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Evaluation of clinical signs, gross lesions and antibody response in experimental of indvidual and co-infection of H9N2 avian influenza and Ornithobacterium rhinotracheale in SPF chickens

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ABSTRACT

In this study, clinical signs, gross lesions and antibody titer of (A/Chicken/Iran/ m.1/2010) H9N2 virus and (ORT - R87-7/1387) Ornithobacterium rhinotracheale bacteria alone and a co-infected group in SPF broiler chickens were investigated. Eighty 1-day-old specific pathogen-free White Leghorn chickens were randomly divided into four equal groups. At the age of three weeks, the chicks in the three experimental groups were inoculated by virus and bacteria, individually or concurrent and in control group allantoic fluid was inoculated. We used PCR for detection of virus in various organs of experimentally infected broiler.The results showed that experimental co-infection of AIV and ORT increased the severity of clinical signs, mortality rate and gross lesions. The HI titer against AIV infection in the co-infected group was higher than the HI titer of other groups, which may indicate that ORT could promote the propagation of H9N2 AIV or stimulate the immune response.

Key words: H9N2 influenza virus, ORT, Co-infection, Clinical signs, SPF chickens

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INTRODUCTION

Avian influenza is a contagious disease caused by type A influenza viruses. In spite of the mild nature of H9N2 low pathogenic avian influenza virus, frequent disease outbreaks with high mortality due to the virus have been observed in different parts of the world including Iran [7,15,19,21] .

Researchers frequent field observation showed that swelling of periorbital tissues and sinuses, typical respiratory discharge and severe respiratory distress in affected chicken flocks resulted in mortality up to 65% in broiler chicken farms and the most prominent lesions in affected dead birds were respiratory airway hyperemia and severe exudation, which lead to tubular cast formation in the tracheal bifurcation, extending to the lower bronchi [6,7] .

Co-infection with other respiratory pathogens may complicate the respiratory disease syndrome during outbreaks of non-highly pathogenic avian influenza viruses and cause severe disease and high mortality [1,9,14] .

The objective of this study was to investigate the clinical signs and gross lesions in SPF White Leghorn chickens associated with ORT and H9N2 alone and co-infected with H9N2 and ORT. The antibody titer of the affected chicks were also measured.

MATERIALS AND METHODS

Virus

The virus isolate used in this study was A/Chicken/Iran/ m.1/2010 (H9N2). It was obtained from Razi Vaccine and Serum Research Institute, Karaj. The virus was passaged 2 times in 11- day -old embryonated eggs by the chorioallantoic route. The dose of inoculum used in birds was 0.1 mL of 1×10^6 mean 50% egg infective dose/mL per bird [10] .

Bacteria

The Iranian isolate R87-7/1387 (JF810491) of ORT was used in this study. This isolate was obtained from Razi Vaccine and Serum Research Institute with 0.5mL of 1×10^{10} CFU.

Chickens

Eighty 1-day-old White Leghorn were obtained from the specific pathogen free (SPF) embryonated chicken eggs from Venky's company (Venky's, India), were divided randomly into four groups (20 chicks per group). They were kept in separate positive pressure isolators at Animal Research Unit of Razi Vaccine and Serum Research Institute and received feed and water *ad libitum* during the experiment.

Experiments design

Prior to challenge, all birds were serologically tested using hemagglutination inhibition test (HI) and they were negative for antibodies to H9N2 influenza virus antigens. At the age of 21 days, all birds in ORT group were inoculated intratracheally with 0.5 mL of PBS containing 1×10^{10} CFU of ORT/ mL. Birds in H9N2 group were inoculated with 0.1 mL of chorioallantoic fluid containing containing 1×10^6 mean 50% egg infective dose of H9N2 /bird by eye drop. Birds in AI and ORT co-infected group were inoculated with chorioallantoic fluid containing 10^6 EID50/0.1 ml AIV by eye drop and intratracheally with PBS containing 1×10^{10} CFU/0.5 ml of ORT. Each bird in group four was inoculated with 0.1 mL of sterile PBS by eye drop as a sham-inoculated control. After challenge, all the chickens were monitored daily for 15 days for clinical signs, antibodies to H9N2 and mortality. No vaccine was used in the experiment. On days 2, 10 and 16 post inoculation (PI), three chickens from experimental and control groups were randomly selected and used for sample collection. Then, they were bled before euthanasia. Gross lesions were recorded. Samples of different tissues including trachea, lungs and liver were aseptically collected for virus detection using RT-PCR technique and ORT was isolated from swab samples. Sera of the birds were collected at the above mentioned days.

