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Preparation of a trivalent oil-emulsion vaccine of inactivated Newcastle disease virus, infectious bronchitis virus and Haemophilus paragallinarum

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Monvalent inactivated oil-emulsion, Newcastle disease virus (NDV), infectious bronchitis virus (IBV) and *Haemophilus paragallinarum* vaccines as well as a trivalent one (NDV + IBV + HP) were prepared. The cellular and humoral immune responses of chicken to these vaccines were evaluated by using lymphocytes blastogenesis assay, haemagglutination-inhibition, serum neutralization, tube agglutination and challenge tests. The results showed that the trivalent vaccine was safe, efficient as monovalent vaccines and protected chicken against Newcastle, Infectious bronchitis and infectious coryza.

Respiratory diseases of poultry remain of major importance. Many of these diseases or infections once re-emerging or introduced into a geographic area can explode into an epidemic and may have a significant negative effect on international trade. Respiratory diseases as Newcastle (ND), infectious bronchitis (IB) and infectious coryza (IC) are continuing to cause high economic losses in many areas world-wide duo to increased mortality rates, decreased weight gain, increased medication costs and increased feed conversion rates (Hafez, 2006).

Newcastle disease virus became endemic in Egypt (Sheble and Reda, 1976) causing great economic losses duo to high mortality rates, reduction of meat and drop in egg production (Biswal and Morril, 1954). Infectious Bronchitis disease is an acute contagious disease of chicken characterized primarily by respiratory signs, and allows secondary invasion of the lungs and air sacs with bacterial infections which is mostly accompanied with significant losses duo to condemnation at slaughter. Weight gain and feed conversion efficiency may be impaired as well as problems in egg production (Cavanagh and Nagi, 2003).

Haemophilus paragallinarum is the etiological agent of infectious coryza, an upper respiratory tract infection affecting chicken, the disease characterized by sneezing, nasal discharge and facial swelling causes major losses to the poultry industry because of increased culling rates in meat chicken and a reduction (10-40 %) in egg production in laying hen in multi-age farms (Blackall and Matsumoto, 2003).

Respiratory diseases of chicken due to mixed infections by different organisms are very common , therefore, using of either bivalent, trivalent or polyvalent vaccines were preferable than that of monovalent vaccines for prevention of the common respiratory diseases as well as they have the advantages of reducing vaccination expenses and saving time and labor costs . Several authors as (Otsuki and Iritani, 1974; Winterfield 1982; Xie and Stone, 1990; Gergis *et al.,* 1994; Soliman *et al.,* 1995) prepared oil-emulsion vaccines with single or mixed antigens of *Haemophilus paragallinarum* , *Pasteurella multocida* , Newcastle disease virus (N DV) , infectious bronchitis virus (IBV) , egg drop syndrome virus (EDS) , avian influenza virus (AV), and fowl pox .

The aim of the present study is the preparation and evaluation of a trivalent vaccine against Newcastle disease virus, infectious bronchitis virus and *Haemophilus paragallinarum* to protect chicken against the three diseases simultaneously.

Material and methods

Viral and bacterial strains.

Newcastle disease virus. La Sota strain of NDV was supplied by the Central Veterinary Laboratory, Weybridge, England. Its initial titer was $10^{10.8}$ EID_{50} /ml. And used for reparation of the vaccine and HI test.

Infectious bronchitis seed virus. IB 274 strain:

it was supplied by Prof. Dr. Magdy El-Kady, Department of Poultry Disease, Fac. Vet. Med., Beni-Suef University, its initial titer was 10^7 EID_{50}/ml IB M41 strain: it was supplied by Arkansas University, USA its initial titer was 10^7. 2 EID_{50} /ml. IB H 120 strain: it was supplied from Delware, University, USA. Its titer was $10^{7.8}$ EID_{50} /ml. All these strains were used for preparation of IB vaccines.

***Haemophilus paragallinarum* strains.** Standard W (serovar A) , Modesto (serovar C) and 0222 (serovar B) strains as well as a locally isolated strain (serovar A) of *Haemophilus paragallinarum* were used in this study .

