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Preparation And Evaluation Of Combined Polyvalent Inactivated Vaccine For The Protection Against Chronic Respiratory Disease In Chickens

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ABSTRACT

Chronic respiratory disease (CRD) is one of the major problems within the poultry farms. In this study, a polyvalent inactivated M. gallisepticum and Escherichia coli vaccine was prepared then evaluated using both serological and vaccination challenge tests. Results of this vaccine was promising, where the peak of humoral immune response against M. gallisepticum as measured by ELISA were 2418 and 5600 when taken in either single or booster doses respectively while were 2240 and 3576 for E. coli. As regards to the protection against challenge with virulent bacterial strains, the achieved protection percentages against M. gallisepticum virulent strain were 62 and 72 post single and booster doses while were 66.67 and 77.47 for E. coli. The achieved results could help to be concluded that the locally prepared polyvalent M. gallisepticum and E. coli vaccine may help and minimize the problem of the chronic respiratory diseases in poultry farms.

INTRODUCTION

Mycoplasma gallisepticum and Escherichia coli are the most important disease problems commonly encountered in poultry in Egypt, especially when complicated. M. gallisaepticum is the aetiological agent of chronic respiratory disease of chickens and infectious sinusitis of turkeys in Egypt *(1)*. *E. coli* infection is mainly affecting chicken, resulting in significant economic losses through mortality, morbidity, cost of treatment and condemnation at processing plant *(2)*. Nevertheless control of *E. coli* infection was thought to be possible through the use of the antibiotic drugs but transferable resistance to these products and their prohibitive costs have shown this type of control to be deficient *(3)*. Vaccination has therefore proposed as a rational alternative method of control *(4)*. The monovalent *E. coli* vaccine did not offer protection against other *E. coli* serotypes associated with colibacillosis. Therefore, an effective vaccine against colibacillosis should contain the serotypes of *E. coli* commonly associated with this infection *(5)*. At the same time, the use of combined vaccine have the advantage of providing protection against more than one disease, and reduced vaccination expenses and the number of vaccination per farm as well as saving time and labor costs beside reducing the stress reactions.

Therefore this study aimed to prepare and evaluate a polyvalent vaccine give protection against M. gallisepticum and the most commonly pathogenic E. coli serotypes infected chickens.

MATERIAL AND METHODS

Microbial strains

Mycoplasma gallisepticum: *M. gallisepticum* S_6 strain which was used in preparation of the targeted polyvalent *M. gallisepticum* and *E. coli* vaccine while *M. gallisepticum* R strain was used in the challenge test for the evaluation protocols for the under testing locally prepared vaccines.

E. coli: *Escherichia coli* O1, O_2, O_{78} were used in the preparation of the vaccine and also in the challenge test.

Laboratory animals

Embryonated chicken egg: Twenty five embryonated specific pathogen free (SPF) chicken eggs, (6 days old) were used in the propagation of *M. gallisepticum* organisms via yolk sac inoculation to increase their virulence for preparation of vaccine and challenge culture seeds.

Chickens: A total of 510 one day old H and N breed chicks were obtained from private farm and reared under complete hygienic measures in special isolators. These birds were examined to ensure that, they are free from bacterial pathogens and they had neither a history of mycoplasma nor *E. coli* infections.

At least other 20 chickens from the same source, 2-3 weeks old were used in safety test of prepared vaccine

Vaccine preparation

Culturing and inactivation of *M. gallisepticum*: The selected seed culture of *M. gallisepticum* (S6 strain) was inoculated into Frey´s medium, incubated under the optimum conditions, then harvested and the antigen concentration was adjusted to 3x 10^{10} ***(6)*** then inactivated with 0.5% formalin with frequent agitation during three hours incubation at room temperature.

Culturing and inactivation of *E. coli*: *E. coli* serotype O1, O2 and O78 were grown separately onto brain heart agar and incubated at 37°C for 48 hr. The colonies were collected in normal saline then mixed and bacterial suspension was adjusted to contain $3.8x10^9$ cells /ml. The bacteria were then inactivated by adding 0.5% formalin with agitation ***(7)***.

