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Original article

EFFECT OF INFECTIOUS BRONCHITIS AND NEWCASTLE DISEASE VACCINES ON EXPERIMENTAL AVIAN INFLUENZA INFECTION (H9N2) IN BROILER CHICKENS

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Summary

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Despite the fact that H9N2 avian influenza virus (AIV) is considered a low-pathogenic agent, frequent outbreaks of this subtype have caused high mortality and economic losses in poultry farms around the world including Iran. Coinfection with a respiratory pathogen or environmental factors may explain the exacerbation of H9N2 AIV infection. In this study, the role of infectious bronchitis (IB) vaccines (H120 and 4/91) and Newcastle disease (ND) vaccines (B1 and LaSota) on experimental H9N2 AIV infection was investigated in 180 broiler chickens allotted into 6 groups (n=30). At the age of 18 days, groups 3 and 4 received H120 and 4/91 infectious bronchitis live vaccines (IBLVs) and groups 5 and 6 received B1 and LaSota Newcastle disease live vaccines (NDLVs), respectively. At the age of 20 days, all birds in the experimental groups except the negative control group (group 1), were inoculated intra-nasally with H9N2 AIV. After the inoculation, clinical signs, gross and microscopic lesions, and viral detection were examined. The results of this study revealed that clinical signs, gross and microscopic lesions were more severe in the AIV challenged groups which had been previously vaccinated with IB vaccines. In addition, AI viral RNA from tracheal and faecal samples in IB vaccinated birds were recovered at a higher rate. Moreover, in the 4/91 IB vaccinated group, the AI virus shedding period was longer than the other challenged groups. In conclusion, infectious bronchitis live vaccines (IBLVs) exacerbated the H9N2 AIV infection; also, 4/91 IBLV extended AI virus shedding period and increased the recovery rate of AI virus from feaces. However, the coinfection of Newcastle disease live vaccines (NDLVs) had no considerable adverse effects on AIV infection in broiler chickens.

Key words: avian influenza, coinfection, infectious bronchitis live vaccine, Newcastle disease live vaccine

INTRODUCTION

Avian influenza viruses (AIVs), the members of the *Orthomyxoviridae* family, belong to the genus influenza virus A and can cause an infectious disease in the sus-

ceptible species (Swayne & Suarez, 2000). AIVs are classified as either high-pathogenic or low-pathogenic based on the existence of multiple basic amino acids at the cleavage site of the HA precursor protein and virulence in chickens (Swayne *et al*., 2013). Despite the low-pathogenic nature of H9N2 AIVs, there have been frequent outbreaks of this subtype, sometimes with high mortality, in Asian countries including Iran (Nili & Asasi, 2002; 2003; Capua & Alexander, 2004). Coinfections or environmental factors may explain the exacerbation of H9N2 AIV infection. Furthermore, some studies have shown that the coinfection of H9N2 AIV with infectious bronchitis (IB) could increase the clinical signs, pathologic lesions, mortality, and shedding period of H9N2 AIV infection (Haghighat-Jahromi *et al*., 2008; Seifi *et al*., 2012). IB and Newcastle disease (ND) are the most important and prevalent diseases in Iran. To control these diseases, both live and inactivated vaccines are extensively used in poultry farms. The live vaccines propagate within the birds and cause various extent of cellular damages, which are referred to as vaccination reaction. Although the reaction in the healthy birds has a minimum effect, in some circumstances such as concurrent diseases and poor management facility, it can prompt pathologic injuries followed by clinical symptoms (Smits *et al*., 1976; Glisson, 2013). Previous studies have revealed that even IB live vaccine could enhance the severity of H9N2 AIV infection (Haghighat-Jahromi *et al*., 2008; Tavakkoli *et al*., 2009; Hassan *et al*., 2017). Since there are limited studies comparing the effects of different live vaccines on H9N2 AIV, this question yet has remained unclear whether only live IB vaccines can exacerbate the H9N2 AIV infection or other respiratory vaccines such as ND vaccines may have the same detrimental effects. Hence, the aim of this study was to evaluate the effects of infectious bronchitis live vaccines (IBLVs) and Newcastle disease live vaccines (NDLVs) on the experimental H9N2 AIV infection in broiler chickens.

