PRELIMINARY TRIALS FOR PREPARATION OF TETRAVALENT INACTIVATED OIL VACCINE AGAINST NEWCASTLE, INFECTIOUS BRONCHITIS, EGG DROP SYNDROME AND FOWL CHOLERA

By

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ABSTRACT

Inactivated Newcastle disease virus (NDV), infectious bronchitis virus (IBV), egg drop syndrome (EDS) and *P. multocida* (PM) were incorporated into water in oil emulsion vaccines alone (monovalent) or as in combined (tetravalent) vaccine. Immunological response to the new tetravalent vaccine was evaluated by injecting groups of susceptible chickens with either tetravalent or monovalent vaccine. Results revealed that humoral immunity with the new tetravalent vaccine were never inferior to those obtain with the monovalent ones. There were no practical differences in the percentage and duration of the protective immunity induced with NDV vaccine and *P. multocida* vaccine upon challenge with velogenic viscerotropic Newcastle disease virus (VVNDV) or PM antigen. The tetravalent (NDV + IBV + EDS and FC) inactivated vaccine was safe, immunogenic and produced satisfactory dual protection against NDV, IBV, EDS and *P. multocida* infections.

INTRODUCTION

Viral disease of poultry constitute one of the most major problems facing the rapidly expanding poultry industry in Egypt causing considerable economic losses due to serious mortality associated with different infectious viruses. Newcastle and infectious bronchitis diseases are among the highly contagious diseases of the respiratory tract of chickens (**Hofstad, 1984**). Mass vaccination against both diseases has become necessary especially in high density of the poultry population to minimize economic losses.

EDS-76 is a disease of laying hens characterized by a sudden and frequently large drop in egg production, with the laying of soft-shelled eggs (**Holmes *et al.*, 1989**). An oil adjuvant inactivated vaccine is widely used and gives good protection against clinical disease. Fowl cholera (FC) is typically an

acute septicaemia or chronic disease of many avian species. It is caused by *Pasteurella multocida* (PM) and characterized by high morbidity, mortality and egg production losses. The effective prevention of the disease is still based upon live and inactivated vaccines. The use of oil emulsion inactivated vaccine induce a satisfactory immunity as the vaccination resulted high level of antibodies in addition to the freedom of vaccinated birds from disease and drop in egg production (**Box and Furminger, 1975**). Combined vaccines have the advantages of providing protection against more than one disease, reducing vaccination expense and number of vaccination per farm as well as saving time and labor costs besides reducing the stress reactions. Also, the more manual capture and restraint needed to inject vaccines into poultry especially egg laying hens. So, the objective of this study was to prepare and evaluate the immune response of tetravalent vaccine of NDV, IBV, EDS and *P. multocida* in single and combined form for protection against diseases caused by these agents.

MATERIAL AND METHODS

Seed Viruses:

1. Newcastle disease seed virus:

Hitchner B1 strain (supplied by the Central Veterinary Laboratory, Weybridge, England).

2. Infectious bronchitis disease seed virus:

Strain H120 was obtained as allantoic fluid from Department of Animal Science and Agricultural Biochemistry, University of Delmare, New York, USA.

3. Egg drop syndrome disease seed virus:

EDS-76 live virus product code PA0081 handled by **Prof Dr. Nadia Mohamed Hassan, from Weybridge, England.**

4. P. multocida vaccine culture:

A virulent local strain of *P. multocida* serovars A and D (5:A, 8:A, 9:A and 2:D) were propagated separately in trypticase soya broth at 37°C aerobically for 24 hours to obtain a dense cultures containing approximately 3.25 x 10^{10} colony forming unit (CFU) of each strain per ml. After that, cultures were equally mixed together then preserved with 0.01% of thiomersal and stored at 4°C until ready for preparation of emulsion vaccine.

Embryos:

- Embryonated duck eggs were obtained from United Company for Poultry Production. It is used for propagation of the virus, testing of complete inactivation in the prepared batch of EDS virus vaccine.
- Nine to eleven days old embryonated SPF eggs (SPAFAS) Inc., Norwich, Comn, were used for propagation and testing of complete inactivation in the prepared batches of ND and IBV vaccines.

Experimental chicks:

One day old Hubbard chicks (United Company for Poultry Production) were reared under complete hygienic measures in isolated and disinfected wire floored cages, commercial broiler ration was used.