Isolation and identificationof ORT

Swab samples from trachea and lungs of birds of the experimental groups were streaked onto 5% sheep blood agar with 10 µg/ml of gentamicin. Plates were incubated at 37°C under 5–10% CO2 atmosphere for 24–48 hrs [2].Colonies, which were circular and small (1–3 mm in diameter), opaque to greyish, and nonhaemolytic were selected [16] . Colonies with characteristics of ORT were stained by Gram's method, identified biochemically to confirm the main phenotypic traits, and antigenically tested by agar gel precipitation (AGP), as previously described [18] .

The biochemical characterization was performed with oxidase, catalase, Mac- Conkey, arginine, lysine, ornithine, phenylalanine, urea, indole, H2S, Vogues- Proskauer, and carbohydrate fermentation. On carbohydrate fermentation tests, tubes containing phenol red broth, supplemented with 1% (w/v) glucose, mannose, lactose, sucrose, sorbitol, maltose, and dulcitol were each inoculated with ORT-suspected overnight cultures. All inoculated tubes were incubated at 37°C for 24–48 hrs and observed or tested for biochemical characterizations [2] .

RNA extraction

All tissue samples were homogenized with triptose phosphate buffer and centrifuged for 5 min and then the supernatant liquid stored at −70°C until using. RNA was extracted from the samples using High Pure Viral Nucleic Acid Kit (a commercial RNA extraction kit, CinnaGen, Iran) following the manufacturer's instructions.

RT-PCR

The cDNA was synthesized using AccuPowder®RT PreMix kit (BioNeer, South Korea) according to the manufacturer's instruction. The primers were specific for H9 protein gene and are shown in Table 1 [5] . Five µl of total RNA and 20 pmol of each H9- specific primers were used for cDNA preparation. PCR was performed to amplify 486-bp fragment of H9 protein gene of avian influenza virus using the AccuPower PCR PreMix kit (BioNeer, South Korea) in a 20 µl reaction mixture containing 5 µl cDNA and 10 pmol of each primers. The reaction mixture was subjected to 35 cycles of 94ºC, 53ºC and 72ºC each for 1 min, followed by a final extension at

72°C for 10 min [11] .The PCR products were separated in 1% agarose gel and visualized under ultraviolet light(Fig. 1).

Serology
Serum samples were tested for the presence of antibodies to the challenge virus antigen using the HI test . The sera were also tested, by HI test, for Newcastle disease virus.

Statistical analysis
The data of HI titers were analysed using one-way ANOVA. Differences among means of the groups were analysed by the Duncan test using SPSS for windows version 13. A P value less than 0.05 was considered significant.

RESULTS

Clinical findings
AIV (H9 subtype) and ORT co-infected group
Some chickens of the this group showed ruffled feathers, depression, reduced appetite and respiratory distress (coughing, sneezing and gasping) on days 2 PI. One birds showed cyanosis of wattles and combs in this day. By day 3 PI, a few chickens exhibited diarrhea. The most remarkable clinical signs appeared on day 3 PI. But on day 6 PI the number of chickens showing clinical signs reduced. The clinical signs disappeared at 10 day PI. The infected birds died among days 2, 3 and 5 PI. There was 15% (3/20) mortality in this group.

AIV (H9 subtype) group
Clinical signs such as depression and ruffled feather appeared on day 4 PI. These clinical signs disappeared on day 6 PI. Birds infected with AIV alone showed no mortality.