MATERIALS AND METHODS

Animal research ethics

This experiment was performed under the approval of the state committee on animal ethics, Shiraz University, Shiraz, Iran. Also, the recommendations of European Council Directive (86/609/EC) of November 24, 1986, regarding the protection of animals used for experimental purposes, were considered.

Experimental design

One hundred and eighty one-day-old Arbor Acres broiler chicks were randomly divided into six groups; and raised for 42 days under equal and controlled conditions in six different isolated poultry rooms. The chickens were provided with food and water *ad libitum* in the Animal Research Unit of Shiraz University Veterinary School. At the age of 18 days, groups 3 and 4 received H120 and 4/91 IBLVs (Ceva Company, Budapest, Hungary), while groups 5 and 6 received B1 and LaSota NDLVs (Ceva Company, Budapest, Hungary), respectively, via eye drop according to the manufacturer's instructions. The negative control group (group 1) and H9N2 AIV infection group (group 2) did not receive any vaccines. At the age of 20 days, all birds of the experimental groups, except group 1, were inoculated intra-nasally (0.1 mL/bird) with the allantoic fluid containing AIV (A/chicken/Iran/SH-110/99(H9N2), 1×10^6

EID_{50}). The EID_{50} was calculated based on the Reed and Muench formula (Reed & Muench, 1938). All birds in the negative control group were inoculated with the same volume of sterile normal saline. Prior to vaccination (day 17) and challenge (day 19), five birds of each group were tested for AI and ND antibodies by haemagglutination inhibition (AI H9N2 and ND antigens, Razi Vaccine and Serum Research Institute, Karaj, Iran) and IB antibody by ELISA (IDEXX: ELISA IBV Test kit, USA) respectively, to show the antibody status of the birds against AI, ND and IB viruses.

Clinical signs and gross lesions

After the inoculation, birds from all groups were monitored daily and specific clinical signs were recorded for 10 days. The clinical signs were scored as described by Jackwood *et al*. (2010) with some modification (Table 1). On days 3, 5, 7, and 11 PI, five birds from each group were euthanised by an overdose of sodium thiopental. At post-mortem inspection, based on the presence of lesions including tracheal discharge/hyperaemia/cast, syringeal cast, air sacs opacity, lung discharge/hyperaemia/cast, kidney hyperaemia, spleen hyperaemia, bursa of Fabricius atrophy/hyperaemia and thymus atrophy/hyperaemia, the birds were scored from 1 (normal) to 4 (severe).

Histopathology

Trachea, lung, kidney, thymus, spleen, and bursa of Fabricius were taken from five euthanised birds on days 3, 5, 7, and 11 PI; and collected in 10% neutral buffered formalin until the histopathology examination. The tissues were processed into paraffin; then, 5 µm sections were cut for haematoxylin and eosin (H&E) staining. The microscopic lesions were scored based on the previous studies (Swayne & Slemons, 1995; Mo *et al*., 1997; Alvarado *et al*., 2003) with some modifications (Table 1).

RT-PCR

Faecal and tracheal samples were aseptically collected at days 0, 3, 5, 7, 11, and 15 PI from five birds of each group for AIV detection by reverse transcriptase-polymerase chain reaction (RT-PCR) assay. All samples were stored at −70 °C until used later in the study. For the total RNA extraction, RNX^{TM}-Plus solution (Cinnagen, Tehran, Iran) was used according to the manufacturer's protocol. Briefly, 1 mL of the ice cold RNX solution was added to about 100 mg of homogenized tissue; then, 200 µL of chloroform was added into the mixture and centrifuged at 12000 rpm at 4 ºC for 15 min. Later, the aqueous top phase was transferred to an equal volume of isopropanol and was centrifuged at 12000 rpm at 4 ºC for 15 min. After the washing step with 75% ethanol, the pellet was completely dissolved in 50 µL of sterile distilled water and stored at −70 °C to be used later in the study. The cDNA was synthesised using a PrimeScript™ RT reagent Kit (Takara Bio INC, China) according to the manufacturer's instruction. The primers were specific for H9 protein gene of H9N2 AIV (Forward primer 5´-CTY CAC ACA GAR CAC AAT GG-3´and Reverse primer 5´-GTC ACA CTT GTT GTT GTR TC-3´) (Lee *et al*., 2001). Five microliters of total RNA and 10 pmol of H9-specific forward primer were used for cDNA synthesis. The PCR test was performed to amplify a 488-base-pair fragment of H9 protein gene of H9N2 AIV using the PCR MasterMix kit (Cinnagen, Tehran, Iran) in a 25 µL-reaction mixture containing 5 µL cDNA and 10 pmol of the