Viruses propagation:

1. NDV propagation:

The method of **Allan *et al.*, (1973)** was conducted. Obtained virus was titrated according to the standard methods described in **FAO Publication (1978)**. The titre was 10^{11} EID_{50}/ml.

2. IBV propagation:

Propagation and titration was carried out after the method described by **Cunningham (1973)**. The titre of the virus was 10^{9} EID_{50}/ml.

3. EDS propagation:

Virus propagation in embryonated duck eggs was applied according to **Allan *et al.*, (1973)**. The titre of the virus was 10^{5} EID_{50}/ml.

Inactivation of viruses:

Inactivation of virus suspension ND, IBV and EDS was carried out using formalin in a final concentration of 0.1% and 0.5% formalin for culture of Fowl cholera. The fluids were blended using magnetic stirrer for about 18 hours at room temperatures for NDV, IBV and 48 hours of EDS virus. Samples from the inactivated viruses were tested for safety in 9-11 days old embryonated chicken and duck egg (0.2ml/egg). Two successive blind passages were carried out before they were considered safe. Titration of the vaccine was done according to the standard method described in the **United State Code of Federal Regulations (1987)**.

Preparation of oil emulsion vaccines:

A. Monovalent vaccine:

The adjuvant for preparation of water in oil consists of 88 parts Marcol 52, 10 parts Span-80 (Sorbital monooleate) and was gradually added. All components were thoroughly mixed, then sterilized by autoclaving at 121°C for 10 minutes (15 lb of pressure). Monovalent vaccines were prepared as described by **Thayer *et al.*, (1983)**. 100 ml of inactivated virus suspension or inactivated *P. multocida* culture were added to 300ml of adjuvant while it was stirred and the mixture was emulsified for 10 minutes.

B. Tetravalent vaccine:

The tetravalent vaccine was prepared by mixing 50ml of aqueous phase emulsified into 150ml of oil adjuvant while it was being stirred and emulsified for 10 minutes. The prepared vaccines were dispensed into sterile bottles and stored at 4°C till used.

Quality control of the prepared vaccines:

a. Purity and sterility tests:

The prepared vaccines were tested to be free from contaminants according to **Code of Federal Regulations, USA (1987).**

Measurement of toxicity of *P. multocida*:

The toxicity of the extract was tested in broiler chickens and Swiss Webster mice as described by **Mukkur *et al.*, (1982).**

ELISA test for viruses and *P. multocida* antibodies:

The IDEXX ELISA kits (flock check system) were used to determine the level of serum antibodies against IBV. Cypress diagnostic kits used in case of NDV according to manufacturer's recommendation.

Virus neutralization test (SNT):

It was carried out estimating the neutralizing antibodies against IBV and EDS according to the method described by **Rossiter *et al.*, (1985).**

Indirect haemagglutination test (IHA):

P. multocida level was assayed by IHA test as described by **Carter and Rappy, (1962).**

Haemagglutination inhibition test (HI):

Used for estimating the haemagglutinating antibodies against ND virus, it was done according to **Majujabe and Hitchner, (1977).** The beta procedure of HI test was employed for antibody assay in case of adenovirus strain 127 using micromethod of **Takatsy, (1956)** using virus suspension containing 4 HA units.

Challenge test:

Against *P. multocida*:

The immunity of vaccinated and unvaccinated birds to *P. multocida* was tested by intramuscular challenge with 0.1ml of 10^6 dilution of virulent strain of serovars A and D. Clinical signs, mortality rates and gross lesions were recorded for ten days post challenge. Reisolation of the viable organisms were also tried from liver, blood and bone marrow of dead challenged birds.

Experimental design:

Three hundred and twenty, one-day-old Hubbard chicks were reared in isolated conditions. These birds were as far as could be examined free from bacterial pathogens. The sera of these chickens when examined serologically were free from antibodies against NDV, IBV, EDS and fowl cholera and divided into groups as follows:

Group (1): Contained (30) birds which were vaccinated with monovalent oil emulsion NDV vaccine.

Group (2): Contained (30 birds) which were vaccinated with monovalent oil emulsion IBV vaccine.

Group (3): Contained (30) birds which were vaccinated with monovalent oil emulsion EDS virus vaccine.

Group (4): Contained 30 birds which were vaccinated with fowl cholera monovalent oil emulsion vaccine.

Group (5): Contained 100 birds were vaccinated with polyvalent oil emulsion vaccine (NDV+IBV+EDSV+fowl cholera).

Group (6): Contained 100 birds kept as a control (unvaccinated) group.

