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Experimental Evaluation for the Dual Infection of Low Pathogenic Avian Influenza Virus H9N2 and Avian Pathogenic *Escherichia Coli* in Commercial Broiler Chickens

Sameh Abdel-Moez Amer*, Hagar Magdy Ahmed, Asmaa Mahmoud Maatouq and Mohamed Mahmoud Abdelbaki

Department of Poultry Diseases, Veterinary Research Institute, National Research Centre, P.O. Code 12622, Dokki, Cairo, Egypt.

LOW PATHOGENIC Avian Influenza Virus (LPAIV) subtype H9N2 became latterly endemic in Egypt with major losses from complicated infections particularly with Avian Pathogenic *Escherichia coli* (APEC) in commercial broilers. In the present study, forty broilers' chickens were emerged into four equal groups (G1, G2, G3 and G4) where groups G1 and G2 vaccinated with LPAIV H9N2 vaccine at 7 days-old, while groups G3 and G4 act as non-immunized positive controls. By the day 28 of age groups G1 and G3 challenged with LPAIV H9N2 alone while groups G2 and G4 were co-infected with both H9N2 and *E.coli*. LPAIV H9N2 Seroconversion, clinical picture, mortality record and virus shedding estimation via QrRT-PCR were all evaluated. The results revealed a significant mortality in co-infected groups G2 and G4 of 50% and 90% deaths respectively with severe clinical illness in compared to H9N2 infected groups only G1 and G3 with 0% and 20% mortalities respectively. Furthermore, positive elevated serological humoral response was estimated in H9N2 vaccinated birds pre- and post-infection. Moreover, virus shedding was significantly elevated in co-challenged non-vaccinated group G4 followed by mono-infected non-immunized group G3 and the lesser significant detected viral load was in vaccinated H9N2 challenged group G1. In conclusion, the usage of H9N2 vaccines can significantly protect the broilers from mortality and minimize viral shedding post-challenge with H9N2 virus challenge only. Whereas, *E.coli* infection can precisely aggravate the virulence and pathogenicity of H9N2 when dually infected in terms of higher mortalities, inferior clinicopathological picture and elevated viral shedding even in vaccinated birds raising the importance about the necessity to control the secondary bacterial infection in broiler chicken flocks.

Keywords: Low Pathogenic Avian Influenza Virus (LPAIV) H9N2, Avian Pathogenic *Escherichia coli* (APEC), Co-infected, Secondary bacterial infection, Broiler chicken.

Introduction

H9N2 subtype of low pathogenic avian influenza virus (LPAIV) is an endemic viral disease of poultry in almost all Middle East regions including Egypt with the G1 sub-lineage the most prevalent genotype which possibly arised from wild birds [1, 2]. The first report of LPAIV H9N2 isolation in Egypt was in the late 2010 whereas antibodies against H9N2 were detected in domestic poultry sera during a serological surveillance in 2009 [3, 4]. Despite of AIV H9N2 is classified pathologically as low pathogenic disease, recent studies in the last years revealed high mortalities and serious clinical disease from this subtype in broiler chickens resulted in mass economic losses which may be possibly due to combined infection

*Corresponding author: Sameh Abdel-Moez Amer, E-mail: drsamehnrc@hotmail.com, Tel.: 01019022002
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with other pertinent respiratory pathogens [5, 6]. H9N2 viruses are categorized into two lineages that prevalent in both domestic poultry and wild birds in which G1 lineage shows the greatest geographic widespread in Asia and Middle east countries with four distinct groups A, B, C and D [7]. In Egypt particularly has possessed the G1-like clade since 2010 with endemic established status in Egyptian poultry flocks causing significant economic losses. Latterly in 2014, a new reassortant of LPAIV H9N2 was emerged from pigeons and rapidly circulated within backyard chickens in 2015 [8]. The egyptian LPAIV H9N2 is of group B G1 lineage which commonly circulated among Saudi Arabia, United Arab Emirates, Jordan and Israel which is recognized that poultry trade and water fowl migration are the main role in LPAIV spread [9].

