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Trial for preparation and evaluation of combined vaccine against ND, IB and M. gallisepticum diseases in chickens

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In this study, a combined Trivalent vaccine against ND, IB and *M. gallisepticum* was locally prepared and evaluated in comparison with other locally prepared Bivalent ND and IB and monovalent *M. gallisepticum* vaccines. The obtained results were promising for this locally prepared Trivalent vaccine and the immune response was outstanding starting at the 2nd week post vaccination and showed extended raising allover the experiment period. The immune response of chickens vaccinated with the Trivalent was shoot up post boostering at the 8th week post 1st vaccination. These results were confirmed and supported by the challenge tests using the virulent strains of the three pathogens. So it could be recommend that the production of this Trivalent ND, IB and *M. gallisepticum* will help in the control of the three diseases and their complications.

There are many common and important respiratory diseases that can affect the respiratory system of poultry. Newcastle disease (ND) and infections bronchitis (IB) are the most common viral respiratory diseases of chickens (Decich, 1998). Newcastle disease is one of the highly contagions and lethal disease affect all birds of all ages and so cause great economic losses meanwhile the Infections bronchitis is a contagions disease and the severity of its infections is influenced by the age and immune status of the flock, environmental conditions and the presence of other diseases (Shankar, 2009). At the same time Mycoplasmosis is one of the most important poultry diseases and causes significant economic losses either directly or indirectly caused by *Mycoplasma gallisepticum* (MG) infection with or without complicating factors (Faruque and Christensen, 2007). As poultry industry developed, Almost the chickens are grown in crowded manner are with low air condition houses. In such situations many of the flocks which infected *by M. gallisepticum* become predisposed or their disease condition have been aggravated and the chronic respiratory disease (CRD) complex occurs (Fotina- Tatiana, 2004).

Combined vaccines have the advantage of providing protection against more than one disease causing the same symptoms, reducing vaccination cost and number of infections per farm as well as saving time and reducing the stress reactions. So the objective of this study was to develop a locally prepared combined inactivated vaccine comprising both NDV and IBV in addition to *M. gallisepticum* strains, to overcome and solve the field problems caused by theses pathogens.

Materials and methods

Infections bronchitis virus strains. H120 strain was obtained as allantoic fluid from department of animal science and agricultural biochemistry, university of Delwar, New York USA and used for vaccine preparation. Classical IBV strain M41 was supplied through the University of Arkansas USA. It was used after propagation and titration in embryonated chicken eggs and was used for vaccine preparation and evaluation.

Newcastle disease virus strains. La Sota strain was obtained from Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo, and used for vaccine preparation. Velogenic Viscerotropic Newcastle disease virus (VVNDV) was obtained from the Vet. Sera and vaccine research institute; Abbasia Cairo: containing 10^6 EID_{50} used as challenge virus.

***M. gallisepticum* strain.** *M. gallisepticum* R. strain was obtained from the Central Lab. For Evaluation of Vet. Biologics, Abbasia, Cairo; It was used for the preparation of the monovalent *M. gallisepticum* and Trivalent ND, IB and MG vaccines, and as a challenge strain.

Embryonated chicken eggs (ECE). 9-11 day old embryonated chicken SPF eggs were

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purchased from the SPF Eggs Farm, Koum Osheim, Fayoum, Egypt. These eggs were used for propagation, preparation, titration and testing complete inactivation of the prepared batches of both bivalent ND and IB vaccine and Trivalent ND, IB and MG vaccines.

Vaccine preparation.

Culturing, inactivation and vaccine preparation of *M. gallisepticum*. The seed culture was grown in Frey's media at 37°C for 36-48 hours, then harvested by centrifugation at 13000 xg then resuspended in 0.01 M PBS (PH 7.2) to give a final concentration of $1x10^{10}$ CCU/ml just prior to inactivation with B-propiolacton using the methods described by Yoder and Hopkins, (1985).

ND and IB bivalent vaccine. It was vobtained from viral avian vaccines production and research Dept., Abbasia, Cairo.

Trivalent ND, IB and MG Vaccine preparation. The combined vaccine was prepared by mixing 50 ml of aqueous phase of each Microbial strains emulsified into oil adjuvant.(The adjuvant for preparation of water in oil emulsion consists of paraffin oil, Span 80, All components were thoroughly mixed then sterilized by autoclaving at 121°C for 10 minutes) as described by Thayer *et al.*, (1983). The vaccine component are then stirred and emulsified for 10 minutes. The prepared vaccine dispensed into bottles stored at 4°C till used as described by Stone *et al.*, (1978).