Each chick of vaccinated groups received 0.5ml of its appropriate vaccine I/M at 21 days of age. Ten random blood samples were collected weekly from each group for 8 weeks post vaccination. Sera were collected and stored at -20°C until used for detection of corresponding antibodies against ND, IB, EDS viruses and fowl cholera.

RESULTS AND DISCUSSION

Dealing with the result of HI antibody titres as presented in Table (2) revealed no noticeable difference between the former groups especially at the 3rd week post booster vaccinal dose of tetravalent vaccine. Our results come in agreement with that obtained by **Nadia *et al.*, (1993)** where they found that no significant difference were noted in the HI antibody titre in groups of chickens vaccinated either with the inactivated monovalent ND or bivalent ND and fowl cholera oil emulsion vaccine.

Dealing with absorbance mean value of ELISA antibody against NDV in Table (3). Groups of birds that took booster dose of vaccination of tetravalent vaccine gave higher values than that had not taken booster tetravalent vaccine and also than that took monovalent ND vaccine from the 4th week till the end of the experiment.

Dealing with the neutralizing antibody against IBV in Table (4) showed that no noticeable difference was detected between groups vaccinated with monovalent IBV vaccine and that vaccinated with tetravalent vaccine with booster dose. These results come in contact with that obtained by **Kolchi and Yoshikazu, (1973)** where they found that no increase in antibody titre demonstrated in birds vaccinated with IBV vaccine alone compared with the mixed trivalent vaccine against ND, IB and Haemophilus gallinarum in the same time.

Our results of ELISA for IBV in Table (5) revealed a noticeable difference between antibody titre in group vaccinated with booster dose of tetravalent vaccine and that took monovalent vaccine. The aforementioned results disagree with that obtained by **Gough *et al.*, (1977), Lamia, (1996)** where they found that no practical difference between groups of chickens vaccinated with monovalent inactivated oil emulsion IBV vaccine and inactivated oil emulsion NDV, IBV and infectious coryza vaccine. Increase of titre in group of birds vaccinated with booster dose of tetravalent vaccine from the 5th week post 1st vaccination and 1st week from the booster vaccine.

Also, there is no noticeable difference between group of birds vaccinated with booster dose of tetravalent vaccine and that had not taken it from the 5th week till 7th week post vaccination.

Dealing with the results of HI test (Table 6) in groups of birds vaccinated with EDS vaccine revealed no difference between HI titre of group vaccinated with monovalent vaccine and that took tetravalent. Also, slight difference noticed between group of birds vaccinated with booster dose of vaccine and that had not taken it by one log till the 7th week. This result agrees with **Szeleszczuk, (1987)**.

Dealing with neutralizing antibodies against EDSV in Table (7) noted that there is an increase in group vaccinated with tetravalent vaccine than that vaccinated with monovalent vaccine at 7th, 8th weeks post 1st vaccination. This result disagree with **Khodeir *et al.*, (1999)** who found SN antibody titre in group vaccinated with monovalent vaccine at 7th week reached (256) while group vaccinated with combined vaccine (EDS + fowl cholera) reached (128).

Data presented in Table (8) showed that inoculation of chickens with tetravalent vaccine gave best result in IHA antibody titre than group vaccinated with monovalent fowl cholera vaccine from the 1st week post vaccination till the end of the experiment. These results are in agreement with **Marshall and Zeigler, (1991)** and **William and John, (1989)** who used IHA to evaluate the immune response of fowls to fowl cholera inactivated oil vaccines and obtained good levels of immunity which increased after the booster dose of vaccination.

Regarding the bioassay test, Table (9) revealed that the immunity of chickens vaccinated with tetravalent vaccine gave good protection rate not less than that of group vaccinated with monovalent vaccine that gave 95% and 90%, respectively.

As regard to the prepared tetravalent inactivated oil emulsion (NDV + IBV + EDS and fowl cholera vaccine, it was found that such vaccine is safe and potent where it protected vaccinated fowls against these diseases and there were no excreted virus or bacteria where contact birds remain healthy. Also, no one of viruses or fowl cholera antigen affected the immune response of birds to the other. These findings come to be confirmed by those of **Winterfield, (1982); Gergis *et al.*, (1994); Samira *et al.*, (1995) and Khodeir *et al.*, (1999)** who used fowl cholera vaccine in combination with other viral vaccines as NDV, IBV, fowl pox and EDS. They stated that there was no interference between bacterial and viral inactivated antigens in the immune response of vaccinated fowl to each other.