Table 1. Scoring system of the clinical signs and histopathology lesions

Score	Description
	Clinical signs
1	normal
2	watery eyes/nares
3	watery eyes/nares with/or sneezing/coughing
4	watery eyes/nares, sneezing/coughing with/or tracheal rales
	Histopathology
Trachea	
1	no lesion
2	mild epithelial hyperplasia and lymphocyte infiltration in submucosa
3	moderate epithelial hyperplasia with deciliation and moderate submucosal lymphoid infiltration
4	extensive epithelial hyperplasia and submucosal lymphoid infiltration with the flattened superficial epithelial layer
Lung	
1	no lesion
2	lymphocyte infiltration in submucosa of secondary bronchus
3	lymphocyte infiltration in the interstitial area with haemorrhage and oedema
4	necrosis, and fibrin leakage
Kidney	
1	no lesion
2	epithelial cell swelling and degeneration
3	multifocal nephritis
4	diffuse nephritis and necrosis
Bursa of Fabricius	
1	lack of inflammatory and/or necrotic lesion
2	mild inflammatory and/or necrotic lesion
3	moderate inflammatory and/or necrotic lesion
4	severe inflammatory and/or necrotic lesion
Spleen	
1	no lesion
2	reticuloendothelial cell hyperplasia
3	increase in lymphoid follicle numbers/ increase or decrease in the size of individual lymphoid follicles
4	necrosis
Thymus	
1	no lesion
2	decreased cortical lymphocytes
3	decreased medullary lymphocytes
4	necrosis

forward and reverse primers. The reaction mixture was subjected to 95 ºC for 3 min, followed by 35 cycles of 94 ºC for 35 s, 50 ºC for 45 s, and 72 ºC for 45 s, and a final extension at 72 °C for 10 min (Lee *et al*., 2001). The PCR products were sepa-

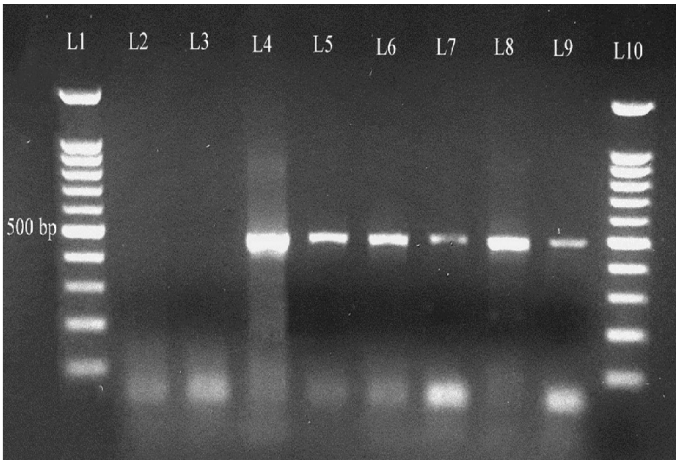

Fig. 1. Results of the AIV PCR assay. *H9* gene of H9N2 AIV (488-bp product) in the 1% agarose gel. Lane L1 and L10: DNA marker (100-bp), L2: negative control (RNA of negative chicken), L3: negative sample from group 1 (negative control), L4: positive control (RNA of the challenging AIV), L5, L6, L7, L8 and L9: positive samples from groups 2 (H9N2 AIV), 3 (H9N2 AIV + H120 IBLV), 4 (H9N2 AIV + 4/91 IBLV), 5 (H9N2 AIV + B1 NDLV), and 6 (H9N2 AIV + LaSota NDLV), respectively.

rated in the agarose gel (1%) and visualised later under ultraviolet light (Fig. 1).

Statistical analysis

The data from clinical signs' scores were analysed using the chi-square test and Fisher's exact test. The gross and microscopic lesions' scores were analysed by the Kruskal-Wallis test. Following a statistically significant result on the Kruskal-Wallis test, the Mann–Whitney U test was used for pair-wise comparison between the treatment groups. The statistical analysis was performed by SPSS software, version 16.0 (SPSS Inc., Chicago, IL). P values of less than 0.05 were considered statistically significant.