Avian Pathogenic *Escherichia coli* (APEC) is one of the family Enterobactiracae members which pathotyped according to its Somatic (O) and Flagellar (H) antigens [6]. The local and systemic pernicious infection caused by *E.coli* in poultry is called colibacillosis which is usually seen as respiratory manifestations and gastrointestinal lesions accompaigned with severe septicemic clinical disease [10]. APEC strains cause local and systemic disease in chickens, turkeys, ducks and other poultry species [11]. The major systemic infections caused by APEC in chickens are arthritis, air saculitis, pericarditis, perihepatitis, omphalitis, cellulitis, salpingitis, egg peritonitis and swollen head syndrome [12]. *E.coli* infection is usually comes secondary to a lot of other primary pathogens including viral agents (AIV, Newcastle disease, Infectious bronchitis virus), Bacterial (Mycoplasma species) and protozoan pathogens (Eimeria and Histoplasmosis) which have led to many difficulties in treating such pathological effect of *E.coli* including the inability of vaccine processing and despicable therapeutics against this devastating secondary infector [13].

Multiple pathogens also have a synergistic effect with H9N2 which severely impacted the prevention and control strategies in the poultry field [14]. Broiler chickens experimentally challenged with H9N2 alone exhibited mild clinical picture with low or even no mortalities [15]. The AIV H9N2 and *E.coli* is considered one of the most common integration of mixed respiratory infection in the poultry field which usually complicates the treatment protocol for such respiratory complex causing severe economic losses [16]. Previous studies have mentioned that H9N2 infection in chicken broilers increase the sensitivity of birds to *E.coli* secondary infection and also can enhance *E.coli* adhesion to the cells of infected host [17]. Furthermore *E.coli* as well intensify the virulence and the pathogenicity of AIV H9N2 and can easily triggers the mortality rates and clinical disease also along with long term H9N2 viral shedding in dually infected birds with both pathogens [18, 19, 20]. Moreover, infection of broiler chickens with AIV H9N2 either pre, post or synchronously with *E.coli* causing elevated mortalities and exacerbates the virulence of both diseases which complicates the poultry field situation [21].

Therefore**,** the aim of the current study is to discuss and evaluate the experimental effect of AIV H9N2 and *E.coli* co-challenge in H9N2 vaccinated and non-vaccinated broiler chickens on the base of Clinicopathological picture, mortality, humoral immune response and virus shedding post-challenge.

Material and Methods

Ethical approval and Biosafety

Birds experimental challenge was approved by the Medical Research Ethics Committee (MREC) of the National Research Centre, Dokki, Cairo, Egypt with an approval ethical code: 0620223-1 and in parallel with the firm protocols of animal rearing and handling.

Challenge virus and bacteria

LPAIV subtype H9N2 *(A/Chicken/Egypt/NRC1/2023/H9N2)* was decently supplied by Veterinary Vaccines Technology lab, Central lab, National Research Centre, Egypt and characterized as G1 Lineage with Genbank accession number (OR742072). The virus was propagated in 11 days-old chicken embryo and adjusted to 10^6 embryo infective dose 50 (EID_{50}) / 0.1 ml as mentioned by Reed and Muench method [22] for challenge of broiler chickens with 100 µ1 via intranasal route.

E.Coli O78 strain was provided by poultry diseases department, Veterinary Research Institute, National Research Centre, Egypt. The bacterial broth was cultured on MacConkey agar plates and the pink colonies were calculated and counted at a concentricity of 10^8 colony-forming units (CFU) / ml as mentioned by Circella et al. [23] to be used in challenge experiment with 1 ml / bird via oral route.

Vaccines

The commercial broilers in experimental protocol was immunized by inactivated H9N2 vaccine (Nobilis® influenza H9N2+ND) for influenza A, subtype H9N2 strain *(A/CK/UAE/145/99)* with $10^{8.2}$ EID_{50} / ml which provided by local agency and was used by 0.3 ml / bird subcutaneously as advised by vaccine manufacturer.

Assessment of serology by Haemagglutination Inhibition test (HI)

The HI test was employed to screen post-immunization immune response pre and post-infection in which blood serum was assembled in 7, 14, 21, 28, and 35 days of age randomly from five birds per group. Two fold serial serum dilutions were homogenized with equal volume of local H9N2 viral antigen consists of 4 HA units as referenced by OIE [24].

