Evaluation of the timing of the *Escherichia coli* co-infection on pathogenecity of H9N2 avian influenza virus in broiler chickens

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Summary

Bacterial co-infections can probably influence the pathogenicity of H9N2 low pathogenic avian influenza virus (AIV). This study aimed to evaluate the effect of exposure time to *Escherichia coli* (O:2) on the pathogenicity of H9N2 AIV in broiler chickens. Three hundred and sixty broiler chickens were randomly allocated to six equal groups. At the age of 26 days, all chicks except groups 5 and 6 were inoculated intra-nasally with H9N2 virus. At the same time, the birds in groups 1 and 5 were infected with *E. coli* via spray route. Birds in groups 3 and 2 were infected with *E. coli* three days prior to and three days post AI challenge, respectively. Mortality rates, clinical signs, gross and microscopic lesions, excretion and duration of virus shedding in faecal and tracheal samples and seroconversion to H9N2 virus were assessed in the challenged groups. The highest mortality rate was observed in chickens inoculated with H9N2 followed by *E. coli*. The most severe clinical signs, gross lesions, mortality rate and virus detection were observed at day 6 post challenge (PC) in birds of group 2, while the duration of virus shedding was longer in group 3 (*E. coli* followed by H9N2) than other groups. In conclusion, *E. coli* infection prior to, after or concurrently with H9N2 virus infection could exacerbate the adverse effects of the virus. Our results indicate that *E. coli* and H9N2 together can mutually exacerbate the condition of either disease in broiler chicks as compared to single infected birds.

Key words: Avian influenza virus, *Escherichia coli*, Exposure time, H9N2, Pathogenesis

Introduction

H9N2 avian influenza viruses (AIVs) are identified regularly in domestic poultry populations in many Eurasian countries (Alexander, 2002; Śmietanka *et al*., 2014). Although the disease caused by H9N2 low pathogenic avian influenza (LPAI) virus is relatively mild in SPF (specific pathogen-free) chickens (Gharaibeh, 2008), considerable information indicates that outbreaks are very costly due to increased mortality, production losses and control measures in commercial poultry farms (Nili and Asasi, 2003). Many questions remain concerning the risk factors which exacerbate the adverse effects of H9N2 virus in field conditions. The presence of other pathogens in birds may be one of the predisposing factors related to the manifestation of a severe disease following LPAI H9N2 infection. A co-infection study is an approach used for the identification of possible synergistic effects of different organisms. The co-infection of H9N2 virus with other respiratory pathogens can complicate respiratory disease syndromes and cause severe disease and high mortality (Pan *et al*., 2012; Seifi *et al*., 2012). Different studies showed that many organisms such as *Staphylococcus aureus* or *Haemophilus paragallinarum*, *Escherichia coli*, *Ornithobacterium rhinotracheale* (ORT), infectious bronchitis virus or infectious bronchitis live vaccines enhance the virulence of H9N2 and increase the mortality rate of infected birds (Bano *et al*., 2003; Kishida *et al*., 2004; Haghighat-Jahromi *et al*., 2008; Barbour *et al*., 2009; Tavakkoli *et al*., 2009; Karimi-Madab *et al*., 2010; Pan *et al*., 2012; Seifi *et al*., 2012; Azizpour *et al*., 2013). Umar *et al*. (2015) demonstrated that in the presence of aflatoxin, H9N2 infection led to more prominent virus transmission, enhanced pathogenesis of H9N2, higher mortality rate and poor seroconversion in the infected turkeys.