RESULTS

Clinical signs

Prior to inoculation, the birds of all groups were monitored and no clinical signs were seen. Respiratory clinical signs, such as discharge from eyes/nares, sneezing/coughing and tracheal rale, were observed after the post-challenge with AIV in the birds of the challenged groups. The typical respiratory clinical signs started to appear at day 2 post inoculation (PI) in the challenged groups. As shown in Table 2, the clinical signs in the birds of groups 3 (H9N2 AIV + H120 IBLV) and 4 (H9N2 AIV + 4/91 IBLV) were significantly ($P<0.05$) more severe than those of group 2 (H9N2 AIV inoculated only) at day 2 PI. The highest severity of the clinical signs was seen in the birds from group 4 at days 5 and 8 PI. The severity of the clinical signs in all the challenged groups diminished after days 8–10 PI. Birds in the negative control group did not show any respiratory clinical signs. No mortality was observed in all the experimental groups during the post-challenge days.

Gross lesions

Tracheal mucosal discharge and hyperaemia were seen in the all challenged groups

during the post-inoculation days (3, 5, 7 and 11). The fibrinous cast was seen in the trachea of birds from groups 3 and 4 at days 3 and 7 PI, respectively. Slight syringeal cast in the group 2 at day 5 PI, group 3 at days 5 and 11 PI, and group 4 at days 3 and 7 PI was also observed. The fibrinous cast in the lung was only observed in a bird from group 4 at day 7 PI. Mucosal discharge and hyperaemia in the lung were also observed in the challenged groups. Air sacs' opacity among the challenged groups, was significantly ($P<0.05$) more severe in group 3 at day 3 PI. In the kidney, spleen, and thymus, hyperaemia was observed in all challenged groups. Slight atrophy in the thymus and bursa of Fabricius was also seen among all the challenged groups. However, hyperaemia in bursa of Fabricius was significantly more in group 4 at day 7 PI in comparison with groups 1, 2, and 5 ($P<0.05$). The birds in the negative control group were normal at necropsy examination.

Histopathology

Mean microscopic scores of different organs in all groups are shown in Table 3. In the trachea, microscopic lesions varied from slight lymphocytic infiltration in submucosa to necrotic tracheitis in the challenged groups (Fig. 2). Tracheal lesions were significantly ($P<0.05$) more severe in group 4 compared to those of groups 1, 2, 5, and 6 at days 3 and 7 PI, and compared to the groups 1 and 2 at day 11 PI. And also these lesions were significantly ($P<0.05$) more severe in the group 3 at day 5 PI compared to the other groups (1, 2, and 5). Mild renal epithelial cell swelling and degeneration accompanied by slight congestion were seen in the kidneys of the challenged groups. In the group 4 at day 5 PI, the kidney lesions were more severe and significantly ($P<0.05$) different with those of group 2 among other challenged groups. The bursa of Fabricius lesions such as congestion, lymphocyte depletion and necrosis, were seen in the challenged groups. However, the severity of these lesions in group 6 (H9N2 AIV + LaSota NDLV) was significantly ($P<0.05$) higher than that of group 2 at days 5 and 11 PI. In the spleen, an increase in both the number and size of lymphoid follicle was observed in the

Table 2. Mean score of clinical signs of the experimental groups at different days post-inoculation

Group	Clinical sign score					
	Day 2 PI	Day 3 PI	Day 4 PI	Day 5 PI	Day 8 PI	Day 10 PI
Control	1^{b}	1^{b}	1^{b}	1^{b}	1^{b}	1^{b}
AIV	1.2^{b}	1.6^{a}	1.6^{a}	2.4^{a}	2.7^{a}	1.8^{ab}
AIV+H120 IBLV	1.9^{a}	1.6^{a}	2.2^{a}	2.7^{a}	2.7^{a}	2^{ab}
AIV+4/91 IBLV	2.1^{a}	2.2^{a}	2^{a}	3^{a}	3^{a}	2^{ab}
AIV+B1 NDLV	1.5^{ab}	1.7^{a}	1.8^{a}	2.4^{a}	2.5^{a}	1.7^{ab}
AIV+LaSota NDLV	1.7^{ab}	1.9^{a}	2.1^{a}	2.4^{a}	2.8^{a}	2.5^{a}

The scores are from 1 (normal) to 4 (most severe changes). PI: post-inoculation. Different superscripts in each column indicate significant difference ($P<0.05$).

