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EXPERIMENTAL STUDY ON TISSUE TROPISM AND DISSEMINATION OF H9N2 AVIAN INFLUENZA VIRUS AND ORNITHOBACTERIUM RHINOTRACHEALE CO-INFECTION IN SPF CHICKENS

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ABSTRACT

Forty-two, one-day-old specific pathogen-free (SPF) chickens were randomly divided into two groups. On 21st day of study, experimental group was infected intraocularly with 1×10^6 EID_{50} of H_9N_2 and intratracheally with 1×10^{10} CFU of Ornithobacterium rhinotracheale (ORT). Control group was inoculated with sterile PBS intraocularly. The samples from various tissues were collected on days 2, 4, 6, 8, 10, 12, 14 and 16 post-inoculation (PI). Experimental group chickens exhibited more severe respiratory signs, depression, anorexia and 15% mortality. The ORT was isolated in the trachea from days 2–4 PI and in the lungs on day 4 PI. The H_9N_2 virus was detected in the lungs and trachea from days 2–4 PI. The virus was also detected in the bursa of fabricius on days 2 and 6 PI and in thymus and liver on day 2 PI. The virus was found only on day 8 PI in kidneys. The virus detection from organs was reduced with increasing antibody titer on day 8 PI. The results of this study indicates that concurrent infection with H_9N_2 virus and ORT can exacerbate virulence and lesions of H_9N_2.

Key words: H_9N_2 influenza virus, Ornithobacterium rhinotracheale, Co-infection, Tissue tropism, SPF chickens.

INTRODUCTION

Avian influenza (AI) is a viral disease of respiratory system, caused by type A influenza viruses; it is also a member of the family *Orthomyxoviridea* (Swayne and Halvorson, 2008). Avian influenza viruses may cause two different diseases in the poultry based on the severity of clinical signs. Highly pathogenic avian influenza viruses (HPAIV) may cause up to 100% mortality. These diseases are caused by H_5 and H_7 subtypes (Capua *et al*., 2002). Moreover, low pathogenic avian influenza viruses (LPAIV) cause mild to moderate infections in various domestic and wild bird species (Alexander, 2000).

Although H_9N_2 AI virus has been classified as low pathogenic AI (Pourbakhsh *et al*., 2000) since 1990s, there are numerous reports regarding the involvement of broiler flocks in different parts of world with H_9N_2 AI where this subtype has caused extensive losses to the poultry industry (Naeem *et al*., 1999; Guo *et al*., 2000; Nili and Asasi, 2002; Alexander, 2007b). However, in experimental and field conditions this type was able to cause only mild disease (Pourbakhsh *et al*., 2000; Kwon *et al*., 2008; Mosleh *et al*., 2009; Seifi *et al*., 2012). An increase in the virulence of H_9N_2 AI virus caused by concurrent infections with infectious bronchitis live vaccine virus, and Staphylococcus, Haemophilus paragallinarum and Ornithobacterium rhinotracheale has been documented (Lee *et al*., 2007; Haghighat-Jahromi *et al*., 2008; Pan *et al*., 2012).

In recent years, researchers have reported existence of co-infection of H_9N_2 AI and ORT in complex respiratory disease with high mortality in Iran (Banani *et al*., 2002). However, thorough studies on co-infection of these two diseases have not been carried out yet. Thus, pathogenesis of H_9N_2 and ORT co-infection has not been clearly described. The aim of the present study was to investigate the pathogenic traits of H_9N_2 and ORT co-infection in SPF chickens as well as to determine tissue tropism and dissemination in various organs. Clinical signs and gross lesions with serologic response against H_9N_2 virus were evaluated.

MATERIALS AND METHODS

Virus: The Iranian isolate A/Chicken/Iran/m.1/2010 (H_9N_2 serotype) of AIV used in this study was isolated in Iran. The titer of virus was determined by inoculation of 0.2 ml of each 10-fold serial dilution (10^{-3}-10^{-9}) with phosphate-buffered saline (PBS) of the virus stocks into the chorioallantoic cavity of 11-day-old SPF embryonated eggs.The inoculated SPF embryonated eggs were checked twice a day, with mortality between days 2 to 7 after inoculation considered being virus-specific. The end of 7 days post inoculation, the allantoic fluid of dead embryos was harvested, and the 50% embryo-infective

dose (EID_{50}) was determined using the Reed and Muench method (Reed and Muench, 1938).