One of the common microorganisms that are ubiquitous in the poultry farms is *E. coli* and colibacillosis is the most frequent respiratory disease of broilers in most countries (Dobrindt, 2005) which often co-infected with other agents and increases their pathogenicity. Co-infection of colibacillosis with diseases such as salmonellosis and histomoniasis has been reported (Ganapathy *et al*., 2000). Little has been done to understand the interactions between AIV and *E. coli* when infecting poultry. Bano *et al*. (2003) showed a significant role of intratracheal exposure of *E. coli* in aggravating the clinical condition of the birds earlier infected with H9N2 AIV. Moreover, it has been reported that the presence of LPAI H9N2 virus predisposes chickens to secondary *E. coli* infection (Barbour *et al*.,

2009). The contribution of *E. coli* co-infection in development of clinical signs, gross and histopathological lesions, duration of viral shedding and death in H9N2 viral infection remains uncertain. The present study was conducted to evaluate the effect of *E. coli* (O:2) infection on pathogenesis (clinical signs, gross and microscopic lesions, mortality rate), duration of virus shedding in faecal and tracheal samples and seroconversion to H9N2 virus in commercial broilers pre and post challenge (PC) as well as simultaneous infection with *E. coli*. The information obtained from the study helps in understanding the interaction of these two pathogens in poultry production facilities.

Materials and Methods

Virus

The virus strain used in this study was A/chicken/Iran/772/1998(H9N2), obtained from Razi Vaccine and Serum Research Institute propagated in 10-day-old embryonated chicken eggs and the 50% egg infectious dose (EID50) was determined by the Reed and Muench (1938) method.

Bacteria

Escherichia coli serotype O:2 was used.

Experimental design

Three hundred and sixty one-day-old cobb-500 commercial broiler chickens (both sex) were divided randomly into six equal groups (sixty birds per group in three replicates of twenty birds). Birds of each group were reared in separate rooms in Animal Research Unit in School of Veterinary Medicine, Shiraz University under strict biosecurity conditions to prevent cross contamination and received standard feed and water *ad libitum* during the experiment. The experiment was carried out in accordance with the guidelines for use of animals in research approved by School of Veterinary Medicine, Shiraz University.

As shown in Table 1, at 26 days of age, all chicks except group 5 and 6 (negative control) were challenged via intra-nasal route with 10 µL of infectious allantoic fluid containing 1×10^9 EID 50/ml H9N2 virus. Moreover, birds in groups 1 and 5 infected with *E. coli* (O:2) (1×10^9 CFU/ml) via spray route (20 ml/48 m^3) at the same time. The room temperature was 24-26°C. Birds in groups 3 and 2 infected with *E. coli* by the same route on three days prior to virus challenge and three days post virus challenge, respectively (Table 1). Chickens were monitored daily for their general condition, clinical signs and mortality. On days one, twenty one and at the end of the experiment, sera of chickens were tested for presence of hemagglutination inhibition (HI) antibodies against H9N2 influenza virus (Pedersen, 2008) by employing 4 hemagglutination units of H9N2 antigen (Pasouk Biological Research and Manufacturing Co., Tehran, Iran).

Table 1: Experimental plan (day 26) for inoculation of broiler chickens with H9N2 AIV and/or *E. coli*

Groups	H9N2	*E. coli*
1	+	+(at the same time with H9N2 challenge)
2	+	+(3 days post H9N2 challenge)
3	+	+(3 days prior H9N2 challenge)
4	+	-
5	-	+(at the same time with group 1)
6 (negative control)	-	-

Pathologic examination

On days 3, 6, 9, 12, 15 and 18 post AIV challenge five birds from each group were randomly selected and euthanized for evaluation of gross and histological lesions in various organs including trachea, lung, kidney and spleen. A score of (0) was assumed for tissues without any gross lesions; +1 mild; +2 intermediate and +3 severe gross lesions. The severity index was then calculated as follows:

$$(\sum \text{number of birds with lesion} \times \text{severity score}) / (\text{number of sampled birds})$$

To determine histological lesions, tissue samples were collected in 10% neutral buffered formalin and embedded in paraffin. Sections were made at 5 µm thickness and stained with hematoxylin and eosin and studied under light microscope.

