella multocida vaccine:

A virulent local strains of P. multocida serovars 5:A, 9:A, 8:A & D:2 were propagated in trypticase soya broth at 37°C aerobically for 24 hours to obtain a dense culture containing approximately $3.25X10^{10}$ colony forming unit (CFU) of each strain/ml. After inactivation by addition of 0.5% formalin, each culture was tested for purity, safety and sterility as mentioned by (Mukkur et al., 1982). Finally, cultures were equally mixed together then preserved with 0.01% of thiomersal and stored at 4°C until use.

c- Preparation of combined inactivated NDV and P. multocida vaccine by using ISA206:

It was prepared by mixing of 50ml of inactivated NDV vaccine with 50ml of formalized cultures of fowl cholera. The mixture was emulsified into 100ml of Montanoid ISA206 adjuvant (volume / volume) according to Barnett et al., (1996).

d- Preparation of combined inactivated NDV and FC vaccine by using paraffin oil:

It was prepared according to Stone et al., (1978) by mixing previously prepared inactivated fluids (ND & FC) by equal volumes, they were emulsified in oil with an aqueous phase to oil phase in a ratio of 1:2 mineral oil was used as an adjuvant and sorbitan monoleate and tween 80 respectively were used as oil phase and aqueous phase emulsifiers.

N.B. During vaccine preparation the bacterial and virus contents within each dose (0.5 ml) were adequately adjusted to be the same in both vaccines.

e- **Quality control of the prepared vaccines:**

The two types of vaccines prepared in this study were tested for sterility and safety following the standard international protocols as described by Code of American Federal Regulation (1985).

f- **Characterization of vaccines:**

The two formulated oil experimental vaccines were subjected to the following tests to evaluate the emulsification process:

- **Drop test:**

It was done according to Geneidy et al., (1971) by expressing a single drop of the oil vaccine from the tip of a needle into a beaker containing cold water. A drop of water disperses.

- **Emulsion viscosity:**

According to Becher (1965) it was measured as the of discharge (flow time) of the emulsified vaccine from the orifice of vertically mounted 1ml serologic pipette in relation to glycerol flow time.

- **Emulsion stability:**

According to Cessi and Nardelli (1973), a vaccine sample was centrifuged at 1000 xg for one hour to calculate the percent of emulsion phase to excess oil and water phases.

4- Experimental design:

Chickens were divided into three equal groups (50 /each). Groups (1) and (2) were vaccinated intramuscularly at the age of six weeks with 0.5ml / bird with each combined inactivated ND and FC vaccine using ISA_{206} and white oil adjuvants, respectively. Such chicken were boostered with the same dose at 3 week after the initial vaccination. Group (3) was kept as non-vaccinated control group. Serum samples were collected up to 20 weeks post vaccination for evaluation of immune response.

5- Serological tests for evaluation of humoral immune response:

a- Haemagglutination inhibition test (HI):

It was carried according to Majujabe and Hitchner (1977) for ND antibody titre.

b- Indirect haemagglutination test (IHI):

It was carried out according to Carter and Rappy (1962) for determining P. multocida antibody levels.

c- Enzyme linked immunosorbent assay (ELISA):

It was carried according to the method adopted by Marshall et al., (1981) for P. multocida antibody titre.

6- Challenge test:

a- Challenge with virulent ND virus:

All vaccinated and non-vaccinated control chickens were challenged against velogenic viscrotropic Newcastle disease virus (VVNDV) at 3 weeks

post second vaccination using 0.25ml /bird intramuscularly containing 10^6 EID_{50}/ml.

b- Challenge with virulent P. multocida:

Chickens of vaccinated as well as non-vaccinated groups were challenged by the inoculation with 0.1ml of 100 LD_{50} of virulent P. multocida serotype 5:A & D:2 used in the preparation of the vaccine. Challenge infection was conducted 3 weeks after booster dose. All chickens were observed for 10 days and mortalities were recorded.

RESULTS

The data obtained in this investigation are illustrated in tables 1, 2, 3, 4 and 5.

Table (1): Comparative results of anti P. multocida antibodies in sera of chickens vaccinated with FC and ND vaccines using different adjuvants as measured by IHA test.

