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Experimental Co-infection of Low Pathogenic Avian Influenza Virus (H9N2) and *Escherichia Coli* in SPF Broiler Chickens

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Abstract

The work was implemented to evaluate the pathogenicity of H9N2 when co-infected with the *E.coli*. Avian influenza H9N2 virus strain A/chicken/Egypt/1618F/2016 which was isolated from Luxor province during 2016 and *E.coli* serotype O_{78} were used in this research. Sixty SPF birds were divided into 6 groups of ten birds each. At age 21 days, group 1 was inoculated with H9N2 only, group 2 received *E.coli* and three days later received H9N2, group 3 inoculated with H9N2 and after three days received *E.coli*, group 4 was challenged with *E.coli* and H9N2 at the same time, group 5 was inoculated with *E.coli* only, and group 6 was designed as a negative group. Clinical signs, postmortem examination, and serological examination were monitored for 15 days. The H9N2 virus presence was assessed in tracheal and cloacal swabs using real-time PCR. The most severe signs and lesions were observed in groups (3, and 4), with also high mortality rate than other groups with a percentage of 20%. Groups 3, and 4 showed a longer duration in virus shedding in the tracheal and cloacal samples. The hemagglutination inhibition test, group 4 showed high HI antibody titer against AIV-H9N2 antigen than other groups, while group 2 showed the lowest HI antibody titer against AIV-H9N2 antigen than other groups that received only the H9N2 virus. In conclusion, the outcomes of this study revealed that the infection of H9N2 with *E.coli* can exacerbate the clinical outcomes and mortality rates which leads to higher economic losses in chicken flocks.

Keywords: Co-infection, *E.coli*, LPAI (H9N2), Pathogenicity, SPF.

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Avian influenza virus is an important poultry disease causing great epidemics resulting in higher economic losses. Avian Influenza virus related to the type of (A), *Orthomyxoviridae* family and possess 18 hemagglutinins and also have 11 sub-neuraminidases (Tong et al., 2013). LPAIV has distributed in a large scale in domestic poultry around the world (Karimi et al., 2010). One possible illustration for this high mortality rates and significant economic losses can be mixed with causes of infection of other respiratory diseases (Seifi et al., 2010). In Egypt, the first introduction of H9N2 LPAIV was reported in May 2011 in commercial clinically healthy bobwhite quail farm (El-Zoghby et al., 2012), then the virus was detected in commercial chicken broilers, breeders and layer flocks (Ahmed et al., 2013).

Infections with H9N2 viruses in domestic poultry are commonly correlated with the reduced feed intake and consuming water and drop in egg production, and moderate respiratory symptoms with low mortality rate (Swayne and Halvorson, 2008). But over the past ten years, the epidemic spread of H9N2 infections with severe clinical symptoms, excessive numbers of mortalities (20-65 %) and loss in production reached to 75 % which have been mentioned in commercial poultry flocks (Suarez, 2008).

The co-infection of the H9N2 virus with other pathogens which cause mainly respiratory disease can complicate respiratory disease syndromes and cause acute disease condition and high numbers in mortalities (Pan et al., 2012; Hassan et al., 2017; Ismail et al., 2018; Mahana et al., 2019).

Colibacillosis is the most significant infectious disease in poultry. The responsible causative factor of the Colibacillosis is *E.coli* that belonged to *Enterobacteriaceae* family, it is a gram-negative in stain and rod in shape, and it is an anaerobic and non-pathogenic bacteria which lives commensally in the digestive system. Avian Pathogenic *E.coli* may infect chickens and cause variable disease forms of *E.coli* e.g. yolk sac infection, coligranuloma and colisepticaemia. The most common respiratory origin of *E.coli* is colisepticaemia, (Saif et al., 2008; Persoons et al., 2011). Risk factors that affect the infection of colisepticaemia respiratory origin and primary respiratory infections caused by Mycoplasma species or viral infections of both wildtype and vaccinal strains of unfavorable housing conditions such as high concentrations of air ammonia overcrowding and a high infective dose (Lutful Kabir, 2010).

This study was carried out to determine the effect of the co-infections of the H9N2 virus and *E.coli* O_{78} in SPF chickens, also understand the impact of the *E.coli* on the H9N2 pathogenesis (clinical pictures, and lesions), virus shedding during single H9N2 infection or *E.coli* co-infection).

Materials and Methods

I. Determination of the EID50 of H9N2:

The virus strain used in this study was A/chicken/1618F/2016 with (Gene bank Acc. No., MH734794). H9N2 field strain was isolated and characterized in Reference Laboratory for Veterinary Quality Control on Poultry production (RLQP), Animal Health Research Institute, Agriculture Research Center. This virus was propagated in embryonated 10 days old chicken eggs and the 50% Egg Infectious Disease (EID_{50}) was determined as recommended by (Reed and Muench,1938). The virus strain was

examined for confirmation that its freedom from other respiratory viral diseases.