Evaluation of virus shedding period

Faecal and tracheal samples were collected from 5 euthanized birds of each group on days 3, 6, 9, 12 and 15 post AIV challenge and stored in -70°C. Virus detection was performed by reverse transcription-polymerase chain reaction (RT-PCR) method. RNA was extracted using RNX™ (-plus) (CinnaGen Co., Tehran, Iran) commercial kit according to the manufacturer's instructions. Viral RNA was extracted from 200 µL of the supernatants of a 10% faecal suspension. Extracted RNA was reverse transcribed using an AccuPower® RocketScript RT PreMix (Bioneer Co., South Korea) kit. The primers were specific to the H9 protein gene with the following sequences: H9F: 5′ CTY CAC ACA GAR CAC AAT GG 3′ and H9R: 5′ GTC ACA CTT GTT GTT GTR TC 3′ as described by Lee *et al*. (2001). Five µL of the cDNA were used for PCR amplification. To amplify a 488-bp fragment of the gene encoding the H9 protein gene of AIV, the PCR thermocycling condition was performed as follows: 30 cycles with denaturation at 95°C for 60 s, primer annealing at 53°C for 60 s, primer extension at 72°C for 60 s and a final extension step at 72°C for 10 min (Tajmanesh *et al*., 2006). The PCR product was subjected to 1% agarose gel electrophoresis containing ethidium bromide and visualized under ultraviolet light.

Statistical analysis

HI antibody titers analysis was carried out by calculating a one-way ANOVA and Tukey's multiple comparison as the post hoc and mortality rates analysis using Crosstab (SPSS 11.5 for windows software). Differences were considered significant at $P<0.05$.

Results

Clinical signs

No clinical signs were observed in the negative control group (group 6), indicating that chicks in this experiment were not affected by other pathogens. In five chickens of group 5 (monoinfected with *E. coli*), mild respiratory signs accompanied with mild depression and ruffled feathers were noticed on day five post bacterial inoculation. Chickens challenged only with H9N2 virus (group 4) showed depression, ruffled feathers, coughing, sneezing, conjunctivitis and nasal and ocular discharges from day 4 to 12 post H9N2 challenge. Ruffled feathers, inactivity, reduced appetite and mild respiratory signs were observed in some birds of groups 1 (inoculated with *E. coli* and H9N2 virus simultaneously) and 3 (inoculated with *E. coli* followed by H9N2) on day 2 post influenza infection. After that time, the frequency and severity of respiratory signs increased in chickens of both groups. On day 5 post influenza infection, respiratory signs were observed in most birds of groups 1 and 3. The predominant clinical signs included facial edema, sneezing, coughing, gasping, conjunctivitis and nasal and ocular discharges which were more severe in chickens of group 3 than group 1. In group 3, symptoms reduced from day 9 post H9N2 infection. A few birds showed mild depression until day 12. Chickens in group 1 recovered more quickly than those of groups 2 and 3.

Clinical symptoms in chickens of group 2 included mild respiratory signs and depression which were noticed from day one post *E. coli* infection. By day 3 post *E. coli* infection, most of the chickens in group 2 abruptly showed clinical signs of respiratory disease, including respiratory distress, sneezing, coughing, conjunctivitis, nasal and ocular discharge. Five infected birds died on day 3 post *E. coli* infection (6 days post influenza challenge). Respiratory signs were more pronounced and more frequent compared to other challenged groups and declined from day 6 post *E. coli* infection in this group. A few birds showed depression, facial edema and sneezing to the end of the experiment.

Mortality rates

Total death rates in chickens of groups 1, 2, 3, 4, 5 and 6 during the experiment were 6.45, 17.7, 9.6, 4.8, 1.61 and 1.61%, respectively. The highest mortality rate was observed in chickens inoculated with H9N2 followed by *E. coli* (group 2). Although the total death rate of chickens in groups 1 and 4 was higher than groups 5 and 6, the difference was not significant. The mortality rate in groups 2 and 3 was significantly higher than groups 4, 5 and 6.