Table 3. Mean score of microscopic lesions in different organs at different days post-inoculation

DPI	Group					
	Control	AIV	AIV+H120 IBLV	AIV+4/91 IBLV	AIV+B1 NDLV	AIV+LaSota NDLV
Trachea						
3	1^{d}	1.4^{cd}	2.6^{ab}	3^{a}	2^{bc}	2^{bc}
5	1^{e}	1.2^{de}	3.4^{ab}	3^{ab}	1.8^{cd}	2.6^{bc}
7	1.2^{c}	2^{bc}	2.8^{ab}	3^{a}	1.8^{c}	1.8^{c}
11	1^{bc}	1.2^{bc}	2.4^{a}	2.6^{a}	1.4^{ab}	2.2^{a}
Kidney						
3	1^{a}	1.4^{a}	1.2^{a}	1.4^{a}	1.2^{a}	1.4^{a}
5	1^{b}	1^{b}	1.4^{ab}	1.8^{a}	1.4^{ab}	1.6^{ab}
7	1^{a}	1.2^{a}	1.2^{a}	1.2^{a}	1.2^{a}	1^{a}
11	1^{a}	1^{a}	1^{a}	1^{a}	1^{a}	1^{a}
Bursa of Fabricius						
3	1^{b}	1.4^{ab}	1.8^{ab}	2^{a}	1.6^{ab}	1.8^{ab}
5	1^{b}	1.2^{bc}	1.8^{ab}	1.8^{ac}	1.4^{ab}	2.2^{a}
7	1^{b}	1.8^{a}	2.6^{a}	2.4^{a}	2.6^{a}	2.6^{a}
11	1^{b}	1.2^{b}	2^{ab}	2^{a}	1.8^{ab}	2.6^{a}
Spleen						
3	1^{a}	1^{a}	1.4^{a}	1.4^{a}	1^{a}	1.4^{a}
5	1^{a}	1^{a}	1^{a}	1.4^{a}	1.4^{a}	1.8^{a}
7	1.4^{a}	1.4^{a}	1.8^{a}	2.2^{a}	1.8^{a}	2.2^{a}
11	1^{a}	1^{a}	1.4^{a}	1.8^{a}	1.4^{a}	1.4^{a}
Thymus						
3	1^{a}	1^{a}	1^{a}	1^{a}	1^{a}	1^{a}
5	1^{a}	1^{a}	1^{a}	1^{a}	1^{a}	1.2^{a}
7	1^{a}	1.2^{a}	1^{a}	1.2^{a}	1^{a}	1^{a}
11	1^{a}	1^{a}	1^{a}	1^{a}	1^{a}	1^{a}

The scores are from 1 (normal) to 4 (most severe changes). DPI: days post-inoculation. Different superscripts in each row indicate significant difference ($P<0.05$).

challenged groups, although there was no significant difference among them. A decreased number of cortical lymphocytes and congestion were found in the thymus of the challenged groups at days 5 and 7 PI without any significant difference. Fibrinous exudative material was observed in the lung of a bird from group 4 at day 7 PI. However, the congestion was the only microscopic finding in the lung of the other challenged birds. No microscopic lesions were found in the negative control group.

Virus detection

The results of AIV genomic detection from tracheal tissue and the faeces of five birds from each group at days 3, 5, 7, 11, and 15 PI are shown in Table 4. Prior to the challenge (day 0 PI), AIV was not detected from tracheal and faecal samples of all experimental groups. However, the