Bacteria: The Iranian strain of ORT used in this study was ORT-R87-7/1387 (JF810491), isolated from commercial chicken flocks by M.Banani (Banani *et al*. 2011). The isolate was maintained lyophilized at -20°C. Bacteria were grown at 37°C on sheep blood agar plates in a 5-10% CO2 atmosphere for 48 hours. The colony formation units (CFU) number was calculated post inoculation. The LD_{50} was determined using the Reed-Muench method (Reed and Muench, 1938).

Chickens: Forty-two white Leghorn chickens hatched from specific pathogen-free (SPF) embryonated chicken eggs (Venky's, India) were divided randomly into two groups (21 chickens per group). They were kept separately in isolators under positive pressure at Animal Research Unit of Razi Vaccine and Serum Research Institute, Karaj-Iran. All the chickens were spplied with feed and water *ad libitum*.

Experiments design: Prior to challenge, all birds were serologically tested using hemagglutination inhibition (HI) test and they were negative for antibodies to H_9N_2 influenza virus antigens. At the age of 21 days, experimental group birds were inoculated with chorioallantoic fluid containing 10^6 EID_{50}/0.1 ml of AIV by eye drop and intratracheally with containing 1×10^{10} CFU/0.5 ml of ORT. All the chickens in the control group were inoculated with 0.1 ml of sterile PBS intraocularly. After challenge, chickens were monitored daily for clinical signs, mortality and serologic response against H9N2 virus. On days 2, 4, 6, 8, 10, 12, 14 and 16 post inoculation (PI), three chickens from experimental and control groups were randomly selected and used for sample collection. They were bled before euthanasia. Then, necropsies were performed and gross lesions were recorded. Sera samples were collected simultaneously in the same days. Tissue samples, which included trachea, thymus, lungs, spleen, liver, kidneys, cecal tonsil, bursa of fabricius, and cloaca were aseptically collected for virus detection using RT-PCR technique and the swab samples were aseptically provided from trachea, lungs, liver and heart to bacteria identification using culture and isolation methods.

Isolation and identification of ORT: Swabs of the trachea, lungs, liver and heart of the experimental group birds were cultured onto 5% sheep blood agar Plates with 10 µg/ml of gentamicin and incubated under microaerobic conditions (5-10% CO2) at 37°C for 48 hours (Chin *et al*., 2008). The pinpoint, circular, small opaque to grayish and non-hemolytic colonies with 1-3 mm diameter, were selected (Vandamme *et al*., 1994). Colonies with characteristics of ORT were used by various identification methods such as staining by Gram's method, biochemical identification tests, and finally genetically identified by agar gel precipitation (AGP), as previously described by researchers (Van Empel *et al*., 1997).

For biochemical characterization oxidase, catalase, Mac-Conkey, arginine, lysine, ornithine, phenylalanine, urea, indole, H2S, Vogues- Proskauer, and carbohydrate fermentation was performed. On carbohydrate fermentation tests, tubes containing phenol red broth, supplemented with 1% (w/v) glucose, mannose, lactose, sucrose, sorbitol, maltose, and dulcitol were inoculated with ORT-suspected overnight cultures. All inoculated tubes were incubated at 37°C for 24–48 hours and observed or tested for biochemical characterizations (Hafez, 2002).

RNA extraction: All tissue samples were homogenized with triptose phosphate buffer and centrifuged for 5 minutes and then the supernatant liquid was stored at −70°C until being used. RNA was extracted from the samples using a High Pure Viral Nucleic Acid Kit (Roche Applied Science, Mannheim, Germany) following the manufacturer's instructions.

Briefly, 1 ml of RNX solution was added to 100 µl of each sample of homogenized tissue. After addition of 200 µl chloroform, the mixture was centrifuged at 12000 rpm at 4°C for 15 min. The upper phase was added to equal volume of isopropanol and centrifuged at 12000 rpm at 4ºC for 15 min. After washing step, the pellet was dissolved in a final volume of 50
µl distilled water (DW) and stored at -70°C until used.