Gross lesions

Results related to gross lesions in different groups during days post H9N2 AIV inoculation are briefly shown in Table 2. Birds of the negative control group (6) did not reveal any macroscopic lesions in any of the examined organs. In group 5 (monoinfected with *E. coli*), the macroscopic lesions were characterized as catarrhal exudates in trachea and bronchi. The tracheal mucosal membrane was mildly congested all through the length. Airsacculitis and liver or spleen enlargement were seen in some cases.

In group 4, mild congestion of the tracheal mucosa, catarrhal exudates within the trachea, airsacculitis, mild kidney swelling and petechial hemorrhage in the intestine were noticed from day 3 to 15 post H9N2 challenge. The most remarkable gross lesions were observed on day 6 and 9 PC. The number of chickens showing gross lesions reduced from day 12 PC.

The most frequent lesions in chickens of group 1 were found in their respiratory tracts including tracheal exudates which varied from serous to cast formation, congestion of tracheal mucosa and fibrinous to fibrinopurulent airsacculitis. Moreover, pericarditis, perihepatitis, liver and heart enlargement, ascites and hemorrhages in intestine were noticed. Kidneys were swollen and pale.

From day 3 PC, the infected birds in group 2 displayed typical lesions such as mild to severe tracheal congestion and serous to fibrinous cast formation in tracheal bifurcation which was extended into the primary bronchi, fibrinousairsacculitis, pericarditis, perihepatitis and scattered areas of hemorrhage in the intestine and testis, swollen and pale kidneys, ascites, femoral head necrosis and necrotic foci in liver and spleen upon necropsy from day 6 to 15 PC.

Gross lesions found in birds of group 3 were similar to group 2 and included tracheal congestion, serous to fibrinous cast formation in tracheal bifurcation, airsacculitis, pericarditis, perihepatitis, swollen kidneys hemorrhage in the intestine, femoral head necrosis, liver and heart enlargement and multiple pale necrotic foci in the liver. Most lesions were observed from day 3 to 18 (Table 2).

Microscopic lesions

In the negative control group (group 6), all examined

Table 2: Severity index of lesions in different organs during days post H9N2 challenge

Groups	Days post H9N2 challenge																							
	3				6				9				12				15				18			
	TC	TE	AS	PP	TC	TE	AS	PP	TC	TE	AS	PP	TC	TE	AS	PP	TC	TE	AS	PP	TC	TE	AS	PP
1	0.6*	0.2	1	0.2	1.6	1.6	1.2	1.2	1.2	1.2	1.2	0.8	1.6	0.8	1.6	0.4	0.8	0.4	0.2	0	0	0	0	0
2	0.2	0.8	0.8	0	2.6	2	2.8	1.8	1.4	2.2	2.2	1.8	1	1.6	1.2	0.8	0.8	0.4	0.6	0.4	0	0.2	0.8	0.2
3	1.2	1.4	1.2	0.4	1.2	1.2	2.6	0.8	1.2	1.2	1.6	0.8	0.8	0.4	1.2	0.8	0.6	0.8	0.4	0.4	0.4	0.4	0.4	0
4	0.4	0	0.2	0	1.2	0.6	1	0	0.6	0.4	0.4	0	0.4	0.4	0.4	0	0.2	0.2	0	0	0	0	0	0
5	0	0	0	0	0.8	1.2	0.6	0	1.2	0.4	0.8	0.2	0.4	0.6	0.4	0	0.2	0	0.2	0	0	0	0	0
6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

* Severity index (Σ number of birds with lesion × severity score) / 5 (from 0 to 3). TC: Tracheal congestion, TE: Tracheal exudates, AS: Airsacculitis, and PP: Precarditis and prehepatitis

Table 3: Mean ± SD of HI antibody titer against H9 antigen of different groups throughout the experiment