Quantitative RT-PCR for viral shedding

A total number of 80 cloacal and tracheal swabs were collected at 3 and 7 days post-challenge (5 tracheal and 5 cloacal swabs per group) to be examined by q RT-PCR for detection of AIV H9N2 shedding post-challenge. RNA extraction was conducted by QIAamp mini kits (Qiagen, Valencia) as per recommended by supplier. The Oligonucleotide primer set targeted the matrix protein gene of influenza A virus as mentioned by Ward *et al* [25] as follow;

Forward: 5´AAG ACC AAT CCT GTC ACC TCT GA 3´

Reverse: 5´CAA AGC GTC TAC Real time PCR GCT GCA GTC C 3´

Probe: 5´FAM TTT GTG TTC ACG CTC ACC GT TAMRA 3´

The calculation of standard curve of H9N2 virus was carried out by viral serial dilution from 10^{-1} to 10^{-6} and the quantity of AIV H9N2 in tested swabs was acquired by CT plotting of tested swabs versus the standard curve and then stated as Log 10 EID_{50} / ml. The detection limit and cut-off value of positive samples was 10^{2} EID_{50} / 0.1 ml and the frequency of viral shedding is represented as the number of positive shedders / total number of tested swabs.

Experimental design for vaccination challenge assay

The major goal of the present work is to estimate the effect of LPAIV H9N2 and avian pathogenic *E.coli* combined infection on commercial broilers under laboratory conditions. So as to, forty Ross 208® broiler chicks were divided into four equal groups of ten birds each. In which G1 and G2 immunized subcutaneously with inactivated H9N2 vaccine at 7 days-old, while group G3 and G4 represented as positive infected non-immunized controls. At 28 days of age group G1 and G3 challenged intranasally with AIV H9N2 only, whereas, group G2 and G4 co-infected with both AIV H9N2 and pathogenic *E.coli* (orally) as in supplementary Table.1. All birds were strictly monitored daily for 7 days post-challenge to investigate the morbidity, mortality and clinico-pathological picture.

Statistical analysis

All obtained data were analyzed by one-way ANOVA for analysis of variations, standards of deviations and to detect significance between different treated groups using SPSS program version 22. Significant differences within treated groups were detected at ($P < 0.5$).

TABLE 1. Experimental design for LPAI H9N2 vaccination and challenge trial with H9N2 and/ or *E.coli* in commercial broiler chickens.

Groups	Number of birds	Vaccination regime		Challenge at 28 days old	
		H9N2 vaccine	Age/days	AIVH9N2 [2]	*E.coli* [3]
G1	10	Vaccinated [1]	7	+ Ve	- Ve
G2	10	Vaccinated	7	+ Ve	+ Ve
G3	10	None vaccinated	None	+ Ve	- Ve
G4	10	None vaccinated	None	+ Ve	+ Ve

[1] (Nobilis H9+ND) an oil emulsified inactivated bivalent vaccine. The dose of vaccine equal 8.2-Log_{10} EID_{50} given 0.3 ml / bird by subcutaneous route.

[2] LPAI H9N2 G1.B virus used for challenge and the challenge viral dose equal 6-Log_{10} EID_{50} given 100µl / bird by intranasal route.

[3] *E.coli* serotype O78 was prepared as 1 ml of broth containing 10^{8} colony forming unit (CFU) E. coli / ml given by 1 ml / bird by oral route.

+ Ve: infected. – Ve: non infected

Results

Morbidity and clinical picture

The clinical signs frequency in each of the four groups were recorded daily for 7 days post-infection where as H9N2 vaccinated and challenged group G1 showed almost no clinical illness except with slight depression and little decrease in food and water intake. While, groups G2 and G4 dually infected with AIV H9N2 and *E.coli* represented a marked depression, ocular and nasal exudates, severe diarrhoea, moderate to severe sickness and head swelling along with respiratory manifestations, whereas such clinical disease was more obvious and severe in non-vaccinated co-infected group G4. Moreover, the non-vaccinated H9N2 challenged group G3 showed moderate respiratory illness with marked depression and sickness.