So, it could be concluded that the locally prepared tetravalent inactivated oil emulsion (NDV + IBV + EDS) and fowl cholera vaccine is a safe and potent vaccine.

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Table (1): Experimental Design.

Groups	NDV vaccine	IBD vaccine	EDS vaccine	Fowl cholera vaccine	Combined NDV+IBV +EDS+Fowl cholera	Control
1st vaccinal dose	0.5ml	0.5ml	0.5ml	0.5ml	0.5ml	Non
	Intramuscular route at 3 weeks old at the thigh					
Blood collection	From the 1st week post vaccination till 8th week from the first vaccination					
2nd vaccinal dose (booster dose) 4 weeks post first vaccination	0.5ml intramuscular at the thigh					
Challenge test (4 weeks post booster dose of vaccination				Intramuscular route	20 birds challenged with P.M by I/M route	
	The duration of immunity was determined till the 8th week post 1st vaccination					

Table (2): NDV HI antibody titres in groups of vaccinated chickens.

Groups	Weeks Post Vaccination							
	1W	2W	3W	4W	5W	6W	7W	8W
Group (1)	8	16	128	128	128	256	256	1024
Group (5)	8	32	128	128	256	256	128	128
Group (6)	0	0	0	0	0	0	0	0

Group (1): Vaccinated with the locally prepared monovalent oil vaccine.
Group (5): Vaccinated with locally prepared tetravalent oil emulsion vaccine.
Group (6): Control non-vaccinated.

Table (3): Absorbance mean value of ELISA antibody against NDV in groups of vaccinated chickens.

Groups	Weeks Post Vaccination							
	1W	2W	3W	4W	5W	6W	7W	8W
Group (1)	0.280	0.357	0.407	0.417	0.512	0.603	0.686	0.706
Group (5)	0.213	0.330	0.396	0.453	0.501*	0.630	0.636	0.629
					0.607**	0.796	0.818	0.750
Group (6)	0.166	0.182	0.189	0.173	0.166	0.183	0.182	0.186

Group (1): Chicken group vaccinated with monovalent locally prepared NDV inactivated oil emulsion vaccine.
Group (5): Chicken group vaccinated with tetravalent NDV-IBV-EDS and P.M inactivated oil emulsion vaccine.
Group (6): Unvaccinated chicken group.
* Chicken without booster dose.
** Chicken received booster dose of tetravalent inactivated oil emulsion vaccine.
N.B. Absorbance value of –ve control = 0.166 Absorbance value of +ve control = 0.336

Table (4): The average mean titre of neutralizing antibodies against IBV in vaccinated chickens.

Groups	Titre of neutralizing antibodies ***							
	Weeks Post Vaccination							
	1W	2W	3W	4W	5W	6W	7W	8W
Group (2)	16	64	128	128	128	256	256	256
Group (5)	16	64	128	265	128*	128	128	256
					256**	256	256	256
Group (6)	0	0	0	0	0	0	0	0

Group (2): Chicken group vaccinated with monovalent locally prepared IBV inactivated oil emulsion vaccine.

Group (5): Chicken group vaccinated with tetravalent NDV-IBV-EDS and P.M inactivated oil emulsion vaccine.

Group (6): Unvaccinated chicken group. * Chicken without booster dose.

** Chicken received booster dose of tetravalent inactivated oil emulsion vaccine.

*** The reciprocal of the highest dilution neutralizing the virus.

Table (5): Geometric mean ELISA antibody titre against IBV in groups of vaccinated chickens.

Groups	Weeks Post Vaccination							
	1W	2W	3W	4W	5W	6W	7W	8W
Group (2)	1260	2429	2614	3182	3465	2628	3596	4225
Group (5)	1067	1745	2210	2033	6448*	5912	5725	3420
					3940**	3910	3732	3222
Group (6)	0.089	0.074	0.064	0.089	0.071	0.081	0.074	0.084

Group (2): Chicken group vaccinated with monovalent locally prepared IBV inactivated oil emulsion vaccine.

Group (5): Chicken group vaccinated with tetravalent NDV-IBV-EDS and P.M inactivated oil emulsion vaccine.

Group (6): Unvaccinated chicken group.

* Chicken received booster dose of tetravalent inactivated oil emulsion vaccine 0.5ml IM.