Days	Groups					
	1	2	3	4	5	6
Day 1	6.7 ± 0.3^{a}	6.6 ± 0.2^{a}	6.7 ± 0.1^{a}	6.5 ± 0.3^{a}	6.6 ± 0.2^{a}	6.4 ± 0.1^{a}
Day 21 (prior to challenge)	2.5 ± 0.1^{a}	2.4 ± 0.1^{a}	2.5 ± 0.1^{a}	2.4 ± 0.1^{a}	2.3 ± 0.1^{a}	2.5 ± 0.1^{a}
Day 42 (end of the experiment)	8 ± 0.1^{a}	8.8 ± 0.4^{b}	9.2 ± 0.4^{b}	8 ± 0.1^{a}	1 ± 0^{c}	1 ± 0^{c}

Different superscript letters ($^{a, b, c}$) in each row indicate significant differences ($P<0.05$)

Table 4: H9N2 virus detection in faeces and trachea of chickens in different groups during post inoculation (PI)

Groups	Days PC											
	3		6		9		12		15		18	
	Faeces	Trachea	Faeces	Trachea	Faeces	Trachea	Faeces	Trachea	Faeces	Trachea	Faeces	Trachea
1	1/5*(20%)	5/5(100%)	1/5(20%)	2/5(40%)	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
2	3/5(60%)	3/5(60%)	1/5(20%)	4/5(80%)	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
3	2/5(40%)	5/5(100%)	0/5	1/5(20%)	0/5	2/5(40%)	0/5	0/5	0/5	0/5	0/5	0/5
4	0/5	5/5(100%)	1/5(20%)	1/5(20%)	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
6	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5

* Number of positive samples/total samples taken

organs (trachea, lung, kidney, spleen) were histologically normal.

The main histologic tracheal lesions in groups 1, 2, 3 and 4 consisted of congestion, hemorrhage, hydropic degeneration and necrosis and deciliation of epithelial cells, infiltration of inflammatory cells and cast formation which were more severe in groups 1, 2 and 3 as compared to group 4. The most frequent microscopic lesions in the lungs, were congestion, hemorrhage, edema and pneumonia. Nephrosis and nephritis, infiltration of inflammatory cells and hemorrhage were observed in kidneys of chickens in groups 1, 2 and 3. In group 4, congestion, mild necrosis and infiltration of inflammatory cells were noticed in kidneys. Necrosis of splenocytes, infiltration of inflammatory cells (particular heterophils) and hemorrhage were observed in the spleens of chickens in groups 1, 2 and 3.

Serological findings

Table 3 shows the results of the HI test on chicken sera to detect antibodies against the H9 antigen during the experiment. The maternal antibody titer of birds from all groups decreased from 6.7 ($-\log_2$) on day 1 to 2.5 ($-\log_2$) on day 21 (before H9N2 challenge). Antibody titer of birds from groups 5 and 6 remained very low to the end of the experiment. Antibody titers against H9N2 were significantly higher in groups 1, 2, 3 and 4 compared to the titers in groups 5 and 6. No significant differences in serum antibodies were observed between chickens in groups 1 and 4 and chickens in groups 2 and 3. HI titers of birds in groups 2 and 3 were significantly higher than those of groups 1 and 4.

All chicks lacked antibodies against Newcastle disease and infectious bronchitis viruses at the end of the experiment.

Virus detection in tissues at different days post-infection

The virus detection results are shown in Table 4. All samples from groups 5 and 6 were void of viruses during the whole sampling period which nullifies the possibility of cross contamination among groups. The virus was detected in tracheas of chickens in groups 1, 2, 3 and 4 on days 3 and 6 PC. On day 3 post H9N2 challenge, although tracheal samples from groups 1, 3 and 4 were 100% positive, viruses were detected in 60% of the tracheal samples from group 2. On the 6th day, the percentages of virus positive tracheal samples in group 1, 2, 3 and 4 were 40, 80, 20 and 20%, respectively.

Faecal samples were positive in groups 1 (20%), 2 (60%), and 3 (40%) on day 3. On day 6, although 20% of faecal samples from groups 1, 2 and 4 remained positive; samples from group 3 were void of virus.

