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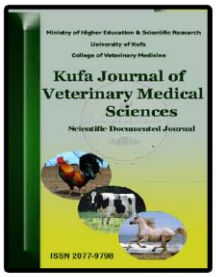

Improved vaccine strategies of infectious bronchitis disease to reduce shedding of virulent virus from infected birds

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Abstract:

Infectious bronchitis (IB) threatens the economies of entire nations, by adversely affecting the backyard farmers, poultry producers and poultry industry throughout the world. Infectious bronchitis virus (IBV), a member of the *Coronaviridae* family, is probably one of the most avian pathogens prevalent in the poultry industry worldwide. In the field which presents difference structures, especially in the S1 spike protein. Complicated immune response against IBV due to few similarities between serotypes. High mutation rate of the virus, in addition to the management and environmental factors, compromise the efficacy of the available vaccines and difficult the control of disease. Prepared effective IB vaccines from any IBV strain stimulates the immunity widely against morbidity and mortality after challenge with virulent IBV strains. On the other hand, vaccinates with live IBV vaccines do not reduce infection or viral shedding after challenge. In order to compare the prepared IBV vaccines taken four different IBV genotypes to determine stimulating immunity and the amount of viral shedding after challenge, two hundred fifty one day broiler chicks divided into five groups and vaccinated with oil-adjuvant vaccines prepared of Iraqi isolates (II, I-II, I- III and I-IV). different inactivated IB viruses including strains QX, CH Baghdad M 2014, CH Baghdad F1 2013, CH Babylon F2 2013, and an allantoic fluid control. Using a hemagglutination inhibition test for serum analysis after challenge with virulent IBV (Variant2) to knowledge antibody content against each of the vaccine antigens, Monitored the vaccinated birds daily for of morbidity and mortality were recorded, and used PCR to determine the amount of viral copy of examined clinical samples in selected periods of viral shedding. All vaccines used in the study gave good protection against morbidity and mortality, except the control group. Homologous vaccines gave good protection and lowest viral shedding in the trachea, kidney and feces compared to the heterogeneous vaccines.

Key word: infectious bronchitis disease, inactivated vaccines, hemagglutination inhibition, viral load, challenge.

تحسين استراتيجيات التلقيح لمرض التهاب القصبات الهوائية المعدي لتقليل طرح الفايروس الضاري من الطيور المصابة

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الخلاصـــة

التهاب القصبات الهوائية المعدية (IB) يهدد اقتصادات دول بأكملها، التي تؤثر سلبا على منتجي الدواجن, وصناعة الدواجن في جميع أنحاء العالم. التهاب الشعب الهوائية (IBV) من الفيروسات المعدية ، وهو عضو في عائلة فيروسات تاجية، هو على الارجح واحد من مسببات الأمراض الأكثر انتشارا في صناعة الدواجن في جميع أنحاء العالم. حقليا

يعرض العديد من التراكيب المختلفة، خصوصا في البروتين الشوكي S1. الاستجابة المناعية معقدة ضد IBV بسبب بعض أوجه التشابه بين الأنماط المصلية. معدل طفرة عالية من الفيروس، بالإضافة إلى إدارة والعوامل البيئية، تقلل من فعالية اللقاحات المتوفرة وصعوبة السيطرة على المرض. وعلى هذا الاساس اجريت مقارنة لقاحات حضرت من اربعة عزلات IBV عزلت محليا وتم تحديد الانماط الجينية لها وحضرت التركيبة اللقاحية الزيتية واجريت لها كافة الاختبارات النهاية لكي يكون اللقاح مطابق للموصفات القياسية للقاحات الزيتية والتي اكدت تلك الاختبارات خلوه من الملوثات البكتيرية والفطرية فضلا عن تميزه بالثبات والاستقرارية والانسيابية في الجريان ونوع المستحلب اللقاحي, لتقييم المناعة المحفزة وكمية الفايروس المطروح بعد التحدي, اخذت مائتين وخمسون فرخة دجاج لاحم قسمت الى خمس مجاميع ولقحت باللقاحات الزيتية المحضرة من العزلات العراقية (I-II,I- III, I-IV و I-IV) تضمنت فايروسات غير الفعالة (CH ,QX CH Baghdad F1 2013,Baghdad M 2014 و CH Babylon F2 2013) ومجموعة السيطرة اخذت سائل اللقانيقي. استعمل فحص اثبات التلازن الدموي لتحليل المصل بعد التحدي بالعزلة الضارية (Variant2) لمعرفة محتوى الاجسام المناعية ضد كل من الانيجينات اللقاحية, وتم مراقبة الطيور الملقحة يوميا لتسجيل نسبة الاصابة و الهلاكات, واستعمل فحص PCR لتحديد الفايروس المطروح باحتساب كمية الفايروس في العينات السريرية المفحوصة في فترات محددة. جميع العزلات المستعملة بالدراسة اعطت حماية جيدة ضد الاصابة ماعدا مجموعة السيطرة, اللقاحات المتماثلة جينيا اعطت حماية جيدة وباقل طرح للفايروس في الرغامي, الكلى والفضلات مقارنة باللقاحات المغايرة