Preparation of polyvalent *M. gallisepticum* and *E. coli* vaccine: Equal parts (V/V) of the inactivated antigens of *M. gallisepticum* (S6 strain) as well as *E. coli* strains (serotype O1, O2, O78) were mixed together using a magnetic stirrer. The inactivated culture suspended in the aqueous phase of water was emulsified in light mineral " extra white " oil to prepare oil adjuvant vaccine. The concentration of the aforementioned polyvalent suspension was adjusted to contain each dose of combined vaccine at least $3x10^{10}$ CFU or (5% packed cell volume) of *M. gallisepticum* (S6 strain) according to ***(6)*** and $3.8x10^9$ per each of *E. coli* serotypes (O1, O2 and O78) according to ***(7, 8)***. An oil emulsion adjuvant was added to batch of inactivated mycoplasma and *E. coli* antigens where the final prepared vaccine contained 1 % packed cell. An aqueous phase was prepared by the adding 40 ml of tween 80 to 960 ml (5%) of concentrated antigen (which was suspended in phosophate buffered saline PH 7.2). Then, an oil phase was prepared by adding 400 ml of span 80 to 3600 ml of mineral " extra white " oil. The 1000 ml aqueous phase represented 1 part and the 4000 ml oil phase represented 4 parts. The aqueous phase and oil phase portions were then mixed together for 10 to 20 minutes in a sterile satainless-metal turbo-emulisifier ***(6)***.

Addition of preservative (thiomersal). The thiomersal was added in a concentration between 0.05 and 0.1mg/liter.

Quality control on the prepared vaccine

The prepared polyvalent vaccines were subjected to quality control measures ***(9)*** as followings:

Sterility tests: The prepared vaccine were tested for the growth of aerobic bacteria, anaerobic bacteria and mould and yeast by culturing the vaccine samples on fluid thioglycolate medium with 0.5% beef extract and Bacto-Sabouraud's Maltose 4% agar Medium.

Safety test: At least 20 SPF chickens, 2 – 3 weeks age were inoculated intramuscularly, with 1.0 ml (double field dose) of the prepared vaccine under test then kept under observation for 14 days.

Potency test

Vaccination and experimental design: A total of 510 pre-reared chickens were divided into 3 groups as following:

Group (1): Consist of 175 chickens vaccinated with single dose of locally prepared polyvalent MG - *E. coli* vaccine. This group divided into 2 main subgroups, the first main subgroup consists of 125 chickens which divided again into 2 groups, the first consists of

50 chickens and were challenged with the virulent strain of MG (R strain) 4 weeks post-vaccination, while the second consists of 75 chickens and were challenged with the virulent strains of *E. coli* (O1, O_2 and O_{78}) 4 weeks post-vaccination. The second main subgroup consists of 50 chickens, from which blood samples were collected weekly post-vaccination up to 13 weeks post-vaccination to be used in sero-evaluation.

Group (2): Consists of 175 chickens and received a booster dose of the same vaccine. This group divided into 2 main subgroups the first subgroup consists of 125 chickens and divided again into 2 groups, the first consisted of 50 chickens which were challenged with the virulent strain of MG (R strain) 4 weeks post-vaccination while the second consisted of 75 chickens which were challenged with the virulent strains of *E. coli* (O1, O_2 and O_{78}) 4 weeks post-vaccination. The second main subgroup consists of 50 chickens, from which blood samples were collected weekly post-vaccination up to 13 weeks post-vaccination to be used in sero-evaluation.

Group (3): Consists of 160 chickens of the same breed, age and condition of rearing to be used as negative unvaccinated control for either sero-evaluation or for challenge test as described in groups (1) and (2).

Challenge test

Against *M. gallisepticum*: each bird from different vaccinated and unvaccinated control groups were challenged with 0.1ml of 3.8 x 10^6 CFU/ml of *M. gallisepticum* (R) strain via intra-air sac route ***(9).***

Against *E. coli*: Each bird from the different vaccinated and unvaccinated control groups with 0.1 ml contain 1x10^8 CFU ***(10)*** for each of serotypes O1, O2 and O78 *E. coli* via intramuscular route.

RESULTS AND DISCUSSION

The results of sterility test of the prepared vaccine indicated that the prepared inactivated polyvalent *M. gallisepticum* and *E. coli* vaccine is free from any contaminants (aerobic bacteria, anaerobic bacteria, mould and yeast). Concerning safety of the prepared vaccine, it was found that chickens vaccinated with even double field vaccine dose didn't show any abnormalities or adverse reaction. As regards to sterility and safety tests, the polyvalent prepared vaccine cover all conditions described previously ***(9).*** Also it cover the requirement reported previously ***(11)*** as the formalin residue was less than 0.05 % and the thiomerthal residue was less than 0.02 mg/ml.