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

Oligonucleotide	Sequence	Gene	Position
Forward primer	5´-TATGGGGCATACAYCAYCC-3´	H9	784
Reverse primer	5´- TCTATGAACCCWGCWATTGCTCC -3´	H9	784

RT-PCR: RT-PCR was performed using the Titan one-tube RT-PCR system (Roche Applied Science, Mannheim, Germany). The primers that were used in the present study were specific for H_9 protein gene (Lee *et al*., 2001) and are shown in Table 1. For the amplification reaction, 4 µl of the extracted RNA in a final volume of 50 µL was used. For the RT reaction, the mixture was incubated at 45 °C for 45 min and then heated to 94 °C for 3 min. The PCR reaction was performed for 35 cycles of 94ºC for 30 sec, 53ºC for 45 sec and 68ºC for 60 sec, followed by a final extension (68°C, 10 min) (Tajmanesh Shokoufeh *et al.*, 2006). The final products

were analysed by electrophoresis in a 1% agarose gel and visualized by UV translumination.

Serology: Serum samples were tested for the response of antibodies against the challenge virus antigen using the HI test (Burleson *et al.,* 1992).

Statistical analysis: The data of HI titers were analysed using independent t-test. A statements of significance was based on the 0.05 level of probability.

RESULTS

Clinical signs, gross lesions and mortality: Some chickens of the infected group showed ruffled feathers, depression, reduced appetite, respiratory distress (coughing, sneezing and gasping) on day 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 on day 10 PI. The infected birds died between days 2, 3 and 5 PI. Feed consumption and weight gain were reduced in the infected group in comparison with the control group. The gross lesions such as tracheal congestion, airsacculitis, pneumonia and swollen of kidneys were observed in birds. These lesions were most prominent at 3 PI. The congestion and hemorrhage in the tracheal mucosa, airsacculitis and lung hyperemia were found in the dead bird at day 2 PI. The the dead bird had swollen of kidneys and liver, fibrinopurulent air sacculitis, tracheal congestion and fibrinous cast formation in this organ on day 3 PI (Fig. 1, 2). Fibrinopurulent air sacculitis and tracheal congestion were also observed in the dead bird on day 5 PI. There were no clinical signs, gross lesions and mortality in the control group during the experiment.

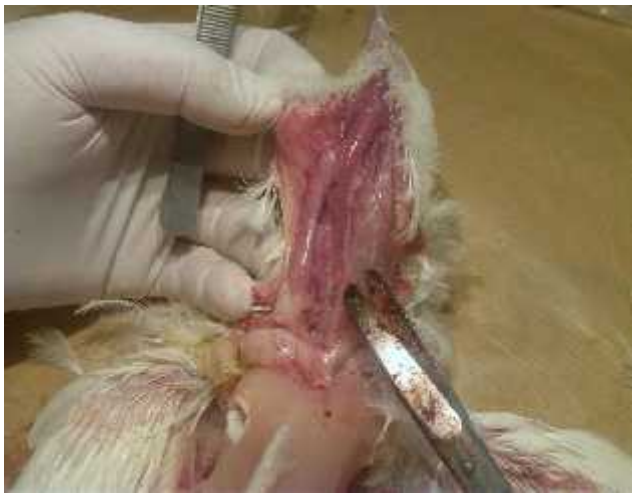

Fig. 1: Diffuse congestion of tracheal mucosa in the bird died on day 2 PI

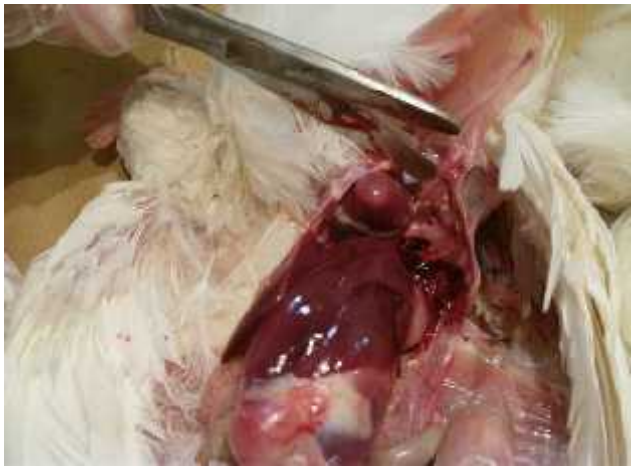

Fig. 2: Air sacculitis in the bird died on day 3 PI

Serological findings: The HI test was used to evaluate antibody titer against H_9N_2 in the serum samples collected on days 2 to 16 PI. All serum samples obtained from two groups were negative to AI on days 0 and 2 PI. As shown in Table 2, the antibody titer against avian Influenza increased at 6 PI and its average reached 7.3 at 16 PI in the challenged group. There were not any changes in the antibody titer in the control group.