Introduction

IB is rapid spread diseases in the world and severe contagious of chickens caused by infectious bronchitis virus (1). The disease characterized by Nephritis, respiratory symptoms, reduced egg reproduction and poor performance (2). The spike glycoprotein (S) of corona virus was determinant the cell tropism (3). Several IBV antigenic variant strains or serotypes have been reported in many countries due to the high mutation rates of glycoprotein S1 gene (2). In general, no cross protection between different IBV serotypes and not confer immunity against each other (4), and the S1 proteins of IBV strains have low cross protection between them (5). On the other hand, some effective strains of the virus are classified in the same protectotypes because ability to induce the cross protection against other genotypes or serotypes (6). In Iraq, since the first report of disease in 1989 still IB continuous problem in poultry industry despite extensive vaccination(7). significant number of IBV genetic variants exist with different characteristics from IBV reference strains from other nations, increasing frequency was reported since the year 2000 (8), indicated the degree of amino acid identity tends to diminish. Recently, Phylogentic analysis showed Iraqi IBV field isolates consists of at least from four clear Phylogentic: I-I, I-II, I-III and I-IV (9). Associated I-I type closely related to the nephropathogenic IBV isolated in China, related type I-II closely with variant nephropathogenic IBV Chinese strain, but related type I-III closely to variant nephropathogenic IBV isolated in Israel, while the type I-IV is associate d closely with Entric IBV isolated in Egypt. Overall, associated the variant nephropathogenic I-II IBV isolate high mortalities and caused huge economic losses in the poultry industry in Iraq, despite available vaccines manufactured nephropathogenic IBV 4/91 (10). Real-time polymerase chain reaction (PCR) is a specific, sensitive and rapid method used for gene expression analysis, nucleic acid detection, quantification of nucleic acid copy numbers and is widely used in the rapid detection of pathogens (11). The present study aimed to find out the protection level resulting of vaccination with genetically four different IBV strains through determined the viral shedding of vaacinated birds, in addition to monitoring the morbidity and mortality rates after challenge with a virulent variant2 strain.

Materials and methods

Virus:

In this study was used four IBV Iraqi field isolate originated from AL-Nahdha Laboratory for Veterinary Vaccines. Use RFLP for virus genotyping and nucleotide sequencing (Genbank: GQ169242) after S1 gene amplification. for propagation of the virus inoculated embryonated chicken eggs at 9-11 days old, were identified infectious dose 50% EID_{50} according to Reed and Muench (12). Formalin was added to the AAF at a rate of 0.12% and then incubated

for 48 hours at 37°C for the inhibition of the virus. Samples preserved in the refrigerator (4°C) in order to use them in other operations.

Sterility and safety testing of AAF

It took 10 ml of AAF and drove for 15 minutes at 6000 rpm, and the sediment on Thioglycholate agar and MacConkey's agar and Mycoplasma broth and agar Sabourad's plans to make sure AAF devoid of any bacterial or fungal contamination. To ensure the inhibition of virus injected embryos of chicken eggs aged 9-11 day in allantoic fluid after 168 hours of injection in the absence of the effect of stunting the embryos demonstrated a clear full inhibition of the effectiveness of the virus (13).