Specific pathogenic free (SPF) eggs. Embryonated chicken eggs (9-11day old) , SPF, were obtained from SPF egg production farm, Koum Oshiem , Fayoum , Egypt, and used for propagation , titration and assurance of complete inactivation of virus inactivation (NDV and IB).

Chicken. Two hundred and fifty one day old chicks were obtained from the Ministry of Agriculture and kept under strict hygienic measures in isolated and disinfectant cages.

Viruses propagation, titration and inactivation. NDV was propagated in 10 day old SPF egg according to (Allan *et al.,* 1973) and titrated according to the method described in (FAO Publication 1978). Its titer was $10^{11.2}$ EID_{50} /ml. Each of the three IB viruses strains (D274 , M41 , and H 120) was propagated separately in 10 day old SPF embryonated eggs according to the method of Cunningham 1973 and the titers of the obtained virus strains were $10^{7.5}$, $10^{7.5}$ and 10^8 EID_{50} / ml respectively. ND and IB virus strains were inactivated using formalin (0.1%) and tested for complete inactivation by two successive blind passage to each virus (N D and IB) in SPF eggs .

***Haemophilus paragallinarum* bacterin preparation.** It was prepared according to the method applied by (Blackall *et al.,*1992).

Vaccines preparation . The monovalent oil-emulsion vaccines against NDV, IB and *Haemophilus paragallinarum* as well as the trivalent vaccine (equal volumes of NDV +IBV+ *H. paragallinarum*)were prepared according to (Stone *et al.,* 1978; Thayer *et al.,* 1983) with 1/3 aqueous to oil ratio

Quality control of the prepared vaccines.

Sterility tests. The prepared vaccines were tested according to (Code of federal regulation 1987) for the freedom of any contaminant.

Safety tests. Ten chicks (4 weeks old) were inoculated subcutaneously (S/C) with double field dose / bird of each monovalent vaccine N D V ,IBV and infectious coryza (IC) as well as trivalent vaccine (NDV + IBV +IC) , The chicken were observed for 15 days post vaccination .

Experimental Design. Two hundreds and ten (4 weeks old) chicken were divided into and vaccinated through intramuscularly injection (I/M) in breast muscles with dose of 0.5 ml / bird from each vaccine as showed in (Table 1).

Three weeks post vaccination 30 chicken of both of groups 1 and 4 were challenged by inoculation of inferaorbital sinus with 0.2 ml (approximately 108 C.F.U.) / bird of16-18 hours broth culture of W,Modesto and 0222 strains of Haemophilus paragallinarum.All chicken were examined daily for clinical signs of Infectious Coryza according to kume *et al.,* (1980); Blackall *et al.,* (1992).

Evaluation of the prepared vaccines, For evaluation of the prepared vaccines the following parameters were carried out:

Cellular immune response. It is measured by using lymphocyte blastogenesis assay where random blood samples were collected at 1, 2, 3, and 4 weeks post-vaccination from vaccinated chicken and subjected for lymphocyte transformation test according to Lucy (1974); Mosmann (1983).

Humoral immune response. Serum samples were collected weekly from vaccinated chicken and used for

Haemagglutination-inhibition test. The test was performed for detection of HI antibodies gainst NDV and Haemophilus paragallinarum according to Majujabe and hitchner (1977); Yamaguchi *et al.,* (1989), respectively.

Tube agglutination test. The test was done for detection of antibodies against Haemophilus paragallinarum according to Irtani *et al.,* (1977).

Serum Neutralization Test (SNT). Used for detection of neutralizing antibodies against IBV according to Cunningham (1973) the antibody titer was calculated as the reciprocal of serum dilution which neutralizes 100-200 ICID50 of IBV.

Table (1) Vaccinated chicken groups

Group No.	No. chicken	Used vaccine
1	60	Trivalent (NDV + IBV +IC) oil-emulsion vaccine
2	30	Monovalent NDV oil-emulsion vaccine
3	30	Monovalent IBV oil-emulsion vaccine
4	60	Monovalent IC oil-emulsion vaccine
5 Control (un-vaccinated)	30	None

Table (2) Cellular immune response of vaccinated chicken with monovalent and trivalent (NDV + IBV + IC) vaccines as measured by lymphocyte transformation test.