Table2. Mean±SD values of avian influenza H9N2 serum antibody titer (HI) in the control and tests groups at different days post-inoculation with H9N2 AI virus.

Groups	Days PI						
	4	6	8	10	12	14	16
Experimental	1.0 ± 0.5^a	4.6 ± 0.8^a	6.2 ± 0.5^a	7.3 ± 0.6^a	7.0 ± 0.5^a	7.0 ± 0.0^a	7.3 ± 0.6^a
Control	0^b	0^b	0^b	0^b	0^b	0^b	0^b

[a,b] In each columns, means with different superscripts significantly different ($P<0.05$)

Bacteria and virus detection from chickens: The existence of the bacteria and virus was evaluated in all samples obtained from the experiment and control chickens at different days PI. The bacteria and virus were not detected in samples taken before inoculation and also control group.

Bacteria detection from swab samples: The results of the culture accomplished for ORT detection are demonstrated in Figure 3. The bacteria were not isolated from swab samples taken from liver and heart of experimental group. In the trachea, bacteria were isolated on days 2 and 4 PI. The positive sample from the lungs was only obtained on day 4 PI (Table 3). The bacteria in the dead bird were isolated from the trachea, lungs, liver and heart on day 2 PI. Except the trachea, in other swabs of the died bird on day 3 PI was not detected. It was not isolated in the died bird at day 5 PI (Table 4).

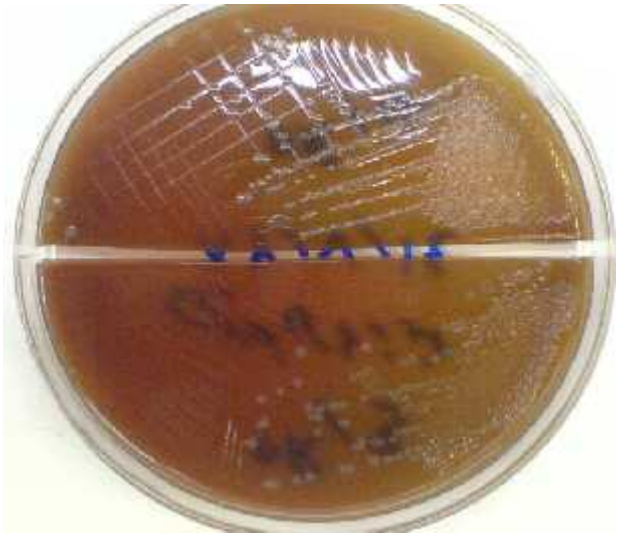

Fig. 3: Growth of Colonies of ORT isolates on blood agar at 37°C for 48 hrs.

Table3. Bacteria detecion from swab samples of chicken after inoculation with H9N2 and ORT

Swabs	Days PI							
	2	4	6	8	10	12	14	16
Trachea	+	+	-	-	-	-	-	-
Lungs	-	+	-	-	-	-	-	-
Liver	-	-	-	-	-	-	-	-
Heart	-	-	-	-	-	-	-	-

*+: Positive samples, - Negative samples

Table4. Bacteria detection from swab samples of dead chicken after inoculation with H9N2 and ORT

Days PI	Swabs			
	Trachea	Lungs	Liver	Heart
2	+	+	+	+
3	+	-	-	-
5	-	-	-	-

*+: Positive samples, - Negative samples

Virus detection in tissues at different days post-infection: The virus was detected in all samples taken from experiment group except for samples taken from spleen, cecal tonsils and cloaca in different periods PI. The typical results of RT-PCR test accomplished for virus detection were demonstrated in Figure 4. Dissemination of virus in various tissues following inoculation is shown in Tables 5 and 6. In the trachea and the lungs, virus was detected on days 5 and 6 PI and in thymus and liver samples virus was detected on day 2 PI. The positive sample from the kidneys only was seen on day 8 PI. The bursa of fabricius samples showed virus on days 2 and 6 PI. In the lungs of the dead bird, virus was detected on days 2, 3 and 5 PI and it was also detected in the trachea, thymus and kidneys of the dead bird from days 2-3 PI and in bursa of fabricius on day 3 and 5 PI. The virus was detected in the liver of the dead bird at days 3 PI.