ORT group
Some chickens infected with ORT alone showed only ruffled feathers on days 3 PI. This sign disappeared on day 5PI. There was not mortality in this group.

Gross lesion findings
The lesions such as congestion and hemorrhage in the tracheal mucosa, air saculitis, pneumonia, fibrinous cast formation in tracheal and swollen kidneys were observed in birds of the AIV + ORT co-infected group.

In this study, no marked gross changes were observed in the AIV infected group except pneumonia. No gross pathological changes were noticed in any of the tissues in birds infected with ORT alone.

There were no clinical signs, gross lesions and mortality in the uninfected control chickens.

Serological findings
HI test was used to measure the antibody titer against H9N2 in the blood samples collected on days 0, 2, 4, 6, 8,10,12,14, and 16 PI. All of serum samples obtained from 4 groups were negative to AI on day 0 and 2 PI. As shown in Table 2, the mean Antibody titer in AI and ORT co-infected group was higher than H9N2 group alone on days 4,8, 10, 12,14 and 16 PI**.** There was no indication of any change in the antibody titer against H9N2 AI virus in the control and ORT chickens. All samples were negative in ND-HI test during the experiment.

DISCUSSION

As during the last decade Similar Asian and Middle Eastern countries [15,21] ,the outbreaks of H9N2 influenza virus with severe clinical signs ,high mortality(20-65%) and low production (up to 75%) have been reported in Iranian commercial poultry farms[6,7] .

However, H9N2 viruses in domestic poultry manifest mild clinical signs and respiratory diseases with low mortality (less 5%). It is proposed that concurrent infection may play a key role in exacerbating mortality in chicken infected with H9N2 influenza virus[1,4,9,12,14 ,22].

One of these bacteria in respiratory complex diseases is Ornithobacterium rhinotracheale that co-infection of AI and ORT in a broiler and laying pullet flock was reported for the first time[1]. Although H9N2 pathogenesis has been studied by various techniques [4,5,6,8,12] . The present study was conducted to determine the clinical signs, gross lesions and antibody titer of indvidual and co-infection of H9N2 and ORT in SPF chickens.

The clinical signs and gross lesions in AIV (H9 subtype) infected chicks were less severe than the lesions reported previously in naturally infected chickens with H9N2 [6] . In this study, no mortality occurred in the experimental group which was infected by H9N2 AIV. Nili and Asasi [6,7] reported that experimental infection of broilers with AIV H9N2 caused severe necrotizing tracheitis and 19% mortality, but it was not shown that the inoculums were free from other pathogens.

In this study, the inoculums used for experimental challenge were negative for bacterial and fungal contamination as well as Newcastle disease virus and *Mycoplasma gallisepticum*.

The chicks in the group infected by ORT showed no gross lesions and mortality. The results of the present study also agree with Thachil *et al.*[13] who reported no gross lesions and mortality in SPF hens

Experimental infections with ORT alone in 2-wk-old [18] and 6-wk-old [20] broiler chickens as well as in 2-wk-old Leghorns [3] resulted in an absence of clinical signs or respiratory lesions. Pronounced lesions were produced in the air sacs, lungs, and liver of 2-wk-old broiler chickens experimentally infected with ORT. However, clinical signs were not evident [20].

The differences found in this study may be attributed to the type or virulence of the strain of ORT used, or to tolerance to ORT by layer chickens.

The chicks in AI and ORT co-infected group showed severe clinical respiratory signs such as coughing, sneezing and gasping and postmortem lesions including tracheal congestion, lung hyperemia, fibrinopurulent air sacculitis and cast formation in tracheal bifurcation. It seems that cast formation was responsible for mortality in dead birds. These clinical signs and gross lesions in infected chicks were similar to lesions reported previously in experimentally infected 3-week-old chickens with co-infection of AI and ORT [9] .

In this study, 15% mortality occurred in the experimental group which was infected by co-infection AI and ORT. Pan *et al.*[9] .reported that experimental co-infection of broilers with AI and ORT caused severe clinical sign of respiratory disease and 70% mortality. In this study, we used for experimental challenge from SPF chicken.