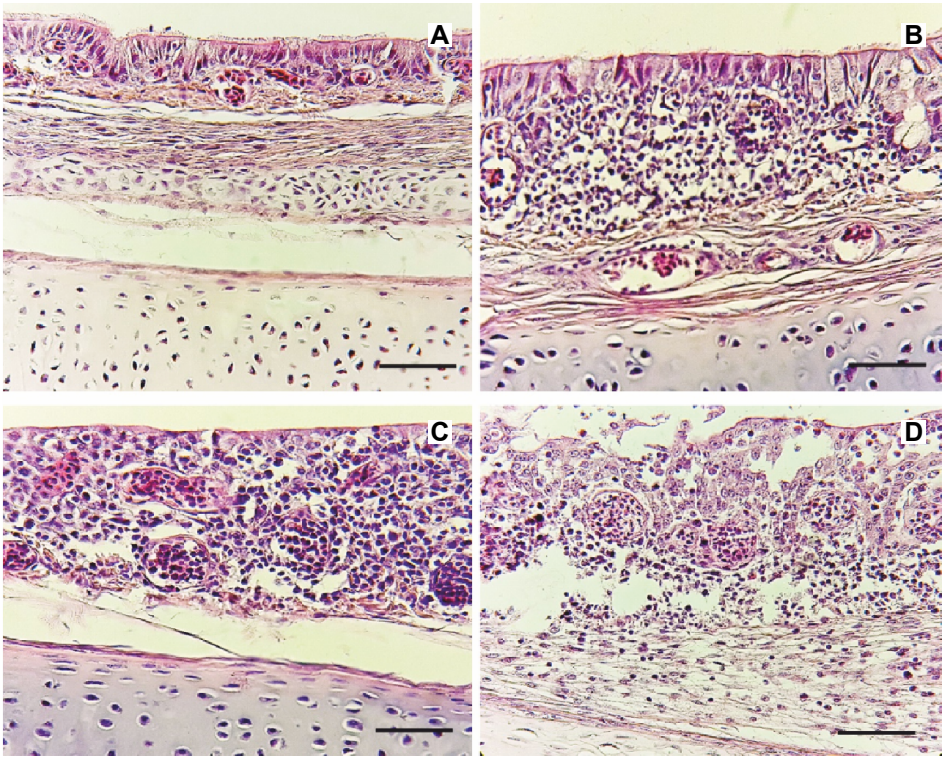

Fig. 2. Microscopic changes of the trachea in chickens experimentally infected with H9N2 AIV and vaccinated against IBVs and NDVs. **A.** score 1: no lesions; **B.** score 2: mild lymphocyte infiltration in lamina propria; **C.** score 3: complete loss of cilia with moderate tracheitis; **D.** score 4: necrotic tracheitis (H&E, bar = 40 µm).

Table 4. Virus detection of H9N2 AIV in trachea and faeces samples at different days post-inoculation

DPI	Tissue	Group					
		Control	AIV	AIV+H120 IBLV	AIV+4/91 IBLV	AIV+B1 NDLV	AIV+LaSota NDLV
3	Trachea	0/5*	1/5	2/5	1/5	1/5	0/5
	Feces	0/5	0/5	1/5	1/5	0/5	0/5
5	Trachea	0/5	2/5	2/5	3/5	1/5	1/5
	Feces	0/5	2/5	1/5	2/5	1/5	2/5
7	Trachea	0/5	2/5	3/5	2/5	2/5	1/5
	Feces	0/5	3/5	4/5	5/5	3/5	1/5
11	Trachea	0/5	1/5	1/5	2/5	0/5	0/5
	Feces	0/5	2/5	2/5	2/5	1/5	1/5
15	Trachea	0/5	0/5	0/5	0/5	0/5	0/5
	Feces	0/5	0/5	0/5	1/5	0/5	0/5

*Number of H9N2 AIV positive samples/total samples. DPI: days post-inoculation.

virus was detected in the trachea of the challenged groups during different post-inoculation days (3, 5, 7 and 11). At day 15 PI, the virus was not recovered from the tracheal tissue of any of the birds from the challenged groups. The virus was also detected within the faecal samples of the birds from the challenged groups. However, at day 15 PI, the virus was only detected in the faecal samples of a bird in the group 4.

Serology

The birds' antibody titres against AI, ND and IB viruses were below the cut off points of theses diseases before the vaccination and challenge days (data not shown).