II. E. coli O_{78} strain:

E.coli serotype O_{78} was used in rate of 1 ml of saline containing 10^8 colony forming unit (CFU) *E. coli*/ ml, and originally isolated from chicken, the inoculum was prepared according to (Fernandez et al., 2002).

III. Experimental design:

Sixty 21-day-old SPF birds were randomly divided into 6 groups with 10 birds in each group. All of the birds were put in negative pressure isolators. Group 1 was administrated 10^7 EID_{50}/ml (Mahana et al.,2019) of H9N2/chicken. Group 2 infected with *E.coli* O_{78} (10^8 CFU /ml) /chicken and, three days later, was received 10^7 EID_{50} of H9N2/ chicken. Group 3 was inoculated with 10^7 EID_{50} of H9N2/ chicken and, three days later, was received of *E.coli* O_{78} (10^8 CFU /ml) /chicken. Group 4 was inoculated with *E.coli* O_{78} (10^8 CFU /ml) /chicken, and at the same once, 10^7 EID_{50} of H9N2/chicken. Group 5, birds were infected with *E.coli* O_{78} (10^8 CFU /ml) /chicken, and Group 6 was taken an intraperitoneal injection of the sterile physiological saline as a control, as shown in Table (1).

Table (1): Experimental design

Group	Design
Group 1	**H9N2# only (at age 21 days).**
Group 2	***E.coli** (at age 21 days) and three days later H9N2.**
Group 3	**H9N2 (at age 21 days) and three days later *E.coli*.**
Group 4	**H9N2 and *E.coli* at the same time at age 21 days.**
Group 5	***E.coli* only (at age 21 days).**
Group 6	**Negative control**

*** *E. Coli*: Intraperitoneally, and # H9N2: intranasally.**

IV. Clinical signs and Postmortem Examination:

Birds were observed two times in the day for fifteen days post-challenge for clinical findings and each one was taken daily clinical score numbers as follows: normal (0); mild signs (1); moderate signs (2) and sever signs (3) (Arafat et al., 2018). All of the birds were scored daily for 15 days post challenge. Birds that was received a score number 3 were euthanized by the dislocation of cervical vertebrae.

V. Detection of H9N2 virus by Real Time PCR:

Tracheal and Cloacal swabs were collected after 6, 9, and 12 days after inoculation and were tested by using real time PCR. RNA extraction by usinga QiaAmp® Viral RNA Mini Kit (QIAGEN, Hilden, Germany) Cat. No. 52904, for swabs according to the manufacturer's guidelines. One step real time RT-PCR (Quantitect prope RT-PCR master mix Kit): cat no. 204443, (Qiagen, Germany) was used for H9N2 Avian influenza type A detection, with specific primers and probe (Ben Shabat et al., 2010) (Table 2). Thermal profile for amplification of HA gene of H9 subtypes was as follows: 50 °C

for 30 min, 95 °C for 15 min, cycling steps of 94°C for 10 s, 54°C for 30 s and 72 °C for 10 s repeated for 40 cycles.

VI. Serological Examination for H9N2 antibodies:

The HI antibody titers analysis was carried out after 15 days Pc (post challenge) by collection of serum from each group. Serum samples were examined for H9N2 antibodies according to (OIE, 2015).

Table (2): Primers and probe used for Real tine RT-PCR identification of H9N2 virus:

Primer ID	Primer sequences	Ref.
H9 subtype	**For: GGA AGA ATT AAT TAT TAT TGG TCG GTA C Rev: GCC ACC TTT TTC AGT CTG ACA TT H9probe: [FAM] AAC CAG GCC AGA CAT TGC GAG TAA GAT CC [TAMRA]**	**(Ben Shabat et al., 2010)**

Results

I. Clinical signs and mortality rate:

The clinical signs of this study are summarized in Table (3). In case of mortality rates, negative control group 6 showed no mortality, also the same results in mortality rates were found in group 1 which challenged by H9N2 only. Groups 3 and 4 showed high percentages of mortalities with 20%, however the mortality rates in groups 2 and 5 revealed 10%.

II. Gross examination

Different gross lesions were noticed during the experiment in the different groups and brief described with score system in Table (4).

Table (3): The score of the clinical signs throughout the experiment.