Necropsy and post-mortem (pm) gross lesions

The necropsy findings of the present experiment revealed no obvious pm lesions in vaccinated and H9N2 challenged group G1 in which all body cavity of scarified birds were found normal without noticeable lesions. While in co-challenged group G2 and G4 necropsy examination of dead birds showed the most sever pm lesions among all groups especially in non-vaccinated co-infected group G4 with; fibrinous air saculitis, hepatitis, arthritis, swollen heads and pericarditis as well as nephritis and severe tracheal congestion with obvious caseated pneumonia and marked enteritis along with splenomegaly, cloudy air saculitis and pancreatitis. While in non-immunized H9N2 infected group G3 the pathological feature declared mild tracheitis, air saculitis, pericarditis and mild nephrosis.

Mortalities post-infection

In the present study, the results related to primary H9N2 viral infection and *E.coli* secondary bacterial challenge at 28 days-old are listed in Table.2. In which, there is a significant higher mortality in non-vaccinated co-challenged group G4 from other groups with 90% (n=9/10) 7 days post-challenge followed by vaccinated co-infected group G2 with 50% (n=5/10) which revealed that *E.coli* secondary infection exacerbates the mortality rate when co-infected with AIV H9N2 even in vaccinated broilers. Moreover, little recorded mortality was demonstrated in non-vaccinated H9N2 challenged group G3 with 20% (n=2/10). While no recorded mortalities at all in vaccinated and H9N2 infected group G1 representing the efficacy of H9N2 vaccine in arresting the deaths in such vaccinated infected group.

Serological immune response

The results of antibody titers monitoring in sera samples collected from the four groups at 7, 14, 21, 28 and 35 days-old against H9N2 are presented in Table.3 and Figure.1. A significant serological response was detected in H9N2 vaccinated groups G1 and G2 along the vaccination course compared to non-vaccinated groups G3 and G4 especially at challenge day (28 days) with GMT 6.3, 6.4, 0.6 and 0.7 in G1, G2, G3 and G4 respectively. Such results revealed the effect of H9N2 vaccine in achievement of humeral immune response against H9N2. Furthermore, after challenge with H9N2 either alone or combined with *E.coli* all groups are elevated in Seroconversion against H9N2 especially vaccinated groups G1 and G2 with significant higher HI titers of 7.4 and 7.9 respectively 7 days post-challenge. As well as, non-immunized groups G3 and G4 recorded 4.2 and 4.9 GMT titers respectively also 7 days post-infection which ensure the effect of *E.coli* co-infection in provoking and enhancement of immune response against H9N2 vaccination especially in non-vaccinated co-challenged group G4 with both LPAIV H9N2 and pathogenic *E.coli*.

TABLE 2. Mortality percent in vaccinated and challenged broiler chickens post-infection .

Group	Daily screening of dead birds post-challenge							Total no. of dead birds	Mortality %	Protection rate %
	1	2	3	4	5	6	7			
G1	None	None	None	None	None	None	None	0/10	0	100
G2	None	None	1	1	2	1	None	5/10	50	50
G3	None	None	None	1	1	None	None	2/10	20	80
G4	None	None	1	2	2	None	4	9/10	►90	10

►**:** denotes significance from groups G1, G2 and G3 at (P<0.05).

None: not recorded.

TABLE 3. Humoral Seroconversion of H9N2 vaccinated broilers and challenged with H9N2 and / or *E.coli* at 28 days old in broiler chickens.

Group	H9N2 Vaccination schedule		HI titer means SD Log-2 of H9N2 at age/days (N = 5)				
	Type	Age/days	7	14	21	28	35
G1	Vaccinated	7	5.2. ±1.01	5.4±0.75	5.7±0.74	►6.3±0.73	►7.4±0.72
G2	Vaccinated	7	5.4±0.89	5.5±0.83	5.6 ±1.16	►6.4±0.82	►7.9±0.88
G3	None vaccinated	None	5.3±1.00	3.3±0.55	2.00±0.71	0.6±0.54	4.2±0.52
G4	None vaccinated	None	5.3. ±1.00	3.4±0.53	2.1±0.72	0.7±0.53	4.9±0.55

None: not treated

►: denotes significance from groups G3 and G4 pre and post-challenge at (P<0.05).