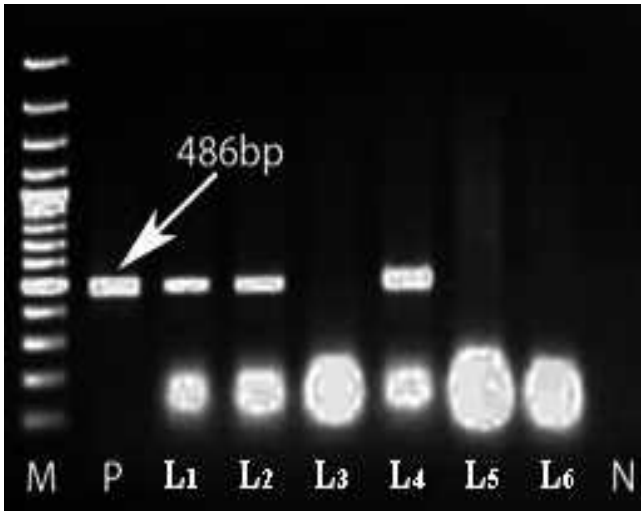

Fig. 4: Results of the RT-PCR assay. Amplifying 486-bp segment of H9 gene of AIV. M: DNA marker (100 bp), P: positive control (RNA of the challenged AI virus), L1-2 and L4: positive samples, L3 and L5-6: negative samples.

Table5. Virus detection from various tissues of chicken after inoculation with H9N2 and ORT

Days PI	Samples								
	Trachea	Thymus	Lungs	Liver	spleen	kidneys	Cecal tonsils	Bursa of fabricius	Cloaca
2	+	+	+	+	-	-	-	+	-
4	+	-	+	-	-	-	-	-	-
6	-	-	-	-	-	-	-	+	-
8	-	-	-	-	-	+		-	-
10	-	-	-	-	-	-	-	-	-
12	-	-	-	-	-	-	-	-	-
14	-	-	-	-	-	-	-	-	-
16	-	-	-	-	-	-	-	-	-

*+: Positive samples, - Negative samples

Table6. Virus detection from various tissues of died chicken after inoculation with H9N2 and ORT

Days PI	Samples								
	Trachea	Thymus	Lungs	Liver	spleen	kidneys	Cecal tonsils	Bursa of fabricius	Cloaca
2	+	+	+	-	-	+	-	-	-
3	+	+	+	+	-	+	-	+	-
5	-	-	+	-	-	-	-	+	-

*+: Positive samples, - Negative samples.

DISCUSSION

The first occurrence of H_9N_2 AI virus in Iran was reported in 1998, which was isolated from the laying hen farms (Vasfi Marandi and Bozorgmehrifard, 1999). Then widespread outbreak of H_9N_2 virus in commercial broiler chickens was reported by other researchers (Nili and Asasi, 2002). Since then, AI has been recognised as an one of the most important respiratory diseases in Iranian poultry industry. Like Asian and Middle Eastern countries (Naeem *et al*., 1999b; Guo *et al*., 2000; Alexander, 2007a), the outbreaks of H_9N_2 influenza virus with severe clinical signs, high mortality (up to 65%) and low production (up to 75%) have been recently reported in Iranian commercial poultry farms (Vasfi Marandi and Bozorgmehrifard, 2002; Nili and Asasi, 2003). However, H_9N_2 viruses in domestic poultry cause mild clinical signs and respiratory diseases with low mortality which usually does not exceed 5% (Swayne and Halvorson, 2008). According to scientific reports, many factors such as management problems, environmental stress, and bacterial and viral co-infections are involved in increasing morbidity and mortality of influenza virus (Liu *et al*., 2003; Hablolvarid *et al*., 2004; Haghighat-Jahromi *et al*., 2008; Pan *et al*., 2012; Seifi *et al*., 2012; Azizpour *et al*., 2013). One of these bacteria in respiratory complex diseases is Ornithobacterium rhinotracheale. This bacterium was isolated by Banani *et al*. (2000) (at the Razi vaccine and serum institute of Karaj-Iran for the first time) from broiler and laying pullet flocks that had respiratory symptoms. Results of several studies showed that some strains of ORT alone cause clinical disease in SPF chickens; moreover, in predisposing conditions such as bacterial and viral infections, the virulence of other pathogens will increase (Van Empel and Hafez, 1999; Van Veen *et al*., 2000; Banani *et al*., 2000; 2002; Marien, 2007; Thachil *et al*., 2009; Pan *et al*., 2012).