Type of vaccine	Type of antigen	Weeks post vaccination												
		Prevacc.	1 W	2 W	3 W*	4 W	5 W	6W	8 W	10 W	12 W	14 W	16 W	20 W
FC and ND vaccine using ISA_{206}	A	14	68	104	388	477.4	1024	2018	2018	5195	1097.5	588.4	445.7	139
	D	14	52	64	181	294.4	724.4	1552.1	1788.9	2018	1024	477.7	388	80
FC & ND vaccine using White oil	A	13	34.3	68	250	315.2	512	1782.9	2019.5	2521	1552.1	891.4	588.4	194
	D	13	29.9	64	238.9	238.9	415.9	1418.2	1910.9	2195	1418.4	724.1	477.7	128
Non-vaccinated control	A	11	11	12	11	12	12	12	-	-	-	-	-	-
	D	11	11	12	11	12	12	12	-	-	-	-	-	-

* All birds received a booster dose at 3 weeks post first vaccination

Table (2): Comparative results of anti P. multocida antibodies in sera of chickens vaccinated with FC and ND vaccines using different adjuvants as measured by ELISA test.

Type of vaccine	Type of antigen	Weeks post vaccination												
		Prevacc.	1 W	2 W	3 W*	4 W	5 W	6W	8 W	10 W	12 W	14 W	16 W	20 W
FC and ND vaccine using ISA_{206}	A	260	1320	2445	3710	4209	5410	6523	7500	8269	6150	5450	4050	2900
	D	260	1187	2198	3201	3789	4189	5694	6901	7889	5908	5013	4560	2654
FC & ND vaccine using White oil	A	213	912	2113	3325	3825	4998	6354	7630	8380	6283	5680	4975	3490
	D	213	628	1918	2981	3305	3677	5980	6234	7890	5891	5260	4789	3129
Non-vaccinated control	A	186	189	195	220	230	195	200	-	-	-	-	-	-
	D	186	189	195	220	230	195	200	-	-	-	-	-	-

* All birds received a booster dose at 3 weeks aftert first vaccination

Table (3): Haemagglutination inhibition (HI) antibody titres of chicken vaccinated with FC and ND vaccines using different adjuvants.

Group	Mean log2 HI titre in weks post vaccination										
Type of vaccine	1	2	3	4	5*	6	8	10	12	16	20
FC and ND vaccine using ISA_{206}	1	3.5	7	9	9.4	10	9	8	7.2	5	4.9
FC & ND vaccine using White oil	2	2	5.5	8	9	9	8	8	7	6.5	6
Non-vaccinated control	0	0	0	0	0	0	0	0	0	0	0

N. B. HI titre pre-vaccination was "0".
* All birds received abooster dose at 3 weeks after the first vaccination

Table (4): Bioassay in chickens vaccinated with F C and ND vaccines using ISA_{206} oil and paraffin oil and challenged with 5:A and D:2 of P. multocida strains.

Type of vaccine	Challenge	No. of chickens	No. of survived	Protection %	Mean death time	Lesion score	Mortality %
FC and ND vaccine using ISA_{206}	5:A	15	15	100	-	-	-
	D:2	15	14	92.8	7	+	7.20
FC & ND vaccine using White oil	5:A	15	14	92.86	5	+	7.20
	D:2	15	13	86.7	6.5	+	13.30
Un-vaccinated control	5:A	15	-	-	1.8	+++	100
	D:2	15	-	-	1.9	+++	100

Challenge was conducted 3 weeks after second vaccination dose 0.1ml of 100 LD_{50} of virulent P. multocida serotypes 5:A and D:2.

Table (5): Protection efficiency of vaccinated chickens against challenge with virulent NDV (3 weeks post second vaccination)

Group	No. of chickens	No. of survived 15 DPC	No. of dead	HI titre*	Protection %
FC and ND vaccine using ISA_{206}	10	10	0	11	100
FC & ND vaccine using White oil	10	9	1	11	90
Un-vaccinated control	10	-	10	0	0

Dose 0.25ml of 10^6 EID_{50}/ml by I/M inoculation.

*Log2 HI titre two weeks post challenge.

$$\text{Protection \%} = \frac{\text{No. of survivors}}{\text{Total No. of challenged birds}} \times 100$$