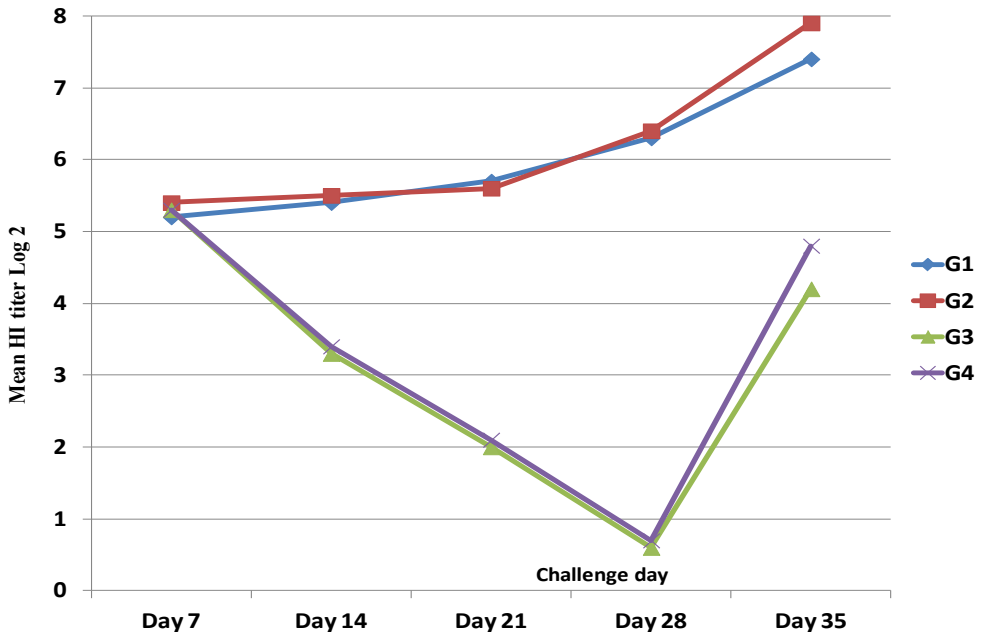

Fig.1. Results of HI antibody dynamics for H9N2 throughout the immunization course before and after challenge with H9N2 and / or E.coli in all experimental groups G1, G2, G3, and G4

Post-challenge viral shedding

Virus shedding is considered one of the premium tools in assessment of vaccination potency as well as reflects how the virus is transmitted among and between infected flocks. Cloacal and tracheal swabs were randomly collected from all infected groups at 3 and 7 days post-challenge and tested by QrRT-PCR then represented as the number of positive shedders / total number of collected swabs as presented in Table.4 and Figure 2&3. The results revealed the positivity of all tracheal and cloacal swabs with significant value in group G4 non-vaccinated challenged with AIV H9N2 combined with *E.coli* at all tested days which demonstrate the role of *E.coli* secondary infection in aggravating H9N2 viral shedding. The non-immunized group G3 challenged with H9N2 comes following to group G4 in viral shedding load in terms of number of positive shedders. While H9N2 vaccinated group G2 co-infected with both H9N2 and *E.coli* comes in the third place with the detectable positive number of tested swabs which revealed also that *E.coli* infection can trigger the shedding to H9N2 even in vaccinated birds. Whereas, the little significant viral load was recorded in group G1 which stop completely from oral swabs 7 days post-challenge reflecting the role of H9N2 vaccination in restriction of viral shedding in vaccinated broiler chickens.