On the 9th day, 40% of the tracheal samples of group 3 were positive while all samples from tracheas and faeces of other groups were 100% negative. This trend continued to the end of sampling period.

Discussion

Considering the significant spread of *E. coli* infections and LPAI viruses in poultry farms, the present study aimed to evaluate possible effects of the time of *E. coli* infection on the pathogenicity of H9N2 AIV in broiler chicks.

In this study, the inoculation of H9N2 virus resulted in a significant increase ($P<0.05$) in the HI antibody response in groups 1, 2, 3 and 4 as compared to non infected groups (groups 5 and 6), clearly demonstrating that birds of the control group were not infected during the experiment. Virus detection studies (Table 4) also revealed the same pattern. The highest antibody titer was observed in birds of group 3. This could be explained by the prolonged H9N2 AIV replication in the respiratory tract (Table 4) and the longer stimulation of the immune system by the H9N2 virus. The next highest HI antibody titer was found in chicks exposed to *E. coli* after AIV infection. Those only inoculated with H9N2 or receiving simultaneous *E. coli* and H9N2 infections showed lower antibody titers than those in groups 2 and 3.

Generally speaking, the rate and duration of virus detection from trachea was much higher than those of faecal samples. In the group pre exposed to *E. coli* (group 3), the duration of viral detection in trachea was

longer than the others. In this group, the lesions' severity indices were higher than others in day 3 PI, and lasted longer up to day 18 PI (Table 2). This finding indicates that the pre-exposure of *E. coli* could provide an opportunity for H9N2 virus to persist longer in the trachea. Although the exact role of pre-exposure to *E. coli* during the virus shedding period could not be deduced from the present study, the immune suppressive effect of *E. coli* (Nakamura *et al*., 1986) or the production of trypsin-like protease (Kato *et al*., 1992; Jiang *et al*., 1998) by this pathogen may have been involved. All H9N2 infected chicks showed much sever clinical signs and higher mortality rates than those infected either with H9N2 or *E. coli* alone. These findings may explain the reason for the higher mortality rate of this virus in field outbreaks due to the fact that *E. coli* being very ubiquitous in chicken farms all around the world. Stipkovits *et al*. (2012) reported that clinical symptoms, pathologic lesions and the reduction in weight gain were much more significant in the group challenged with both *Mycoplasma gallisepticum* (Mg) and LPAI H3N8 virus than that challenged with Mg alone. Vasfi-Marandi *et al*. (2007) also demonstrated that the simultaneous inoculation of chickens with Mg play an important role in the formation of tracheal casts which might have increased the pathogenesis of the H9N2 infection. Concurrent infection with the H9N2 virus and ORT bacteria exacerbates clinical signs and gross lesions in infected chickens (Goudarzi *et al*., 2014). Other researchers have explained that H9N2 AIV infection is a predisposing factor for respiratory disease complexes (Bano *et al*., 2003; Barbour *et al*., 2009). Our results indicate that when *E. coli* and H9N2 are combined, they can mutually exacerbate the condition of either disease in broiler chicks as compared to single infected birds. The loss of cilia on the epithelial surface of the trachea is associated with AI infection and may enhance infections with *E. coli*. Therefore, *E. coli* may gain access to the circulation following damage to the respiratory mucosa (Ginns *et al*., 1998) caused by H9N2 infections and induce severe clinical symptoms. It has been demonstrated that *E. coli* can spread faster and can cause bacteremia, severe airsacculitis, and peritonitis in conjunction with mycoplasma infection (Mészáros and Stipkovits, 1967a, b). The production of type I interferon during influenza A virus infection in mice enhanced susceptibility to gram-negative and gram-positive bacterial pneumonia via immunosuppression (Lee *et al*., 2015).

In conclusion, this study demonstrates that the infection of broilers with *E. coli* prior, post or concurrently with the H9N2 influenza virus induces higher mortality rates and when combined, the two can mutually exacerbate the condition of either disease in broiler chicks as compared to each one alone.

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Conflict of interest

The authors declare no conflict of interest.

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