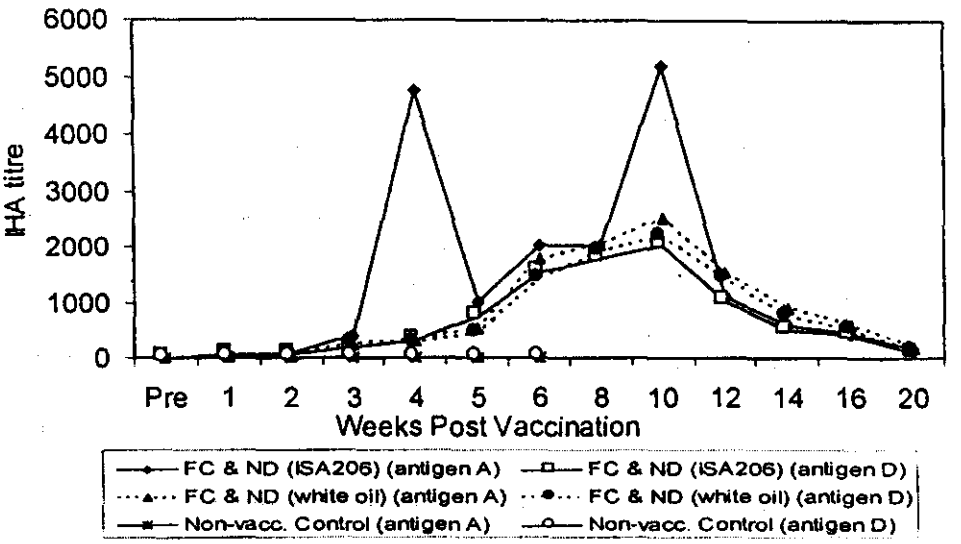

Fig. (1): Comparative results of anti P. multocida antibodies in sera of chickens vaccinated with Fowl Cholera and ND vaccines using different adjuvants as measured by indirect HA test

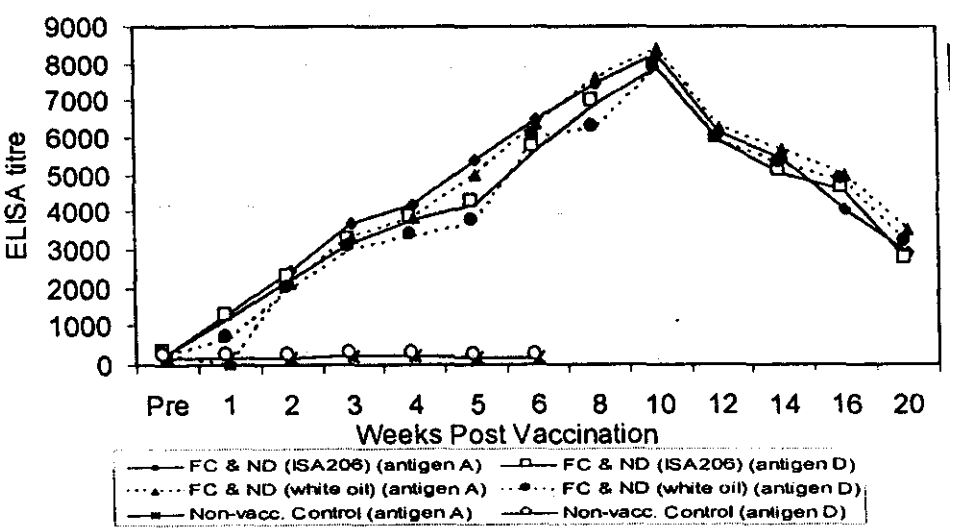

Fig. (2): Comparative results of anti P. multocida antibodies in sera of chickens vaccinated with Fowl Cholera and ND vaccines using different adjuvants as measured by indirect ELISA test

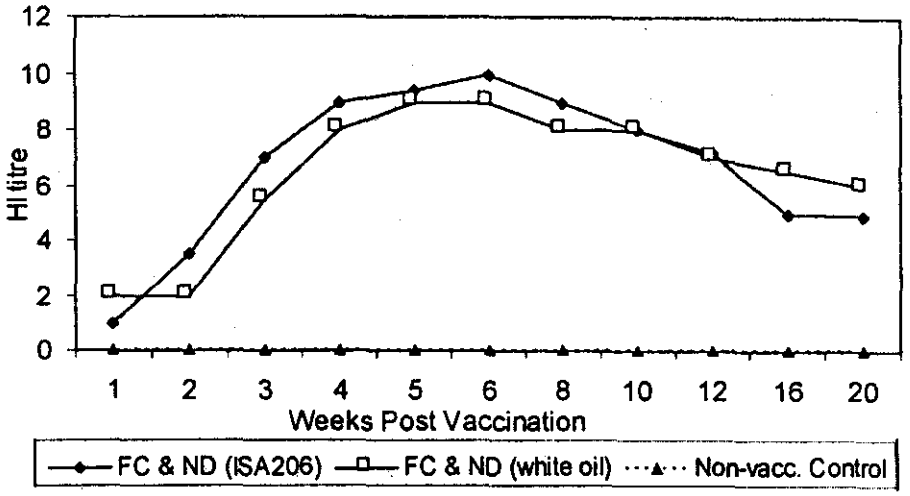

Fig. (3): Haemagglutination inhibitiion (HI) antibody titres of chicken vaccinated with FC and ND vaccines using different adjuvants

DISCUSSION

FC and NDV and their control are still as subject of interest and usually attract the attention of researchers to know more and more about the diseases epidemiology and how to control in chickens.