TABLE 4. Viral shedding after vaccination trial and challenge with H9N2 and / or *E.coli* at 28- days of age in broiler chickens:

Group no.	Birds no.	H9N2 Vaccination program		H9N2 Shedding in days post-challenge *			
		Type	Age / days	Oral swabs		Cloacal swabs	
				3	7	3	7
G1	10	Vaccinated	7	1/5	0/5	0/5	1/5
G2	10	Vaccinated	7	3/5	2/5	2/5	3/5
G3	10	None vaccinated	None	4/5	3/5	3/5	4/5
G4/	10	None vaccinated	None	5/5	5/5	5/5	5/5

*Swabs were collected from five birds in each group as oral and cloacal swabs and evaluated to assess virus shedding via QRT-PCR for H9N2. The frequency of viral shedding post-challenge is expressed as the number of positive shedders / total number of tested swabs.

►: denotes significance from groups G1, G2 and G3 (P<0.05). None: not treated

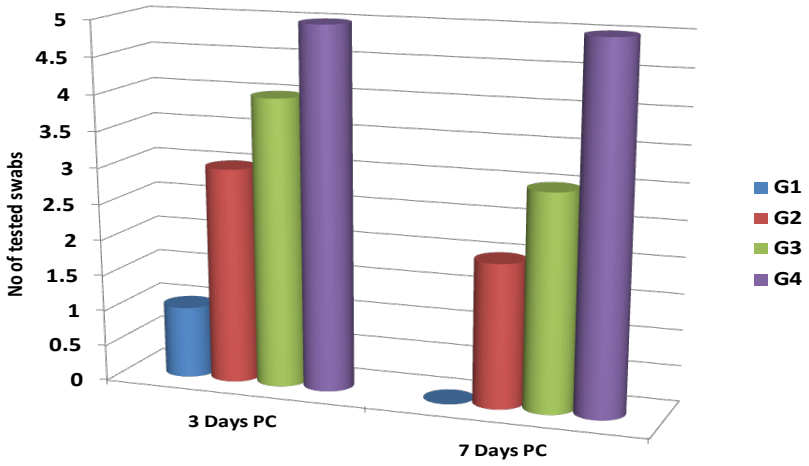

Fig. 2. Oropharyngeal viral shedding by QrRT-PCR for H9N2 at 3 and 7 days post-challenge (PC) in all tested groups .

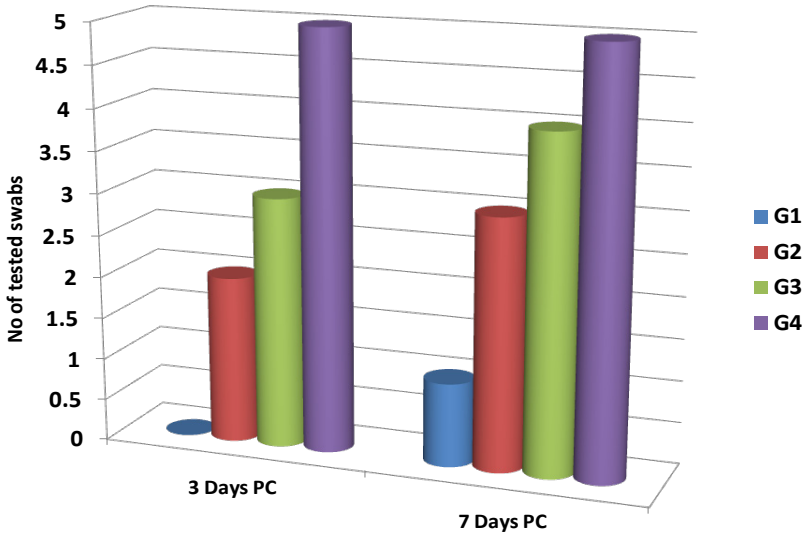

Fig. 3. Cloacal viral shedding by QrRT-PCR for H9N2 at 3 and 7 days post-challenge (PC) in all tested groups .