Group/Clinical signs	Group1	Group 2	Group 3	Group 4	Group 5	Group 6
Coughing and sneezing	**1**	**2**	**2**	**3**	**1**	**0**
Head enlargement	**0**	**1**	**1**	**2**	**1**	**0**
Depression	**1**	**2**	**2**	**3**	**2**	**0**
Respiratory distress	**1**	**1**	**2**	**2**	**1**	**1**
Nasal and ocular discharge	**1**	**1**	**1**	**2**	**1**	**0**
Ruffled feather	**1**	**1**	**2**	**2**	**2**	**0**
Reluctant to move	**0**	**0**	**1**	**2**	**1**	**0**

(0) absence of apparent clinical disease signs, (1) mild, (2) moderate, and (3) severe signs, groups (1,2,3,4,5, and 6).

Table (4): The score of the gross lesions in all experimental groups.

Lesions	Group (G)					
	G 1	G 2	G 3	G 4	G 5	G 6
Air sacculitis	**1**	**3**	**3**	**3**	**2**	**0**
Tracheal congestion	**1**	**3**	**3**	**3**	**2**	**0**
Catarrhal exudates in trachea	**1**	**2**	**2**	**2**	**2**	**0**
Serous exudates in trachea	**0**	**2**	**2**	**3**	**1**	**0**
Serous to cast formation in the bifurcation of trachea	**0**	**3**	**3**	**2**	**1**	**0**
Pericarditis	**0**	**3**	**3**	**3**	**2**	**0**
Perihepatitis and necrosis in liver	**1**	**3**	**3**	**3**	**2**	**0**
Hemorrhages in the intestine	**0**	**3**	**3**	**3**	**1**	**0**
Enlargement and necrosis of spleen	**1**	**3**	**3**	**2**	**2**	**0**
Enlargement of kidney	**1**	**3**	**3**	**3**	**1**	**0**
Synovitis	**1**	**3**	**2**	**3**	**1**	**0**

(0) no lesions, (1) slight legions, (2) moderate lesions, and (3) severe lesions.

III. H9N2 Virus detection by Real time PCR:

All groups were examined during the experiment using real-time - PCR for virus detection in the cloacal and tracheal sample's swabs, the virus detection results with cycle threshold (Ct) are explained in Table (5). Group 5 and group 6 showed negative results in the virus detection in the cloacal and tracheal swabs on 6, 9, 12 days post challenge. It implied that no cross contamination presented between groups. The virus was revealed in cloacal and tracheal samples in groups from 1 to 4 on day 6 after inoculation.

Only groups 3, and 4 still given positive results in tracheal swabs on day 9 post challenge but given negative results in cloacal swabs, however, group 1, and 2 simultaneously (day 9) revealed negative results in both cloacal and tracheal samples. Also, the virus detection and shedding from cloaca wasn't detected in groups 1, 2, 3, and 4 on day 12 post 1st challenge.

IV. Serological examination

In this study the HI titers geometric mean in groups 1, 2, 3, and 4 was done on day 20 prior to challenge revealed low titers in all groups with geometric mean ranged from (2.1 to 2.3). On day 15 post challenge (the age of birds was 36 days), antibody titers against H9N2 revealed high titers in all four groups (1,2,3, and 4) however the groups 5, and 6 showed the lowest as explained in Table (6).

Table (5): H9N2 virus detection in tracheal and cloacal swabs by RT-PCR.

Groups	Days post 1st challenge					
	6 days		9 days		12 days	
	T. S.	C. S.	T. S.	C. S.	T. S.	C. S.
Group 1	+ve (Ct: 24.61)	+ve (Ct: 30.17)	-ve (No Ct)	-ve (No Ct)	-ve (No Ct)	-ve (No Ct)
Group 2	+ve (Ct: 25.73)	+ve (Ct: 34.61)	-ve (No Ct)	-ve (No Ct)	-ve (No Ct)	-ve (No Ct)
Group 3	+ve (Ct: 25.53)	+ve (Ct: 31.44)	+ve (Ct: 34.17)	-ve (No Ct)	-ve (No Ct)	-ve (No Ct)
Group 4	+ve (Ct: 27.27)	+ve (Ct: 30.51)	+ve (Ct: 28.08)	-ve (No Ct)	-ve (No Ct)	-ve (No Ct)
Group 5	-ve (No Ct)	-ve (No Ct)	-ve (No Ct)	-ve (No Ct)	-ve (No Ct)	-ve (No Ct)
Group 6	-ve (No Ct)	-ve (No Ct)	-ve (No Ct)	-ve (No Ct)	-ve (No Ct)	-ve (No Ct)

T. S.: Tracheal swabs; C. S.: Cloacal swabs; +ve: Positive; -ve: Negative, Ct: cycle threshold.

Table (6): The serological findings of HI titers of H9 antibodies of all experimental groups.