Chicken groups	weeks post-vaccination			
	1	2	3	4
1	0.090*	0.101	0.065	0.062
2	0.038	0.090	0.046	0.039
3	0.042	0.100	0.047	0.033
4	0.065	0.098	0.063	0.049
5	0.010	0.053	0.008	0.003

Group (1): Chicken vaccinated with trivalent oil-emulsion (NDV + IBV + IC) vaccine.
Group (2): Chicken vaccinated with monovalent oil-emulsion Newcastle disease virus vaccine.
Group (3): Chicken vaccinated with monovalent oil-emulsion Infectious Bronchitis Disease virus vaccine
Group (4): Chicken vaccinated with monovalent oil-emultion Infectious Coryza disease Vaccine.
Group (5): Non-vaccinated control group.
*: Delta Optical Density (DOD).

Results and Discussion

Prevention and control of respiratory diseases such as NDV, IBV, and IC are still as subject of interest and usually attract the researcher attention to vaccination is still the major control programs which induce specific immunity Fegan *et al.,* (1995) .

Cell- mediated immune response was carried out using lymphocytes blastogenesis and expressed as Delta Optical Denisty (DOD) as shown in (Table 2) and its results indicated that the best highest level of cellular immune response was recorded at two weeks post – vaccination in all vaccinated grougs then decreased gradually till the end of the test (4 weeks postvaccination) .

These findings agree with Abou-Elkhair and Eman (2004) , in addition the trivalent oil emulsion vaccine induced higher level of cellular immune response the monovalent vaccines (NDV , IB and IC) as recorded by Reynolds and Maraqa (2000) .

Dealing with the humoral immune response against NDV , results represented in (Table 3) revealed that no noticable difference was obtained between the overall mean of heamagglutinating antibody (HI) titer either in groups of chicken vaccinated with trivalent vaccine (ND + IB + IC) or in groups vaccinated with monovalent ND vaccine , these findings agree with that reported by (El-Bayoumy and Abd-El Wanis 2004)

Regarding the humoral immune response against IB which was evaluated by using seurm neutralization test as shown in (Table 4) , it was clear that both monovalent IB and trivalent vaccine (ND + IB + IC) can produce protective levels of neutralizing antibodies against IB disease as mentioned by Outsoki and Iritani (1974) .

Results illustrated in (Tables 6,7) indicated that tube agglutination and HI antibody titers in chicken sera against *Heamophilus paragallinarum* (serovars A, C, and B) either in monovalent or trivalent (ND + IB + IC) oil-emultion vaccines reached their peak at 10-12 weeks and at 8-10 weeks post-vaccination respectively and these findings agreed with that reported by Kamal (1996); Awad *et al.,* (2004) .

Table (3): Mean value of NDV Heamagglutinating titer in chicken vaccinated with monovalent oil-emulsion NDV and Trivalent oil-emultion (NDV + IBV + IC) vaccine .

Groups	*HI antibody titer*									Overall
	1**	2	3	4	5	6	8	10	12	mean
1	6*	7	10.5	10.5	10.6	10.5	9.3	8.3	8.3	81
2	6	8	10.8	10.3	10.3	11	9.3	9.3	8.3	83.3
5	0	0	0	0	0	0	0	0	0	0

*Mean Log_2 HI antibody titer **: Weeks post-vaccination

Table (4): Mean value of IB serum neutralizing antibody titer in chicken vaccinated with monovalent oil-emulsion IBV and Trivalent oil-emultion (NDV + IBV + IC) vaccine.

Groups	*IB Neutralizing antibody titer*									Overall
	1**	2	3	4	5	6	8	10	12	mean
1	2*	8	16	21.3	64	64	7406	42.6	21.3	313.8
2	2	8	21.3	26.6	106.6	128	53.3	26.6	26.6	339
5	0	0	0	0	0	0	0	0	0	0

*Mean serum neutralizing antibody titer: mean of the reciprocal of serum dilution which neutralized and inhibited the CPE of 100-200 $TCID_{50}$ of the IB virus.
**: Weeks post-vaccination.