Discussion

The present study aimed to evaluate the effect of single H9N2 infection and combined infection with Avian Pathogenic *E.coli* in both vaccinated and non-vaccinated broiler chickens. The dual infection of H9N2 intranasally and *E.coli* orally in 28 days-old showed 50% and 90% mortalities in both H9N2 immunized and non-immunized birds, respectively which is assisted by recent studies [20, 26]**.** Moreover a higher significant clinical protection was noticed in vaccinated broilers (G1 and G2) in compared to non-vaccinated ones (G3 and G4), thus come in harmony with previous findings [21, 27] who mentioned that mixed infection of both H9N2 and *E.coli* can exacerbate the clinical outcome and mortality levels in broiler chickens and also the report of Wang *et al.* [16] who reported the effect of LPAIV-bacterial synergy in exaggerating the clinical disease in a mouse model. Moreover, lesser mortality was recorded in non-vaccinated H9N2 mono-challenged group G3 with 20% (n=2/10) as in the same findings of Abdel-Hamid et al. [28] who recorded accumulative mortality of 20% in experimentally challenged broiler chickens with H9N2 virus. On the other side El Nagar et al. Have concluded that H9N2 infection in specific pathogen free chickens revealed no mortality with observed clinical signs of mild depression and recorded pm lesions post-infection as; mild tracheitis, pneumonia, spleenomegally and sever nephritis [29].

The present results showed that the most observed clinical illness in challenged broilers either with H9N2 alone or in combination with *E.coli* were; depression, diarrhoea, nasal and ocular exudates, head swelling accompaigned with respiratory signs in which a comparable findings were mentioned by [15, 18, 30, 31] as well as a previous report by Li et al. [32] who observed a gastroenteritis like symptoms as diarrhoea in a secondary *E.coli* infection following to AIV H9N2 challenge.

In the present study, there was no serological immune response to H9N2 virus in non-immunized birds which reflect the virus ability to spread higher than those of immunized chickens and further exclude the significant superior mortalities and clinical disease in non-immunized birds challenged with either H9N2 only or co-challenged with *E.coli*. There was a significant humoral response of H9N2 vaccinated groups G1 and G2 along the vaccination course compared to non-vaccinated groups G3 and G4 especially at challenge day (28 days) with GMT 6.3, 6.4, 0.6 and 0.7 in G1, G2, G3 and G4 respectively. Such results revealed the effect of H9N2 vaccine in achievement of humeral immune response against H9N2 which come in assent with [15, 20] who concluding that a single H9N2 vaccination shoot could provide a good antibody response in broiler chickens. Furthermore, after challenge with H9N2 either alone or combined with *E.coli* all groups are elevated in Seroconversion against H9N2 especially vaccinated groups G1 and G2 with significant higher HI titers of 7.4 and 7.9 respectively 7 days post-challenge, thus analogous with previous findings [19, 21, 33, 34].

Based on our findings, a single shoot from inactivated H9N2 vaccine during the first 10 days of life can provide a better protection to broiler chickens against mono-challenge with AIV H9N2 in terms of clinical protection and mortality in compared with non-vaccinated birds, thus was supported by previous studies [15, 20, 35] who concluded that H9N2 vaccination in commercial broiler chickens supported them with a premium protection against clinical illness and probable deaths from AIV H9N2 infection.

Since viral shedding is considered as an important aspect in assessment of H9N2 vaccine potency post-challenge especially in case of mixed bacterial infection. Our results declared the positivity of all tracheal and cloacal swabs with significant value in group G4 non-vaccinated challenged with AIV H9N2 combined with *E.coli* at all tested days which demonstrate the role of *E.coli* secondary infection in aggravating H9N2 viral shedding. Moreover, the non-immunized group G3 challenged with H9N2 comes following to group G4 in viral shedding load. While H9N2 vaccinated group G2 co-infected with both H9N2 and *E.coli* comes in the third place with detectable positive number of tested swabs which revealed also that *E.coli* infection can trigger the shedding to H9N2 even in vaccinated birds, which come in accordance with recent studies [20, 21, 27]**.** Whereas, the little significant viral load was recorded in group G1 that virus shedding stop completely from oral swabs 7 days post-challenge reflecting the role of H9N2 vaccination in limiting viral shedding in vaccinated broiler chickens as in harmony with previous findings [15, 35] who concluded that H9N2 vaccines can definitely decrease and stop AIV H9N2 shedding in specific pathogen free birds and commercial broiler chickens.