** Chicken without booster dose. N.B. Absorbance value of –ve control = 0.74 – 0.089

Absorbance value of +ve control = 0.411 – 0.409

Table (6): EDS HI antibody titre in groups of vaccinated chickens.

Groups	Weeks Post Vaccination							
	1W	2W	3W	4W	5W	6W	7W	8W
Group (3)	2	8	64	128	128	128	128	128
Group (5)	2	16	16	64	64*	64	128	128
					128**	128	256	128
Group (6)	0	0	0	0	0	0	0	0

Group (3): Chicken group vaccinated with monovalent locally prepared EDS inactivated oil emulsion vaccine.

Group (5): Chicken group vaccinated with tetravalent NDV-IBV-EDS and P.M inactivated oil emulsion vaccine.

Group (6): Unvaccinated chicken group. * Chicken without booster dose.

** Chicken received booster dose of tetravalent inactivated oil emulsion vaccine.

Table (7): Mean value of EDS neutralizing antibody titre in chicken groups vaccinated with different types of inactivated oil emulsion vaccine.

Groups	Titre of neutralizing antibodies ***							
	Weeks Post Vaccination							
	1W	2W	3W	4W	5W	6W	7W	8W
Group (3)	3	6.5	3.2	64	64	128	128	128
Group (5)	2	6	16	32	64*	128	256	128
					64**	128	256	256
Group (6)	0	0	0	0	0	0	0	0

Group (3): Chicken group vaccinated with monovalent locally prepared EDS inactivated oil emulsion vaccine.

Group (5): Chicken group vaccinated with tetravalent NDV-IBV-EDS and P.M inactivated oil emulsion vaccine.

Group (6): Unvaccinated chicken group.

* Chicken without booster dose.

** Chicken received booster dose of tetravalent inactivated oil emulsion vaccine.

*** The reciprocal of the highest dilution neutralizing the virus.

Table (8): Mean P. multocida antibody titres determined by indirect haemagglutination test (IHA) in chickens vaccinated at 3 weeks of age with inactivated PM alone or in combination with (NDV+IBV+EDS) vaccines in oil emulsion.

Group	Pre-vaccination	Mean antibody titre							
		Weeks Post Vaccination at shown ages							
		1	2	3	4*	5*	6	7	8
Group (4)	-ve	160	320	140	180	190	320	398	394
Group (5)	-ve	320	394	394	522	640	686	1040	1114
Group (6)	-ve	6	5	8	5	6	9	6	5

Group (4): Chicken group vaccinated with monovalent locally prepared P. multocida inactivated oil emulsion vaccine.

Group (5): Chicken group vaccinated with tetravalent NDV-IBV-EDS and P.M inactivated oil emulsion vaccine.

Group (6): Unvaccinated chicken group.

* Group (5): Chicken received booster dose of tetravalent inactivated oil emulsion vaccine after 4 weeks.

Table (9): Results of challenge test against fowl cholera.

Group	No. of birds	Dead/Living	Protection %
Group (4)	20	2/18	90 %
Group (5)	20	1/19	95 %
Group (6)	20	20/0	0 %

Group (4): Chicken group vaccinated with monovalent locally prepared P. multocida inactivated oil emulsion vaccine.

Group (5): Chicken group vaccinated with tetravalent NDV-IBV-EDS and P.M inactivated oil emulsion vaccine.

Group (6): Unvaccinated chicken group.

الملخص العربي

محاولات مبدئية لتحضير لقاح رباعى مثبط زيتى ضد أمراض النيوكاسل والالتهاب الشعبى المعدى وتدنى البيض فى الدجاج وكوليرا الطيور

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تم تحضير لقاح رباعى ميت زيتى ضد كلاً من مرض النيوكاسل والألتهاب الشعبى والفيروس المسبب لظاهرة انخفاض البيض وكوليرا الطيور. تم تجربة اللقاح من ناحية الاستجابة المناعية الخلطية وذلك بأخذ عينات دم عشوائية بانتظام. تم إجراء اختبارات التعادل المصلى، التلازن الدموى غير المباشر والمباشر والاليزا. وأثبتت التجارب السابقة كفاءة اللقاح سيرولوجياً. كما أوضحت النتائج أن خلط ميكروب الباستيريلا وفيروسات النيوكاسل والالتهاب الشعبى وتدنى البيض فى لقاح واحد لم يؤثر على الاستجابة المناعية لهذه الأمراض وبناءا عليه يوصى باستخدام اللقاح المركب بأمان وكفاءة عالية.