Table (5): Results of challenge test of chicken vaccinated with monovalent oil-emulsion Infectious Coryza and Trivalent oil-emulsion (NDV + IBV + IC) Vaccines

Chicken group	Vaccine type	Strain used in challenge	No. of chichen	No. of protected chicken	No. of chicken have clinical signs	Protection rates %
4	Monovalent Infectious Coryza	W	10	7	3	70
		Modesto	10	6	4	60
		0222	10	6	4	60
1	Trivalent (NDV +IBV + IC)	W	10	7	3	70
		Modesto	10	7	3	70
		0222	10	6	4	60
5	Control Un-vaccinated	W	10	0	10	0
		Modesto	10	0	10	0
		0222	10	0	10	0

Table (6): Geometric mean of tube agglutinating antibody titers of chicken sers vaccinated with monovalent oil-emulsion Infectious Coryza and Trivalent oil-emulsion (NDV + IBV + IC) vaccines.

chicken group	No. of chickens sera	Vaccine type	Antigen Used	Geometric mean of tube agglutinating antibody titer						
				Pre-v.	2*	4	6	8	10	12
4	10	Monovalent IC Vaccine	W	0	22.62	25.99	27.85	34.29	36.75	39.39
			Modesto	0	22.62	24.25.	25.99	32.00	34.29	34.29
			0222	0	19.69	21.11	22.62	29.85	34.29	32.00
1	10	Trivalent (ND+IB+IC) Vaccine	W	0	21.11	24.25	27.85	32.00	34.29	36.75
			Modesto	0	22.62	22.62	29.85	32.00	34.29	32.00
			0222	0	21.11s	22.62	24.25	29.85	32.00	34.29

*Weeks post – vaccination.

Table (7): **Geometric mean of Heamagglutinating Inhibition antibody titers of chicken sers vaccinated with monovalent oil-emulsion Infectious Coryza and Trivalent oil-emulsion(NDV+IBV+IC)vaccines**

chicken group	No. of chickens sera	Vaccine type	Antigen Used	Geometric mean of HI antibody titer						
				Pre-v.	2*	4	6	8	10	12
4	10	Monovalent IC Vaccine	W Modesto 0222	0	16.00	17.14	21.11	25.99	27.85	24.25
				0	14.92	16.00	18.37	22.62	24.25	21.11
				0	14.92	14.92	16.00	21.11	22.62	19.69
1	10	Trivalent (ND+IB+IC) Vaccine	W Modesto 0222	0	17.14	17.14	22.62	27.85	27.85	25.99
				0	16.00	17.14	21.11	24.25	25.99	22.62
				0	13.92	14.92	17.14	21.11	22.62	21.11

*Weeks post-vaccination

In addition, the results represented in (Tables 6,7) showed that there was no difference of antibody titer against *Haemophilus paragallinarum* between monovalent and trivalent vaccines.

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تحضير لقاح ثلاثى مثبط زيتى من النيوكاسل والالتهاب الشعبى المعدى والكوريزا

تم تحضير ثلاثة لقاحات أحادية مثبطة زيتية من كل من فيروس النيوكاسل وعترات فيروس الالتهاب الشعبى المعدى وعترات ميكروب الهيموفيليس باراجالينارام وكذلك تم تحضير لقاح ثلاثى مثبط زيتى منهم جميعا. تم مقارنة الاستجابة المناعية الخلطية والخلوية للقاح الثلاثى بالنسبة لكل لقاح أحادى على حده فى مجموعات مختلفة من الكتاكيت. وقد أوضحت نتائج الاختبارات السيرولوجية واختبار التحدى كفاءة اللقاح الثلاثى فى حماية الكتاكيت ضد أمراض النيوكاسل والالتهاب الشعبى المعدى وزكام الطيور المعدى (الكوريزا) مثل اللقاحات الاحادية فهو لقاح أمن وفعا ل .