Data available in table (1) showed that the antibody titer against *M. gallisepticum* starts as early as the first week post vaccination with the locally prepared polyvalent mycoplasma and *E. coli* vaccine giving a mean titer of 786 while the peak titer was 2418 and achieved at 7 weeks post vaccination then the antibody titer was decreased gradually up to 2019 at 13 weeks post vaccination in the same aforementioned group. From table (1), a rapid shooting in the antibody titer was observed after vaccination with a booster dose with the locally prepared polyvalent Mycoplasma and *E. coli* vaccine as the antibody titer reached 3840 3 weeks post boostering .The maximum titer level 5600 was observed at 8 weeks post boostering in the same group. From 8 up to 13 weeks post boostering dose, the antibody titer continue at the plateau level showing a slow declining in the antibody up to 5392 in the vaccinated chicken sera. All these results were compared with the negative titers obtained from the non vaccinated control chicken groups all over the experiment.

The same results were obtained by ***(12)*** 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. The obtained protection level of the *M. gallisepticum* vaccine depends on the virulence of the strain used in the preparation of vaccine so the use of *M. gallisepticum* S6 strain may be beneficial in this study ***(13).***

Table 1. ELISA antibody titers against *M. gallisepticum* after vaccination with the locally prepared poly valent MG and *E. coli* vaccine.

Weeks post vaccination	Single vaccination		Control		Weeks post vaccination	Booster vaccination		Control	
	No. of Tested birds	ELISA mean titer	No. of Tested birds	ELISA mean titer		No. of Tested birds	ELISA mean titer	No. of Tested birds	ELISA mean titer
1	50	786	10	211	1	50	3170	10	334
2	50	1103	10	205	2	50	3250	10	332
3	50	1450	10	203	3	50	3840	10	277
4	50	1632	10	220	4	50	4182	10	336
5	50	2032	10	207	5	50	4320	10	216
6	50	2211	10	221	6	50	4918	10	322
7	50	2418	10	253	7	50	5209	10	261
8	50	2404	10	290	8	50	5600	10	313
9	50	2378	10	270	9	50	5580	10	245
10	50	2281	10	246	10	50	5568	10	266
11	50	2195	10	241	11	50	5504	10	274
12	50	2118	10	273	12	50	5424	10	245
13	50	2019	10	300	13	50	5392	10	340

Concerning the antibody titer against *E. coli* after vaccination of chickens with the same polyvalent vaccine, table (2) showed that, the positive titer starts as early as the first week post vaccination in chickens vaccinated with the locally prepared polyvalent mycoplasma and *E. coli* vaccine group giving mean titers 828. Then the antibody titers increased gradually to reach 1640 at 4 weeks post vaccination while the maximum level 2240 was observed at the 7th weeks post vaccination then gradually decreased up to 1660 at 13 weeks post vaccination in the same aforementioned group. Boostering of chickens with the same vaccine was followed by a rapid shooting in the antibody titer as the titer of antibodies reached 2466 at 3 weeks post boostering. The peak of antibody titers was 3689 and was noticed at the 8th week post boostering. These finding are confirmed with that obtained elsewhere *(14)* where the antibody titers in pullets vaccinated once or twice with *E. coli* vaccine were found to be ~ 0.5-3.6 times higher than controls, respectively. In breeder hens, the antibody titer figures were observed ~1.5-4.8 times higher than those from non vaccinated hens. Also the degree of protection conferred by the *E. coli* vaccine was positively correlated with the antibody titer against *E. coli* as measured on day of challenge *(15)*. From the above-mentioned results, it was found that antibody titre has been shown to play an important role in the development of immunity against *E. coli* *(16, 17)*. The same results were previously reported *(18)*, where ELISA was more sensitive than the indirect haemagglutination test and a high correlation was found between ELISA titers and protection against *E. coli* challenge.

Table 2. ELISA antibody titers against *E. coli* after vaccination with the locally prepared polyvalent MG and *E. coli* vaccine

Weeks post single vaccination	Single vaccination		Control		Weeks post boostering	Booster vaccination		Control	
	No. of Tested birds	ELISA mean titer	No. of Tested birds	ELISA mean titer		No. of Tested birds	ELISA mean titer	No. of Tested birds	ELISA mean titer
1	50	828	10	251	1	50	2240	10	324
2	50	1035	10	215	2	50	2380	10	312
3	50	1247	10	303	3	50	2466	10	229
4	50	1640	10	320	4	50	2790	10	336
5	50	1995	10	287	5	50	3109	10	326
6	50	2180	10	221	6	50	3566	10	322
7	50	2240	10	263	7	50	3576	10	214
8	50	2180	10	290	8	50	3689	10	313
9	50	2105	10	260	9	50	3598	10	275
10	50	1979	10	346	10	50	3560	10	266
11	50	1860	10	241	11	50	3548	10	284
12	50	1750	10	273	12	50	3535	10	290
13	50	1660	10	207	13	50	3510	10	244