Preparation of oil emulsified IB vaccines

Use paraffin oil as oil adjuvant. Add the amount of aqueous phase (Tween-80) and the oil phase (span-80) surfactants to 10% of paraffin oil. Then fixed hydrophile lipophile (HLB) of oil emulsion at 7.0 using the following formula

$$z = \frac{ax + by}{a + b}$$, where

Z = the amount of required HLB of the emulsion, a = the amount of surfactant A, b = the amount of surfactant B, x = HLB value of the surfactant A, y = HLB value of surfactant B.

The water in oil emulsion preparation by adding one part of the AAF diluted in four parts of paraffin oil containing surfactants. And then the mixture was homogenized with the help of the homogenizer (Ultra Turrax T_{45}) for 4 minutes at 4000rpm.

Physical properties

All physical properties of the production vaccines containing color, the emulsion type, stability and viscosity (flow time) were conducted. The viscosity measurement by calculate the time required in seconds for the drops the volume of 0.4 ml of a one-ml glass pipette and marked (0). While sure the emulsion type by placing two drops of the prepared vaccine on a glass slide and then mix each drop with mineral oil and distilled water separately, where water mixed easily with emulsified oil compared to mineral oil. The stability examination, the emulsion is divided into three aliquots the first aliquot is placed at a temperature 37°C and puts the second aliquot in the refrigerator at a temperature 4°C while the third aliquot is placed at room temperature 25°C and determine the stability by determine the time required for oil phase-water phase separation (15).

Experimental design:

Two hundred fifty broiler chicks in good condition (strain: Rose 308, a Belgian of Origin) were bought from AL-Afrah-hatchery- Baghdad. Divided randomly into 5 groups (A to E) 50 chicks each group. All birds vaccinated at one day-old subcutaneously with IB vaccines dose of 0.25 ml. Group A maintained as non-vaccinated control. Group B were administered I-I (QX strain) vaccine. Group C were administered I-II (CH Baghdad F1 2014 strain) vaccine. Group D were administered I-III (CH Baghdad F1 2013 strain) vaccine. Group E were administered I-IV (CH Babylon F2 2013 strain) vaccine.

Haemagglutination inhibition (HI):

Collected blood samples randomly five birds from each group weekly, at (0, 7.14, 21, 28, 35 and 42) after vaccination. Serum was separated from blood and inactivated at 56°C for 30 minutes to break down the complement and storage at -20°C until used later in HI test to determine the antibodies titre against IBV. Trypsin is used to help in HA and HI performed these tests in the U-shaped microtitration plates.

RNA isolation and Real Time RT-qPCR:

The total RNA of tracheal, kidney tissue and fecal samples after 2 and day post challenge at 28 days old were extracted with TRIZOL Reagent® (Invitrogen, USA) and then cDNAs were obtained in the RT with a Superscript III Kit (Invitrogen, USA), as described previously. The Cdna samples were submitted to real time quantitive PCR for the absolute

quantification of viral load, and this technique was conducted as recommended Okino *et al*. (17), except that the primers described by Wang and Tsai (18) were used in place of HV+ and HV- primers. A linear regression was determined plotting the logarithmic values of the number of copy of plasmid DNA containing the insert of gene S1 against the cycle in other organs. threshold (Ct) values, in order to convert the Ct values from tissue samples into S1 gene copy number (17).

Challenge test:

For challenge used the virulent field IBV isolate (Variant2) in titration (100 ELD_{50} $10^{4.0}$) determined according to Reed and Muench (12). challenge birds were monitored daily for 10 days post challenge, to record morbidity (respiratory and neurological signs) and the mortality rate.

Statistical Analysis:

Used the SAS system to illustrate the effect of various factors in parameter study (19). Also used Least significant difference-LSD multiple levels to illustrate significant comparison to the means of the current study.

Results

Physical properties:

The emulsion type for all study vaccines were water in oil, and a milky white color, while the time flow (viscosity) 6 seconds. The stability of each vaccine was stable for more than four weeks at 4°C but one week at 37°C and room temperature 25°C.