Quality Control of the Prepared Vaccines. The locally prepared vaccines were subjected to quality control measures according to OIE, (2009).

Sterility Tests. The prepared vaccines were tested for aerobic and anaerobic bacteria, mould and yeast, Enerobacteriacae and Mycoplasma contaminations by culturing the vaccine samples on specific media for each of the abovementioned bacterial species.

Safety Test. Four to six old SPF chicks were inoculated with the double field dose of the prepared vaccines (the prepared field dose is 0.5 ml) . The chicks were kept under observation for any signs or local reactions two weeks post inoculation.

Potency test.

Vaccination. Three hundred thirty one day old chicks were reared under complete hygienic condition till 21 days old. Random blood samples were taken and sera were tested against NDV, IBV and *M. gallisepticum*. Chickens were susceptible to experiment as they were found to be seronegative for the three organisms. The chickens were divided into four groups as follow: Group (1). Consisted of 80 chickens that were vaccinated IM with 0.5 ml of the locally prepared trivalent inactivated vaccine (ND, IB and MG). Of which 30 chicks received booster dose 8 weeks later. Group (2). Consisted of 60 chickens those were vaccinated with IM with 0.5 ml of the locally prepared bivalent inactivated vaccine (ND and IB). Of which 30 chicks received booster dose 8 weeks after the first dose. Group (3). Consisted of 50 chickens and were vaccinated with the locally prepared monovalent MG inactivated vaccine. Of which 30 chicks received booster dose 8 weeks later with the same vaccine using I/M route and dose. Group (4). Consisted of 140 chickens and non vaccinated control group. Random blood samples were collected weekly from each group for 8 weeks after first vaccination and sera were collected and tested for detection of corresponding antibodies against ND, IB and MG. Other blood samples were collected for 8 weeks post- boostering at 2 weeks intervals, sera were collected and tested for evaluation of the immune response.

Challenge test.

Against ND and IB. Ten chickens from groups (1), (2) and (4) were challenged four weeks post first vaccination using 0.5 ml of velogenic viscerotropic Newcastle disease virus (VVNDV) 10^6 EID_{50} intra muscularly (I/M). Chickens were Kept under observation for 15days post challenge. Another ten chickens from the same groups were challenged 4 weeks post boostering. Dead chickens and those showing symptoms throughout the period of observation were subjected to post mortem examination and evaluated as described by Allan *et al.*, (1973). The same procedure was done using standard virulent IBV strain M41with at least $10^{3.5}$ EID50 intranasalyin vaccinated and control chickens and the results were evaluated according to Gough *et al.*, (1981); Zanella *et al.*, (2000).

Against MG. Ten chickens from both group(1), group (3) and group (4) were challenged 4weeks post 1st vaccination using 0.1ml of an overnight virulent R. strain of *M. gallisepticum* containing (3.8×10^6 CFU/ml) according to Longhnane *et al.*, (1993). Second challenge was done 4 weeks post boostering in another 10 chickens from the same groups. Chickens were kept under observation and examined weekly for 4 weeks post challenge.

Results and Discussions

Newcastle disease (ND) and infections bronchitis disease (IB) are among the most important respiratory diseases affecting poultry and cause great economic losses due to high rate of mortality in ND infections and high morbidity rate in IB infections as well as drop in egg production (Field's, 1996). Therfore the aim study was to produce a combined vaccine against both viruses for saving time, effort and money and improving poultry production.

Serological examination of serum samples collected from chickens vaccinated by the locally prepared inactivated oil emulsion vaccines under test was done using haemagglutination inhibition test (HI), serum neutralization test (SNT) and ELISA test. To confirm successful application of the prepared vaccine and development of immune response, the vaccinated birds were exposed to challenge test.

Results of HI titers of sera collected from random samples of chickens vaccinated with the locally prepared vaccine as shown in Table (1), indicated that the highest HI titer of group (1) was 2^8 and recorded at the 5th week post vaccination and gradually decrease till 2^6 at the 14 weeks post vaccination, while in the subgroup (1-B) received boostering at 8th week post vaccination recorded an increasing in HI titer up to 2^{10} two weeks post boostering and persist till 16 weeks post vaccination. On the other hand group (2) recorded 2^7 at the 5th week and gradually decreased till reached 2^5 at 14th week post vaccination, meanwhile subgroup (2–B) which received boostering at the 8TH week post first vaccination showed an increasing in HI titer up to 2^9 two weeks post boostering .These results agree with that obtained by Rofail, (2001); Nadia *et al.*, (1990) who reported that even $\log_2$ HI titer of 3.9 and 3.0 can protect chickens against challenge virus.