Day	Group (G)					
	G 1	G 2	G 3	G 4	G 5	G 6
Day 20 prior challenge (GMT* with SD#)	2.1±0.1	2.2±0.1	2.3±0.1	2.1±0.1	2.3±0.1	2.2±0.1
Day 36 post challenge (end of the experiment (GMT with SD)	6.5±0.2	6.3±0.2	7.1±0.1	7.3±0.1	1.0±0.0	1.0±0.0

***GMT: geometric mean titer**
#SD: standard deviation.

Discussion

H9N2 virus is one of the respiratory viral diseases. It causes economic losses in broiler farms, as it is considered the primary gate for secondary bacterial infection, so the complication of mixed infection with pathogenic bacteria is very common. The natural co-infection problems usually occur simultaneously between LPAIV H9N2 and bacterial infection in poultry farms (Pan et al., 2012, Pu et al., 2012). Co-infection in poultry farms leads to misdiagnosis and complication in the determination of the accurate clinical picture of the problem (Pan et al., 2012, Costa-Hurtado et al., 2014). Considering the high spread of the H9N2 in chicken farms and presence of pathogenic bacteria in the same farms, this study was implemented to assess and determine the effect of bacterial co-infection especially E. coli O_{78} on H9N2 pathogenicity.

In this research, the clinical signs of all 6 groups were studied. Group 6 showed no observed symptoms and lesions which means that the cross infection between groups didn't occur. In the present study,

group 5 was observed with similar findings of *E.coli* infection with 10% in mortality rate and observed lesions which specifically for *E.coli* disease, as mentioned before by (Peighambari et al., 2000). The severity of the symptoms and gross lesions increased in group 4 with higher respiratory distress and mortality rate with 20%, these results match with (Ginns et al., 1998). The results in group 3 revealed the same severity of the clinical symptoms, gross examination, and mortality rate of group 4, however, the infection of the *E.coli* 3 days post the H9N2 inoculation, as previously reported (Barbour et al., 2009 ; Mahana et al.,2019). Converting to the group 2, there were decrease in mortality rate in comparison to group 3, and group 4 which also were co-infected with *E.coli* with mortality rates in both of them, however the clinical findings of the symptoms and post mortem lesions of group 2 very close to group 3, and group 4. This means, the exposure of bacterial infection (before, after, or simultaneously) with the H9N2 LPAIV increases the severity of the clinical findings and mortality rates, these signs are consistent with previous studies (Goudarzi et al., 2014; Stipkovits et al., 2012).

In case of virus shedding, groups 3, and 4 showed prolonged duration in tracheal swabs by real time PCR than other groups, it means that the infection of the *E.coli* with H9N2 prolonged the persistence of H9N2 virus in trachea and upper respiratory tract which extended to 9 days post the 1st inoculation,. However, groups 1, and group 2 showed positive results in tracheal detection only after six days post the 1st inoculation and negative results on days 9, and 12 posts the 1st challenge. Groups 5, and group 6 implies negative results in all days post the inoculation. In the virus shedding in cloaca, the results were negative in groups 5, and 6 in all cloacal examined swabs during the experiment, but groups 1, 2, 3, and 4 were given positive results only after six days post inoculation. However the study reported by (Bano et al., 2003) revealed that the groups which were infected with H9N2 LPAIV before *E.coli* showed prolonged duration in the shedding of the virus until 14 days post-inoculation, in compared to groups which were infected only by H9N2 that the shedding extended up to a week after challenge.

The highest antibody titer was noticed in group 4, however, (Mosleh et al., 2017) demonstrated that receiving simultaneous H9N2 and *E.coli* infections showed lower antibody titer. Also, group 3 show high geometric mean number, which implies that the infection of the *Ecoli* with H9N2 at the same duration or after H9N2 inoculation gave high HI titers which was also observed by previous works (Bano et al., 2003; Mahana et al.,2019). In group 1 which inoculated H9N2 only revealed lower titer than group3 and 4, these results match with (Mosleh et al., 2017). The lowest antibody titer between groups who challenged with H9N2 appeared in group 2, these group was infected by *E.coli* first then after 3 days H9N2 inoculation, but other studies reported by (Mosleh et al., 2017) showed that the highest occurrence of HI antibody titer occurred when inoculating LPAI H9N2 after *E.coli* infection.

Conclusion

It was shown that H9N2 virus is still low in pathogenicity if it comes individually without other respiratory pathogens, however, it appears more serious if co-infected with other bacterial infection especially *E.coli*. In this study, the results revealed that the correlation between H9N2 and *E.coli* in chicken farms

induces severe respiratory signs and high mortality rates in the suspected broiler flocks. Continuous monitoring and diagnosis of H9N2 are very important with good plans and strategies in experimental programs to identify the status of the virus and follow its development, especially in the case of co-infection with *E.coli*.

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Conflict of interest statement

The authors declare that they have no conflict of interest.

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