Post-vaccinal Ab titer against IB

(Table 1) showed no significant differences at level (P <0.05) in antibody titre in vaccinated groups (A, B, C, D and E) on the first day of birds age, that point to the correct random distribution of birds in the five groups. The results of present study showed a highly significant increase in antibodies titre at level (P <0.05) at day 7, 14, 21 and 28 post-vaccination in vaccinated groups B, C, D and E compared to the control group A (non-vaccinated). Day 35 after challenge each of the group C and D had the highest antibody titre compared to the B and E, while both group B and E did not record any significant difference at level (P <0.05). In days 42 recorded all vaccinated groups significant increase in antibodies and group C were vaccinated topping the totals in the significant elevation, while the control group A (non-vaccinated) recorded highly and sudden increase in antibodies compared with vaccinated groups

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Table 1. Antibody titers against IB measured by HI test of different groups (Mean ± SE) in different times

Day	Group A	Group B	Group C	Group D	Group E	LSD
0	34.4±0.6 **a**	35±0.7 **a**	35.6±0.7 **a**	34.4±0.7 **a**	35.2±0.7 **a**	3.09
7	24.6±0.3 **d**	43±0.4 **c**	48±0.5 **a**	45.6±0.3 **b**	41.8±0.7 **c**	2.06
14	19±0.7 **e**	56.6±0.9 **c**	68±0.7 **a**	63±0.6 **b**	52.8±0.9 **c**	3.38
21	12.8±0.6 **d**	131.2±1.4 **c**	161.4±1.5 **a**	143±2.1 **b**	128±1.7 **c**	6.58
28*	5±0.5 **d**	195.2±1.8 **c**	251.4±1.5 **a**	227.4±2.1 **b**	191.4±3.3 **c**	8.65
35	4.2±0.2 **d**	228.4±3.1 **c**	320.4±3.9 **a**	268±2.9 **b**	226.4±3.7 **c**	12.94
42	323±4.4 **b**	294.6±2.05 **c**	374.6±2.8 **a**	328.8±5.1 **b**	278±5.02 **c**	17.12

*: challenge with (Variant2) at 28 days

Group A: Control group.

Group B: Vaccinated with 0.25 ml killed IBV (QX strain) vaccine.

Group C: Vaccinated with 0.25 ml inactivated IBV (Variant2 strain) vaccine

Group D: Vaccinated with 0.25 ml inactivated IBV (CH Baghdad F1 2013 strain) vaccine

Group E: Vaccinated with 0.25 ml inactivated IBV (CH Babylon F2 2013 strain) vaccine

Post challenge viral load distribution:

The results of viral load distribution of different tissues (trachea and kidney) and fecal samples in chickens vaccinated with different inactivated IBV strains and challenged with local IBV virulent Variant2 strain (100 ELD_{50} $10^{4.0}$) at 28 days of age are listed in Tables 2, 3 and 4. The results of the viral load at 2 and 4 days post challenge showed a high significant difference ($P<0.05$) between the 5 groups at 2 days, group A showed the height viral load (viral shedding) as compared with group C showed the most lowest ($P<0.05$) viral load followed by group (D, B and E). At 4 days post challenge the same trends were recorded in the five groups with significantly higher ($P<0.05$) viral load within and between the five groups. However, group A rank in the first place followed by groups (E, B and D) in the second and third rank respectively in compared with group C was recoded less viral shedding.

Table 2. Distribution of viral load (RT-PCR, means ± SE) of the trachea tissue.

Groups	Post challenge at 28 days old			
	2 days		4 days	
A	2286.6±58.36	**a**	4984.4±177.5	**a**
B	468.4±14.3	**b**	906±13.7	**b**
C	188±17.8	**c**	264.4±17.4	**c**
D	293±21.5	**c**	375.2±17.7	**c**
E	508.6±21.7	**b**	1045.6±22.2	**b**
LSD	130.12		337.88	

Number of samples=5.

-The different small letters refer to significant differences between different columns ($P<0.05$)

Table 3. Distribution of viral load (RT-PCR, means ± SE) of the kidney tissue.

Groups	Post challenge at 28 days old			
	2 days		4 days	
A	3010±84.6	**a**	7199.4±152.2	**a**
B	852.8±41.2	**bc**	1370.4±44.7	**b**
C	423.8±25.8	**d**	775.2±28.4	**c**
D	706±20.9	**c**	1159.4±34	**b**
E	901.2±24.8	**b**	1379.4±24.5	**b**
LSD	191.92		310.58	

Number of samples=5.