As regards to the immune response against IB, Results in Table (2) showed that the SNT reached its peak at the 8th week post vaccination with a mean value of 128 for group (1-A) and reached its maximum level at 1024 at the 10th week post vaccination while the subgroup (1-B), which received booster dose showed increasing value (1024) starting earlier from the 2nd week post boostering. Regarding group (2-A), which received one vaccinal dose, the antibody titer reached 256, ten weeks post vaccination, while the subgroup (2-B), which received boostering reached 512 two weeks post boostering. The previously mentioned findings coincide with those of Chedid, (1985); Cardona *et al.*, (1987).

The results of the challenge test for vaccinated and non vaccinated groups post inoculation with the virulent ND or IB viruses as represented in Table (3) showed that the control non vaccinated group showed no protection 4 weeks post vaccination while groups (1) and (2) recorded 90% protection 4 weeks post first vaccination and 100% protection 4 weeks post boostering on challenge with virulent ND virus. Regarding the challenge with virulent IB virus, the same results were obtained in the control group while group (1) gave 100% protection on both challenges. In the same time group (2) gave 90% protection 4 weeks post first vaccination and 100% protection 4 weeks post boostering. The same results were obtained by Ignjatovic and Gall, (1995) when used log 10^4 EID50 of IBV strain M41.

Concerning the antibody titer against *M. gallisepticum* after vaccination, it was noticed that the positive titer start as early as the second week post vaccination on both groups vaccinated with monovalent *M. gallisepticum* and Trivalent ND, IB and MG vaccines giving a mean titer of 670 and 668, respectively (Table 4). These titers were increased gradually in both groups and reaching the Plato at the 7th week post first vaccination.

ELISA Antibody titers were shouted up at the second week post boostering and reach its maximum level at the 8th week post- boostering giving a antibody mean titers of 831 and 823 in chicken groups vaccinated with monovalent or trivalent vaccines, respectively. All these results were compared with the negative titers obtained from the non vaccinated chicken group allover the experiments. The same results were obtained by Aboul-Saoud *et al.*, (2007) where the protective antibody titer appeared as early as the first week post vaccination with *M. gallisepticum* inactivated vaccine in 37.5% of tested samples and reached its maximum level at the sixth week post vaccination. Also Lin and Kleven, (1984) suggested that the obtained protection level of the *M. gallisepticum* vaccine depends on the virulence of the strain used in the preparation of vaccine so we used the virulent *M. gallisepticum* R. strain in preparing of the vaccine in this study. The ELISA antibody titers obtained post vaccinations were correlated with such results obtained after challenge with the virulent *M.*

Table (1): Immune response of chickens against ND as measured by mean log_2 HI titer.

Chicken groups		Weeks Post Vaccination										
		2	3	4	5	6	7	8	10	12	14	16
1	A	3	6	6	8	8	8	7	7	7	6	5
	B								8	10	10	10
2	A	2	3	5	7	7	7	7	6	6	5	5
	B								8	9	10	9
4		0	0	0	0	0	0	0	0	0	0	0

(A) Birds received single dose of the corresponding vaccine.
(B) Birds received a booster dose 8 weeks post first vaccination with the corresponding vaccine.

Table (2): Humeral Immune response of chickens against IB as measured by mean serum reutilization test (SNT) (Antibody titer = the reciprocal of serum dilution which neutralized and inhibited the CPE of 100- 200 TCID 50 of the IBV).

Chicken Groups		Weeks post vaccination									
		2	3	4	5	6	8	10	12	14	16
1	A	8	8	16	64	64	128	1024	512	512	128
	B							1024	1024	1024	512
2	A	4	4	8	32	64	256	256	256	128	128
	B							512	512	512	256
4		0	0	0	0	0	0	0	0	0	0

(A) Birds received single dose of the corresponding vaccine.
(B) Birds received a booster dose 8 weeks post first vaccination with the corresponding vaccine.

Table (3): Protection efficiency against challenge with VVNDV and virulent IBV (M41) in vaccinated and non vaccinated chickens 4 weeks after first vaccination and 4 weeks post boostering.