Conclusion

This study discussed and evaluated the dual infection between LPAIV H9N2 and APEC in H9N2 vaccinated and non-vaccinated broiler chickens. The synergistic action plays a major role in broilers immune response that *E.coli* can definitely aggravates the pathogenicity of H9N2 infection in terms of inferior clinical picture, high mortality and long term virus shedding even in vaccinated birds. While H9N2 infection alone in vaccinated birds was under control without any impact on broiler chickens. On contrary, H9N2 infection in susceptible non-vaccinated birds revealed a noticeable effect on clinical picture and mortality along with detectable viral shedding load. So as to, it strongly recommended controlling the secondary bacterial infection accompaigned with viral challenge in broiler chickens to avoid mass losses in poultry flocks even with vaccination strategies or suitable Antibiotic added programs .

Author's Contribution

All authors equally participated in design, experimental procedure, writing, revised, and reviewing the manuscript.

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Conflict of interest

The authors have declared no conflict of interest.

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التقييم التجريبي للعدوى المزدوجة بفيروس أنفلونزا الطيور منخفض الضراوة H9N2 والإيشيريشيا القولونية في دجاج التسمين التجارى

سامح عبد المعز عامر* ، هاجر مجدى أحمد ، أسماء محمود معتوق و محمد محمود عبد الباقى.
قسم أمراض الدواجن - معهد البحوث البيطرية - المركز القومى للبحوث - الدقى - القاهرة -مصر- الرقم البريدى 12622

لقد أصبح النوع الفرعي H9N2 من فيروس أنفلونزا الطيور منخفض الضراوة (LPAIV) متوطنًا مؤخرًا في مصر مع خسائر كبيرة ناجمة عن حالات عدوى معقدة خاصة مع بكتيريا الإيشيريشيا القولونية المسببة للأمراض (APEC) في دجاج التسمين التجاري. في هذه الدراسة، تم تقسيم أربعين دجاجة تسمين إلى أربع مجموعات متساوية G1 وG2 وG3 وG4 حيث تم تحصين المجموعتين G1 وG2 بلقاح H9N2 عند عمر 7 أيام، بينما عملت المجموعتان G3 وG4 كمجموعة ضابطة إيجابية غير محصنة. في عمر 28 يومًا، تم تحدي المجموعتين G1 وG3 باستخدام H9N2 وحدها بينما تم عدوى المجموعتان G2 وG4 بكل من H9N2 والإيشيريشيا القولونية. تم تقييم رد الفعل المناعى والصورة السريرية وسجل النفوق وتقدير الذرف الفيروسى عبر QrRT-PCR. كشفت النتائج عن معدل وفيات كبير في المجموعتين المصابتين G2 وG4 بنسبة 50% و90% على التوالي مع أعراض سريرة حادة مقارنة بالمجموعات المصابة بفيروس H9N2 فقط G1 وG3 مع وفيات 0% و20% على التوالي. علاوة على ذلك تم قياس نسب مناعة مرتفعة تدريجيا فى المجموعات المحصنة لفيروس H9N2 قبل وبعد العدوى .أيضا كان الذرف الفيروسى مرتفعًا بشكل ملحوظ في المجموعة G4 غير المحصنة ، تليها المجموعة G3 غير المحصنة المصابة بعدوى أحادية، وكان الحمل الفيروسي الأقل في المجموعة G1 المحصنة ضد H9N2. في الختام، فإن استخدام لقاحات H9N2 يمكن أن يحمي الدجاج اللاحم بشكل كبير من الوفيات ويقلل من تساقط الفيروس بعد تحدي فيروس H9N2 فقط. وحيث أن عدوى الإيشيريشيا القولونية يمكن أن تؤدي بدقة إلى تفاقم الوضع الوبائى عند الإصابة به بشكل مزدوج من حيث ارتفاع معدل الوفيات، والصورة المرضية الإكلينيكية السيئة وارتفاع الذرف الفيروسى حتى في الطيور المحصنة، مما يزيد من أهمية ضرورة السيطرة على العدوى البكتيرية الثانوية في دجاج التسمين التجاري.

الكلمات الدالة: فيروس أنفلونزا الطيور (H9N2 (LPAIV، الإشريشيا القولونية الممرضة للطيور (APEC)، العدوى البكتيرية الثانوية، الدجاج اللاحم.