-The different small letters refer to significant differences between different columns ($P<0.05$)

Table 4. Distribution of viral load (RT-PCR, means ± SE) of the fecal samples.

Groups	Post challenge at 28 days old			
	2 days		4 days	
A	4817.4±150.9	**a**	9834±234.5	**a**
B	1226.2±22.3	**b**	1749.8±26.9	**b**
C	316.8±29.6	**d**	646±27	**c**
D	716.4±17.9	**c**	1088.8±26.3	**c**
E	1336.6±34.2	**b**	1867.6±42.1	**b**
LSD	298.65		452.65	

Number of samples=5.

-The different small letters refer to significant differences between different columns (P<0.05)

Protection test

The data in Table 5. showed that the morbidity rate in group C had significant lower (P<0.05) rate (10)% followed groups (D, B, and E) were recorded (15, 20 and 25)% respectively as compared with group A was recorded 100%. While the no mortality rate was recorded in vaccinated groups (C, d, B and E) in compared with group A (control group) was recorded (100%) mortality rate.

Table 5. The protective levels

Groups	Morbidity %	Mortality %
A	100 **a**	100 **a**
B	20 **bc**	0 **b**
C	10 **b**	0 **b**
D	15 **b**	0 **b**
E	25 **c**	0 **b**

*Number of chicks groups= 250

Discussion

The study aimed to determine the antigenic distance of vaccinal strains, which have a big effects on the amount of viral shedding post challenge with virulent IBV strain, therefore can create the final decision in forming of vaccine structure and challenge virus depending on the potency examination. So homologous IBV vaccines with the challenge virus (Variant2) give the highest titre of antibodies compared to the amount of antibodies produced from heterogeneous vaccines with challenge virus (Table 1). These results agree with Ali *et al.,* (20). The increase in HI Abs titre at 21 and 28 days age return to immune response of inactivated vaccine these results are in agreement with Farhan, (21) who confirmed that the inactivated vaccine which was used in early age of chicks led to elevation immune response with progress of the time especially after 14-21 days of vaccination, also, Grimes, (22) mentioned that the inactivated vaccine needed for long time about 21 days to reach a high level of antibody production. The results of the current studies showed high Abs titre in vaccinated group after challenge These results agreed with the findings of researchers Okino, et al., (17), who reported that the rise in the titre of antibodies after challenge due to the capacity of the tracheal memory cells to induced rapid immune response represented Lachrymal IgG and IgA from 1 to 5 days after challenge in the tracheal mucosa after infection or challenge with local IBV strain. The results agree with Liu, *et al*., (23) who mentioned the positive relationship between high level dose of vaccine and antibody titers, then chicks will protect after challenge test, therefore, the group C and D group showed a significant ($P \leq 0.05$) increased antibody titers as compared with other vaccinated groups of inactivated vaccine, may be due to increase in the dose of homologues antigen in the prepared vaccine these finding agreed with finding of (23 and 24). Recently, tended researches to improve the protection of vaccinated birds by improving the ability of the vaccine on reduce significantly viral shedding of the challenge virus in tracheal and kidney tissue as well as feces samples marked by antigenic homologous between the vaccinal and challenge strains. The aim of the study, which evaluated the immune status of vaccinated birds with inactivated IBV vaccine in different strains against challenge by variant Iraqi IBV isolate. The results of the study inducted lower viral shedding was obtained in the tracheal tissue in the fact that the IBV challenge strain is less pathogencity to tracheal tissue compared to the kidney tissue. But the best protection was obtained in group C was vaccinated homologous antigenically to challenge strain these findings agreed with (25 and 26). The change that is happening

in the tissue tropism of the IBV to the infection of some tissues but not others is due to the difference in the amino acids located in some sites of S1 glycoprotein, which has the highest role in virulence and antigencity of the IBV and escape from the immune defenses of the host (27, 28). In addition to the decline viral shedding in environment, so the vaccinated birds against IBV become more resistant to the virus and require large amounts of the virus to become infected.

Conclusion

our studies show that virus shed can be controlled by choosing vaccines that are more genetically similar to the challenge virus and suggest that minimizing virus shed may be a useful strategy to limit the spread of the disease.

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