In this study, ORT infection alone had little effect on White Leghorn chickens, but coinfections with AI caused more severe disease.

It has been reported that infectious bronchitis live vaccine(H120 strain) also exacerbates the manifestation of experimental H9N2 AIV infection in broiler chicken[4,12] .

It has been demonstrated that co-infection of IBV virus and ORT bacteria exacerbates clinical signs and gross lesions in adult White Leghorn hens [13] .

Post translational proteolytic activation of the precursor of HA molecule (HA0) into HA1 and HA2 subunits by host proteases is essential for infectivity and for the spread of the virus. Thus, virus activation by the host proteases plays a vital role in the spread of infection, tissue tropism and pathogenicity of LPAIV [15] .ORT co-infection may have provided the protease enzymes and enhanced H9N2 pathogenicity in this experiment.

Avian influenza HI titer in the group co infected with AIV and ORT IBV was significantly more than the group infected with AIV alone on 4,10,12 and 16 ($P<0.05$). This finding may show that ORT could enhance the propagation of AIV, immunostimulation and consequently an increase in AIV HI titer. It has been demonstrated that stimulation of host cells to produce or secrete more protease and the destruction of endogenous cell protease inhibitors may increase trypsin-like protease activity and enhanced influenza virus pathogenicity [8] .

Our field observation indicates that flocks that are positive to H9N2 have shown increase in mortality in recent years that could be due to increased pathogenicity of the virus or due to other undetected field infections. This study demonstrated that ORT infection increased the pathogenicity of H9N2 AIV in broiler chickens.

Table1. RT-PCR primer sequences and positions of the oligonucleotide.

Oligonucleotide	Sequence	Gene	Position
Forward primer	5´-TATGGGGCATACAYCAYCC-3´	H9	486
Reverse primer	5´- TCTATGAACCCWGCWATTGCTCC -3´	H9	486

Table 2: Mean±SD values of avian influenza H9 serum antibody titer (HI) in the control and experimental groups

Days PI	Groups		
	ORT/Control	H_9N_2	H_9N_2 + ORT
4	0±0.00 [a]	0±0.00 [a]	1.0±0.57 [b]
6	0±0.00 [a]	5.3±0.33 [b]	4.6±0.88 [b]
8	0±0.00 [a]	6.1±0.33 [b]	6.2±0.57 [b]
10	0±0.00 [a]	6.6±0.33 [b]	7.3±0.66 [c]
12	0±0.00 [a]	6.6±0.33 [b]	7.0±0.57 [c]
14	0±0.00 [a]	6.6±0.33 [b]	7.0±0.00 [b]
16	0±0.00 [a]	6.3±0.33 [b]	7.3±0.66 [c]

a, b, c In each rows, means with different superscripts significantly different (P<0.05)

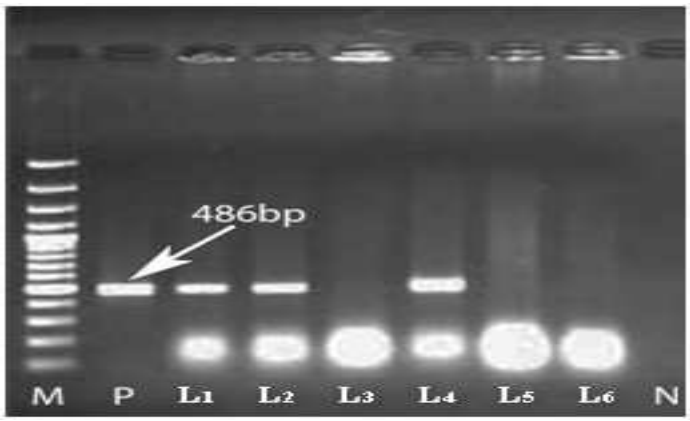

Fig 1: Results of the RT-PCR assay. Amplifying 486-bp segment of H9 gene of AIV. M: DNA marker (100 bp), P: positive control (RNA of the challenged AI virus), L1-2 and L4: positive samples, L3 and L5-6: negative samples, N: negative control

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