Infection with H_9N_2 AI and ORT from 26 industrial flocks with severe respiratory disorders and high mortality rate was reported during 2000 in Iran (Banani *et al*., 2002). However, complete studies on H9N2 AI virus and ORT co-infection have not been conducted yet. Since these two diseases alone are not serious complications in chickens (Azizpour *et al*., 2013), it is necessary to study experimental co-infection of H_9N_2 AI virus and ORT. The present study was conducted to determine the pathogenesis, tissue tropism and dissemination in co-infected SPF chickens with H9N2 and ORT .

Results of a recent study demonstrated that the concurrent inoculation of AI and ORT in 3-week-old chickens caused ruffled feathers, inactivity and reduced appetite on the second day post inoculation and respiratory distress on third day PI and increasing mortality in the second to fifth days after inoculation. Also in necropsy airsacculitis, pericarditis, peritonitis, diffuse hemorrhage and congestion in the respiratory tract (trachea and lungs) were reported (Pan *et al*., 2012). Seifi *et al*. (2012) indicated that 50% of inoculated chickens between 2 to 8 days PI in experimentally infected broilers with H_9N_2 and 4/91 infectious bronchitis had severe clinical and gross lesions. In an experimental study, a group was inoculated simultaneously with infectious

bronchitis virus (IBV) and *E. coli*, and it was exposed to secondary ORT infection 5 days later. Then this group was compared to groups of infectious bronchitis, IBV + E.coli and IBV + ORT clinical signs, and, in the end, gross lesions were severe (Thachil *et al*., 2009). Haghighat-Jahromi *et al*. (2008) demonstrated that not only did the co-infection of H_9N_2 virus with IBV live vaccine (H120 strain) exacerbate the clinical signs and gross lesions results from the H9N2 but also it increased mortality rate and long-term excretion of AI virus in broiler chickens. In experimental study, with inoculation crude extracts from tracheal of chickens suspected to H_9N_2 AI in broilers, reported mortality up to 19%. The presence of other pathogens in this crude extract might have caused this mortality rate (Nili and Asasi, 2002; 2003). In the present study, adding several antibiotics by clear proportional to crude extract precluded the possibility of the presence of bacterial pathogens. Also the inoculums of experimental challenge were assessed for other bacterial and fungal contamination such as Newcastle disease virus and *Mycoplasma gallisepticum* where all terms turned out negative.

In the present study, the clinical signs and gross lesions were observed in experimental group similar to lesions previous reports (Haghighat-Jahromi *et al*., 2008; Thachil *et al*., 2009; Pan *et al*., 2012; Seifi *et al*., 2012). In previous studies, mortality rates from 19% (Nili and Asasi, 2002; 2003) to 20% (Seifi *et al*., 2012) and also up to 70% (Pan *et al*., 2012) in concurrent infection AI with IBV and ORT have been reported. The results of present study indicated that co-infection of AI and ORT could cause 15% mortality in SPF chickens. This finding is different from some previous reports that had reported higher mortality in concurrent infections. It seems that some factors such as poor management, nutrition**,** concurrent bacterial or viral infections, immune suppressant agents, ORT and virus strains, species, age and strain of birds and inoculation method of samples were the main causes of pathogenicity differences of H9N2 and ORT isolates.