The ELISA antibody titer obtained post vaccination was correlated with such results obtained after challenge with the virulent *M. gallisepticum* R-strain as shown in table 3. It was found that birds received single dose of vaccination with locally prepared combined MG and *E. coli* vaccine showed marked and significant lower scores than the control unvaccinated chicken group starting one week post challenge up to the fifth week post challenge. At the same time, bird received booster dose showed marked and significant lower scores than the control unvaccinated chicken group starting one week post challenge up to the fifth week post challenge.

Table 3. Air sac lesion scores in chicken vaccinated with single and booster dose after challenge with virulent M. gallisepticum strain

weeks post challenge	Air sac lesion scores post *M. gallisepticum* challenge			
	Single vaccination	Control	Booster vaccination	Control
1st	0.50	1.14	0.30	1.5
2nd	0.90	1.97	0.64	2.1
3rd	1.20	2.96	1.0	3.2
4th	1.29	3.27	1.1	3.5
5th	1.54	3,50	1.3	3.75

It is clear that air sacculitis depends on the immune status of the birds as the grads of air sacculitis were significantly lowered after the challenge post boostering than after the challenge post first dose of vaccination.

Data described in table 4 showed that, the gross lesions in the air sacs, pericardial sac and livers of chickens vaccinated with locally prepared polyvalent MG and *E. coli* vaccine had lower grades than unvaccinated control chickens group. Also, *E. coli* was recovered from birds received single dose of the vaccine at a percentage of 24% while the unvaccinated control chickens demonstrated a relatively more extensive and severe lesions with average scores of 77.7% from the unvaccinated control challenged birds. At the same time, birds received booster dose showed lower lesion grades than unvaccinated control chickens group and from those received only single dose. Also, *E. coli* was recovered from these birds in at a percentage of 17.33%, while it was recorded at a percentage of 86.6% in the unvaccinated control chickens group. These results indicated that score lesions could be considered as a parameter on which the estimation of protection could be greatly depend to some extent as cleared by *(8, 19)*.

Table 4. Lesion scores in vaccinated chicken with single and booster doses of polyvalent Mg and *E. coli* vaccine and challenged with virulent *E. coli* strain

Weeks post challenge		Single vaccination						Booster vaccination					
		Vaccinated			Control			Vaccinated			Control		
		AS	PC	PH	AS	PC	PH	AS	PC	PH	AS	PC	PH
1st	Score	1.2	1.2	1.1	1.5	1.7	1.6	1.0	1.2	0.9	1.5	1.8	1.6
	Mean		1.1			1.6			1.03			1.63	
2nd	Score	1.3	1.4	1.2	2	2.4	2.8	1.2	1.1	1.0	2.5	2.4	2.7
	Mean		1.3			2.4			1.1			2.53	
3rd	Score	1.4	1.5	1.3	2.9	3	3.1	1.6	1.5	1.4	3	2.9	3.4
	Mean		1.4			3			1.5			3.10	
4th	Score	2.1	1.9	2	3.4	3.2	3.5	1.8	1.5	1.7	3.5	3.7	3.2
	Mean		2			3.4			1.7			3.46	
5th	Score	2.6	1.9	1.8	3.5	3.7	3.6	2	2.4	2.6	3.9	3.3	3.7
	Mean		2.1			3.6			2.3			3.76	
Recovery			**24%**			**77.7%**			**17.33%**			**86.6%**	

As= airsacculitis Pc=pericarditis Ph=perihepatitis

The same finding was achieved by *(20)* who found that daily mortality in vaccinated birds with *E. coli* vaccine is lesser than in non vaccinated birds. Also the birds vaccinated with *E. coli* aro-A live vaccine can have a reduction in morbidity and mortality rates *(21)*. Moreover the birds in the challenged control group had a significantly higher air sac lesion score and also a higher number of birds with arthritis than the birds in the vaccinated group *(22)*. Additionally, the chickens in the vaccinated group tended to show lower morbidity and fewer pericarditis and perihepatitis lesion scores than the chickens in the control group. The same results were stated previously *(23)* where more than 95 percent of chickens in control group showed colibacillosis and 70% of them were died after challenge with O78:K80, while in vaccinated groups with monovalent and polyvalent *E. coli* vaccine just 3.7% mortality was observed.