Challenge	Chicken group	ND			IB		
		No of challenged birds	No of survival birds	Protection %	No of challenged birds	No of survival birds	Protection %
4 weeks post first vaccination	(1)	10	9	90%	10	10	100%
	(2)	10	9	90%	10	9	90%
	(4)	10	0	0%	10	0	0%
4 weeks post boostering	(1)	10	10	100%	10	10	100%
	(2)	10	10	100%	10	10	100%
	(4)	10	0	0%	10	0	0%

Table (4): ELISA mean titer of serum samples obtained from vaccinated chickens groups with monovalent MG. and Trivalent ND, IB and MG vaccines.

Groups	Weeks post										
	1st vaccination							Boostering			
	2	3	4	5	6	7	8	2	4	6	8
1	668	672	681	718	721	720	714	736	762	801	823
3	670	679	690	717	726	726	718	734	768	810	831
4	195	181	145	157	161	149	119	182	191	165	148

Table (5): Air sac lesion scores in chickens vaccinated with combined and single MG vaccine and challenged with virulent MG - R strain.

Chicken Groups	Air sac lesion scores / weeks post							
	1st dose challenge				Boostering challenge			
	1	2	3	4	1	2	3	4
1	0.34	0.76	0.95	1.03	0.26	0.54	0.83	0.98
3	0.33	0.75	0.96	0.97	0.24	0.55	0.81	1.00
4	1.16	2.1	2.99	2.27	1.14	2.01	2.89	3.31

gallisepticum R- strain as shown in Table (5). It is clear that air saculitis depends on the immune status of the birds where the grads of air saculitis were significantly lowered with the challenge post boostering than after first dose of vaccination. On the other hand the non vaccinated chicken group showed high grade of air saculitis in comparison with the vaccinated groups. These results agreed with that obtained by Yoder, (1986), who noticed that the chicken groups either vaccinated with monovalent *M. gallisepticum* or Trivalent ND, IB and MG vaccines and challenged with virulent *M. gallisepticum* R- strain showed a good degree of protection. Also Sundquist *et al.*, (1996) reported that the inactivated immunostimulating complex vaccine of *M. gallisepticum* induced protective immunity and significantly reduced lesions in air sacs after challenge with virulent *M. gallisepticum* strains.

It could be concluded that the locally prepared Trivalent ND, IB & MG vaccine is of great value because it gave acceptable significant levels of protection in vaccinated chickens against NDV, IBV or *M. gallisepticum* infections in comparison with the non vaccinated chickens. Depending on this study, it could be recommended that, the production of this vaccine will be useful to avoid ND, IB and MG infections and their complications in the poultry field.

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محاولة لتحضير و تقييم لقاح مركب ضد أمراض النيوكاسل , الإلتهاب الشعبى المعدى والميكوبلازما فى الدجاج

فى هذه الدراسة تم انتاج وتقييم لقاح ثلاثى مركب للوقاية من أعراض النيوكاسل ، الإلتهاب الشعبى المعدى و الميكوبلازما جاليسيبتيكم فى الدجاج وذلك بالمقارنة بلقاحين محضرين محلياً أحدهما ثنائى للنيوكاسل والإلتهاب الشعبى والآخر احادى للميكوبلازما جاليسيبتيكم ، وكانت النتائج المتحصل عليها واعدة بالنسبة لهذا اللقاح الثلاثى وكانت الاستجابة المناعية له فى الطيور المحصنة به هائلة بداية من الاسبوع الثانى بعد التحصين واستمرت فى الزيادة طوال فترة التجربة والتى استمرت حتى ستة عشر اسبوع. كذلك كانت الاستجابة المناعية للقاح الثلاثى المحضر محلياً زادت بشكل قوى بعد التحصين بجرعة ثانية اضافية فى الاسبوع الثامن بعد اتحصين بالجرعة الأولى ، وتم تأكيد هذه النتائج باجراء اختبار التحدى باستخدام عترات ضارية للمسببات المرضية للأمراض الثلاثة. لذلك من خلال هذه الدراسة يمكننا التوصية بانتاج هذا اللقاح الثلاثى ضد أمراض النيوكاسل والالتهاب الشعبى المعدى والميكوبلازما جاليسيبتيكم للمساهمة فى السيطرة على هذه الأمراض الثلاثة ومضاعفاتها فى حقل صناعة الدواجن .