Vaccination is still considered one of the major tools for controlling both of the two diseases (Nadia et al., 1993) vaccine efficacy depend on many variables, such as the nature, the amount of antigen administrated and the presence of adjuvants to enhance immunogenicity (Stone et al., 1978).

Adjuvant is a substance that when added to a vaccine will enhances immunogenicity of an antigen in the stimulation and evaluation humoral immune response. In some instance adjuvants also stimulate a cell mediated response by trapping antigens at sites where they are accessible to reactive lymphocytes and they induce antigen presenting cells to express costimulatory molecules such as CD80. Also adjuvant can reduce the quantity of antigen needed to generate a protective immune response and enables the vaccine to be more cheaply, they can prolong the immune response of the vaccinated animals and birds (Stewart, 1994).

In this study we compare between two types of adjuvants (ISA_{206} and white oil) for improving the immune response of the chickens against combined ND and FC vaccine.

The data given in tables (1, 2 and 3) and (Fig.1,2 and 3) revealed that, there was none of the serum samples from all vaccinated and control chickens showed the presence of antibody before vaccination. This mean that they were neither previously exposed to diseases infection nor received FC and ND vaccines before using in this experiment. Also, all chickens of groups (1 & 2) vaccinated with combined vaccines (FC and ND) containing two different adjuvants induced a systemic humoral antibodies as measured by IHA, ELISA and HI tests.

In this work, as showed in tables (1, 2), and (Fig.1,2) the earlier, good immune response and higher antibodies against P. multocida occurred in 1st group of chickens which received vaccination with FC and ND vaccines contained oil ISA206 than 2nd group which received vaccination with FC and ND vaccines contained white oil from first week till six weeks, on the other hand, avian cholera and ND vaccines contain paraffin oil gave higher antibodies values from 2 months post vaccination till the end of the experiment by using IHA, ELISA tests. These results were supported by Hala et al. (2002).

Hala et al. (2002) found that FC vaccine containing oil ISA206 induced earlier response and was responsible for the highest level during 6 weeks post primary vaccination. Also, FC vaccine containing white oil gave higher anti-P. multocida antibodies values for 2 months post vaccination till the end of the experiment (5 months). On the other hand HI titer recorded in Table (3) and (Fig.3) proved that group one which vaccinated by combined inactivated. FC and ND by using ISA206 was earlier and higher allover the experiment till 8th WPV than group two which was vaccinated by combined inactivated FC and ND by using white oil (9 log2 HI titre and 8 log2 HI titre) respectively, become equal at 10 WPV (8 log2 HI titre). After 12th WPV, one can notice sudden decrease of HI titre in group one (5 log2 HI titre) while it was gradually decreased in group two (6.5 log2 HI titre). Our results agree with Abd El-Wanis et al. (2003) who observed that among mineral oils, montanide oil (ISA206) was preferable as it is easily prepared and give good immunological response.

The data shown in table (4) indicated that the results of the challenge test, chickens that received FC and ND vaccine contain oil ISA206 and challenged with 5:A of P. multocida strain showed 100% protection and give 92.8% when challenged with D:2 P. multocida strain, on the other hand, the 2nd group which vaccinated with FC and ND vaccine contain white oil and challenged with 5:A P. multocia strain showed 92.8% protection and 86.7% when challenged with D:2 P. multocida strain. These results are in agreement with Hala et al., (2002) who noticed that chickens that received FC vaccine containing oil ISA 206 showed

90% protection but chickens received FC vaccine containing white oil showed 86.6% protection. This result was confirmed in table (5) which shows that protection percent 100% and 90% in groups one and two respectively when challenged by VVNDV and shows high equal HI titre 3 WPV for both groups (11 $\log_2$ HI titre).

These results are in agreement with Nadia et al. (1993) and Abdel-Wanis et al., (2003) who found that a combined inactivated oil emulsion vaccine for ND and FC in chickens was protective.

The results from this studies indicated that the two vaccines are valid according to specifications of OIE manual (1990).

The conclusively, the two prepared vaccines were protective but ISA206 oil was easily prepared and gave earlier immunity.

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