As regards to the overall protection percent obtained by the vaccination with the locally prepared polyvalent MG and *E. coli* vaccine, results in table 5 showed that, the vaccinated birds challenged with virulent *M. gallisepticum* R-strain and Virulent E. coli

serotypes O1, O2 and O78, showed a good degree of protection up to 62 and 66.67%, respectively when received single dose of vaccination. These rates of protection was raised up to 72 and 77.47%, respectively when birds received booster doses.

Table 5. Protective indexes (PIs) assessment in vaccinated chicken with single and booster dose of inactivated polyvalent MG and *E. coli* vaccine

	Type of vaccine	Single vaccination				Booster vaccination			
		Dead/ Total/5 weeks	survival with lesions /Total/5 weeks	% of birds with lesions	PIs	Dead/ Total/5 weeks	Survival with /Total/5 weeks	%of birds with lesions	PIs
M. gallisepticum	Vaccinated	-	15/50	30.00	62%	-	12/50	24.00	72%
	Control	-	20/25	80		-	22/25	88	
E. coli	Vaccinated	8/75	12/75	26.66	66.67%	6/75	9/75	20.00	77.47%
	Control	16/45	20/45	80		18/45	22/45	88.8	

It is clear that the inactivated immunostimulating polyvalent vaccine of *M. gallisepticum* and *E. coli* induced more protective immunity with the challenge post boostering than after single dose of vaccination.

These results are agreed with that obtained by *(24)*, 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 *(25)* 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.

Concerning protection against virulent *E. coli*, the rates of protection in vaccinated and non vaccinated pullets were found to be 77.8 and 46.7 %, respectively *(14)*. The mortality rates of the chicks from the hens which had been vaccinated with trivalent *E. coli* vaccine (TECA) and combined vaccine or not been vaccinated were as follows: 8.8, 13.3 and 50.3%, respectively.

In conclusion, recommendation of use of the locally prepared polyvalent M. gallisepticum and E. coli vaccine could help in protection against the chronic respiratory diseases caused by M. gallisepticum and complicated by E. coli as vaccine is more efficient and the combination between M. gallisepticum and E. coli in one product not interferes with each other, but may potentiate the humoral immune response against each other. The use of this vaccine showed no adverse effects on growth rate of the birds that were vaccinated and the use of polyvalent vaccines prepared from endemic *E. coli* and M. gallisepticum are immunogenic and protective in broiler chicks.

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الملخص العربى

تحضير و تقييم لقاح مثبط متعدد العترات من ميكروبى الميكوبلازما جالليسبتكم و الإكولاى للحماية ضد مرض الجهاز التنفسى المزمن فى الطيور

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[1] المعمل المركزى للرقابة على المستحضرات الحيوية البيطرية – العباسية – القاهرة .

[2] استاذ الميكروبيولوجى - كلية الطب البيطرى - جامعة بنى سويف

مرض الجهاز التنفسي المزمن (CRD) هو احد من المشاكل الرئيسية داخل مزارع الدواجن فى مصر. في هذه الدراسة تم تحضير لقاح مثبط متعدد العترات من ميكروب الميكوبلازما جالليسبتكم و ثلاثة عترات من ميكروب الإكولاى و تم تقييم هذا اللقاح سيرولوجيا و بإجراء اختبار التحدى على طيور تم تحصينها بهذا اللقاح. النتائج المتحصل عليها لهذا اللقاح كانت واعدة حيث كانت ذروة الاستجابة المناعية الخلطية ضد ميكروب الميكوبلازما جاليسبتكم باستخدام اختبار الإليزا ٢٤١٨ و٥٦٠٠ عد استخدامه فى الطيور المحصنة بجرعة واحدة او او جرعات معززة على التوالي في حين كانت ٢٢٤٠ و ٣٥٧٦ لميكروب الإكولاى. بالنسبة لنتائج اختبار التحدى باستخدام العترات الضارية كانت نسبة الحماية التي تحققت ضد الميكروب الضارى للميكوبلازما جاليسبتكم ٦٢ و ٧٢ عد استخدامه فى الطيور المحصنة بجرعة واحدة او جرعات معززة على التوالي في حين كانت نسبة الحماية ٦٦.٦٧% و٧٧.٤٧% لEـ بالنسبة للعترات الضارية من ميكروب الإكولاى. من النتائج التي تحققت يمكن لهذا اللقاح أن يساعد على أن التخلص من او المساعدة فى حل مشاكل مرض الجهاز التنفسى المزمن الناتج عن الاصابة بميكروبى الميكوبلازما جالليسبتكم و الإيشيريشيا كولاى في مزارع الدواجن.