Researchers in experimental studies reported that bacteria in addition to trachea and lung from heart blood, liver, joints, brain, ovaries and oviduct are isolated (Van Beek *et al*., 1994). Hafez and Beyer (1997) showed that under field conditions, ORT was not detectable from the heart and liver. Research on experimental infection of SPF chickens showed that the bacteria can be isolated from infraorbital sinuses, trachea, liver, air sacs and kidneys in ORT group. The bacteria in concurrent infected groups including ORT +IBV, ORT+ E. coli and ORT+ IBV + E. coli, as well as the above-mentioned organs from lungs and oviducts were identified and isolated (Thachil *et al*., 2009). In the present study, bacteria from the trachea and the lung were identified. Also in the dead chickens, bacteria from trachea, lung, liver and heart swabs on day 2 PI and just from trachea on day 3 PI were isolated. Detection of the bacteria from the lung, trachea, the liver and the heart indicates that infection is systemic.

Studying intranasal inoculation of H_9N_2 AIV in commercial 35-day-old chickens, Mosleh *et al*. (2009) isolated viral RNA from the trachea, lungs and spleen on days 3 and 6 PI, the kidneys on days 3, 6, and 9 PI and feces just on day 6 PI. These researchers, however, were unable to isolate the virus from the blood and pancreas. Following intranasal inoculation of the H_9N_2 virus, Hadipour (2009) showed the presence of virus in the trachea and lungs (2 to 11days PI), and kidney (6-11 days PI) by immunohistochemistry. Kwon *et al*. (2008) detected A/Chicken/HS/K5/01(H_9N_2) virus antigen in the lungs, trachea, spleen, kidneys, bursa, thymus and cecal tonsils of experimentally infected 3-week-old broiler chickens on day 5 post-inoculation. In other studies following intranasal and intravenous inoculations, the virus was isolated from cloaca swabs (Swayne and Slemons, 1992; Noroozian *et al*., 2007). It was reported that H_9N_2 LPAI virus was recovered from kidneys and spleen of experimentally infected commercial layers in 35-week-old chickens (Lee *et al*., 2007). One immunohistopathological study showed A/Chicken/Iran /259/1998 (H_9N_2) virus nucleoproteins in the trachea, lungs and cecal tonsils following intratracheal inoculation of 5-week-old SPF chickens (Hablolvarid *et al*., 2004). Researchers indicated that following the inoculation of A/chicken/Pakistan/31/01 (H_9N_2) virus by different methods to chickens and then challenging them with other infective agents (including IBV, ORT and E. coli), viral antigen in tissues of trachea, lungs, kidneys and bursa fabricius was detected (Bano *et al*., 2003). In experimentally infected 6-week-old Aryan broiler chickens with Iranian H_9N_2 virus, the virus antigens were identified in the trachea, lungs, and kidneys of inoculated chickens using immunoperoxidase assay (Shamseddini *et al*., 2002).

In present study, virus was isolated from trachea (days 2–4 PI), lungs (days 2–4 PI), bursa fabricius (days 2 and 6 PI), thymus (day 2 PI), liver (day 2 PI) and kidney (day 8 PI). On day 8 PI the virus was not detected from any tissues except the kidneys and it might be resulted from increasing of the antibody titer in the blood. Although the H_9N_2 virus was isolated from spleen, cecal tonsils and cloaca in previous reports (Hablolvarid *et al*., 2004; Kwon *et al*., 2008; Mosleh *et al*., 2009),but the virus was not detected in the spleen, cecal tonsils and cloaca swabs in this study. In dead chickens, H_9N_2 virus in the respiratory system (trachea and lungs), urinary system (kidneys), immune system (thymus, bursa fabricius) and liver was detected; it was recovered in lung for a longer time (day 5 PI) in comparison to the other organs. Also on day 3 PI, the number of organs with positive virus detection was more than other experimental days (days 2 and 5 PI).

The results of present study indicated that co-infection of AI and ORT causes change in virus behavior, and similar HPAI viruses replicate systematically. Subsequently, it results in an increase in mortality up to 15% and in an exacerbation of clinical signs and gross lesions in infected chickens. According to the importance of concurrent infection, it is recommended that the role of other pathogens such as Newcastle, E. coli, Haemophilus paragallinarum and Mycoplasma in the complications caused by disease in different commercial broilers be investigated. Consequently, fighting and prevention strategies from respiratory complex disease and decreasing economic losses should be considered.

Conclusion: Our results indicate that co-infection AI and ORT has tropism for the respiratory system (trachea and lungs), urinary system (kidneys), immune system (thymus, bursa fabricius) and liver. Also it causes high mortality (15%) with increased pathogenicity of the H9N2 influenza virus.

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