DISCUSSION

Although H9N2 AIV has been considered to be a mild-pathogenic agent, frequent incidence of this agent has caused increased mortality and resulted in great economic losses in the broiler chicken farms in some Asian and Middle East countries including Iran (Nili & Asasi, 2002; 2003; Capua & Alexander, 2004). Some researchers have attributed the increased severity of the H9N2 AIV infection to the mixed infection with other respiratory pathogens such as IBV (Seifi *et al*., 2012), *Staphylococcus aureus* or *Avibacterium paragallinarum* (Kishida *et al*., 2004), and *Escherichia coli* (Bano *et al*., 2003). Moreover, even live IB vaccine could exacerbate H9N2 AIV infection (Haghighat-Jahromi *et al*., 2008; Tavakkoli *et al*., 2009; Hassan *et al*., 2017). To find out more about the exacerbating factors, we carried out an experimental coinfection with H9N2 AIV with IBLVs and NDLVs. In agreement with the findings of the other studies, the respiratory clinical signs and gross lesions in mixed H9N2 AIV infection with both IBLVs (H120 and 4/91 strains) were more severe than those of other challenged groups. This indicates that even the vaccine strains of IBV could increase the severity of H9N2 AIV infection (Haghighat-Jahromi *et al*., 2008; Seifi *et al*., 2012). However, coinfection with NDLVs and H9N2 AIV had no remarkable effect on the severity of the clinical signs, which is consistent with the results of a study conducted by Fazel *et al*. (2018) on the coinfection with NDV (LaSota) and AIV (H9N2). According to the microscopic findings, the trachea was the most affected tissue in our study and the lesions were most severe in mixed H9N2 AIV and 4/91 IBLV infection. In the single H9N2 AIV infection, similar to our findings, predominant lesions of the trachea were congestion, deciliation and leukocytic infiltration (Nili & Asasi, 2002). In an experimental intravenous AIV (H9N2 subtype) infection in chickens, tubulointerstitial nephritis in the kidneys was the most frequent microscopic findings (Hablolvarid *et al*., 2003) whereas in our study, mild renal epithelial cell swelling and degeneration accompanied by slight congestion were observed in the challenged groups; also, the lesion rates were higher in the mixed H9N2 AIV and 4/91 IBLV infection. Previous studies indicated that H9N2 AIV affected the immune system of broiler chickens by lymphocyte apoptosis, and subsequently, it caused a decrease in the numbers of $CD4^+$ and $CD8^+$ T cells (Hadipour *et al*., 2011; Qiang & Youxiang, 2011). In the current study, congestion, lymphocyte depletion, and necrosis in the bursa of Fabricius were observed in the mixed infection groups. Specifically, the lesions were most remarkable in mixed H9N2 AIV and LaSota NDLV infection. In an

evaluation of H9N2 AIV dissemination in various organs of experimentally infected broiler chickens, Mosleh *et al*. (2009) revealed that the virus had tropism for the lungs, trachea, kidneys, spleen, and the digestive system. In the present study, we detected the genome of H9N2 AIV in the trachea and faeces at days 3, 5, 7, and 11 post-inoculation. In another experimental study, coinfection of H9N2 AIV with IBLV (H120 strain) showed that IBLV enhanced AIV propagation as well as extended the viral shedding period (Haghighat-Jahromi *et al*., 2008). Similarly, in our study the number of positive samples for H9N2 AIV in the groups 3 (H9N2 AIV + H120 IBLV) and 4 (H9N2 AIV + 4/91 IBLV) was higher compared to the other challenged groups. Furthermore, in the group 4, the viral RNA was recovered over a longer period (up to the 15th day PI). It is not yet clear how the IBV increases the viral replication of H9N2 AIV in the chickens, but there are some possible explanations for it. Klenk & Garten (1994) indicated that only trypsin-like proteases which are secreted from respiratory and intestinal tracts cells, could cleave the HA protein of H9N2 AIV and, subsequently, trigger the AI infection. One of the possible explanations for such exacerbation is that IBV may stimulate the chicken host cells to produce or secrete more protease. Interestingly, in our study, we found out that in the mixed H9N2 AIV and both NDLVs (B1 and LaSota strain) infection groups, the viral detection was less than in single H9N2 AIV infection group on day 11 PI, which may explain NDV interference with the influenza virus replication (Umar *et al*., 2015). Similar to our findings, Ge *et al*. (2012) showed that the previous infection with NDV could suppress H9N2 AIV replication.

In conclusion, the results of this study indicate that IBLVs could exacerbate H9N2 AIV infection. In addition, 4/91 IBLV was found able to extend AI virus shedding period as well as to increase the detection rate of AIV from faeces of the broiler chickens. However, NDLVs coinfection had no significant adverse effects on AIV infected broiler chickens.

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