

*Full Length Research Paper*

## **Detection of H9N2 avian influenza virus in various organs of experimentally infected chickens**

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**H9N2 Avian influenza virus (AVI) infection is a major cause of economic losses in poultry industry. Therefore further study to explain the virus pathogenesis is necessary. In this study tissue tropism and dissemination of A/chicken/Iran/11T/99(H9N2) virus in various organs of specific pathogen free (SPF) chickens were investigated. Fifty 2-week-old chickens hatched from SPF eggs were divided randomly into two groups. Forty chicks in the experimental and ten chicks in the control group. Experimental chicks were inoculated intranasally-intraorally with the virus. Samples of lung, trachea, pancreas, thymus, spleen, brain, bursa of fabricius, proventriculus, cloaca and kidney were aseptically collected at 1, 3, 5, 7, 9 and 10 day post inoculation (DPI). A reverse transcriptase polymerase chain Reaction (RT-PCR) test was performed for virus detection. Viral RNA was detected in the respiratory system on days 3, 5 and 7 PI. The virus was also found in the kidney on days 3,5,7,9 PI and in the pancreas on days 3 and 5 PI. Viral RNA was observed only on day 5 PI in cloaca. The virus was not detected in the blood, brain and immune system. The virus was not found from any organs on day 10 PI. These results suggest that H9N2 AIV has tropism for respiratory, digestive and urinary system following intranasal/intraoral inoculation.**

**Key words:** Avian influenza, H9N2, SPF, RT-PCR.

### **INTRODUCTION**

Avian influenza viruses (AIVs) belong to the family orthomyxoviridae and to the type A influenza virus. These viruses are classified into subtypes based on their surface haemagglutinin (H) and neuraminidase (N) glycoproteins. So far, 16 different H subtypes (H1-H16) and 9 different N subtypes have been identified (Fouchier et al., 2005). According to the pathogenicity of AIV to domestic and wild bird species these viruses are categorized into two pathotype groups including Highly Pathogenic Avian Influenza (HPAI) viruses and non-Highly Pathogenic Avian influenza (nHPAI) viruses

(Capua and Alexander, 2006). The H9N2 AIV outbreaks occurred in domestic poultry in Asia and the Middle East since the 1990s, and have caused severe economic losses in many countries. In 1998 an AI outbreak in Iran caused great economic losses in poultry industry and a non-highly pathogenic avian influenza virus (H9N2) has been reported as causative agent (Pourbakhsh et al., 2000). H9N2 AIVs induce significant troubles for the poultry industry in Iran due to decreased production, increased mortality and cost of vaccination. Avian influenza disease due to H9N2 subtype has been markedly common during 1994 to 1999 in many parts of the world (Vasfi Marandi and Bozorgmehrifard, 2002). H9N2 influenza viruses are also discussed to be one of the potential candidate for the next human widespread epidemic disease (Butt et al., 2005). Experimental infection

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in specific pathogen-free (SPF) chickens announced that the H9N2 AIV is not capable to cause pathological lesions, severe clinical signs and mortality by itself (Lee et al., 2007; Pourbakhsh et al., 2000). During outbreaks of non-highly pathogenic AIVs co-infection with other pathogens especially in severe stress conditions may complicate the syndrome and induce signs of respiratory disease and even mortality in field.

Because of widespread incident of the disease and ambiguous behavior of the H9N2 AIV further study to explain the virus pathogenesis is necessary. In a characteristic manner non-highly pathogenic AIVs have been isolated from respiratory exudate and feces of infected birds, and AIV nucleoprotein has been demonstrated in epithelial cells of the intestine, trachea, lungs and air sacs (Shalaby et al., 1994; Swayne et al., 1994). LPAI viruses often need trypsin like enzyme activity to cleave the Hemagglutinin into HA1 and HA2 proteins in order to make the infectious virus particle (Klenk et al., 1975). Hence respiratory and gastrointestinal epithelia that contain these types of enzyme and organs containing epithelial cells like pancreas and kidney are principal places for non-highly pathogenic AIV replication and lesion formation (Klenk et al., 1975; Shalaby et al., 1994). Anyway the pathway of virus distribution into these organs remains ambiguous and it needs more studies to be investigated well. Virus isolation in SPF chickens for identification of AI viruses is time consuming and require specific facilities. Molecular tests like reverse transcription PCR (RT-PCR) are being introduced in order to detection of AIV due to their premium such as rapidity, delicacy and sensitivity (Saberfar et al., 2008). The aim of this study was assessment of the H9N2 virus spreading in various organs of the infected SPF chickens at different days after inoculation. RT-PCR test was performed to diagnose the presence of the virus in different body tissues. It may further help us to investigate the virus pathogenesis.

## MATERIALS AND METHODS

### Virus strain

The influenza virus A/chicken/Iran/11T/99 H9N2 that was isolated from outbreak among poultry in Iran, was provided by Razi Vaccine and Serum Research Institute (Karaj, Iran). The virus was propagated two times in the allantoic cavity of 9 to 11-day-old embryonated chicken specific pathogen free eggs. Hemagglutination (HA) titers of the viruses ranged from 512 to 1024 HA unit, when tested according to the methods as described previously (Burlinson et al., 1992).

### SPF chickens

Fifty 2-week-old chickens hatched from SPF eggs were randomly divided in two groups (forty chicks in experimental group and ten chicks in control group). Both groups were housed in same condition in two separate isolated rooms. Feed and water were

available ad libitum.

## Experimental design

All birds were bled and serologically tested using Hemagglutination inhibition test (HI) (Burlinson et al., 1992). They were negative for antibodies to H9N2 influenza virus antigens. Five chickens from treated group were sacrificed and their organs were investigated from virus detection. All of these samples were also negative for virus detection. Subsequently, chickens of the experimental group were inoculated via intranasal/intraoral routes with 120 µl of infectious allantoic fluid containing  $10^{7.5}$  EID<sub>50</sub> of the applied virus strain diluted in sterile PBS solution. The control group was received sterile PBS with the same manner. All the birds were monitored daily for 15 days to investigate the changes of antibody titre to H9N2 and mortality. Five chickens from the experimental group and one chicken from the control group were randomly selected on days 1, 3, 5, 7, 9 and 10 post inoculation (PI). They were bled and sacrificed. During this period, all chickens were observed if they have clinical signs of disease or not and observations were recorded. Necropsy was done on sacrificed chickens and all gross lesions were recorded. Samples of lung, trachea, pancreas, thymus, spleen, brain, bursa of fabricius, proventriculus, cloaca and kidney were aseptically collected for virus detection and RT-PCR assay. Blood samples were collected in EDTA tubes. Sera of the birds were also collected at the same days for HI test. All tissue samples were immediately stored at -70° until used.

## Serology

Serum samples were collected on the pre-inoculation, first to fifteenth days post inoculation from all chickens and were tested against specific antibodies to H9 antigen by using Haemagglutination Inhibition (HI) test, according to the manual of standards for diagnostic test (OIE, 2008).

## Extraction of viral RNA

RNA of blood and tissue samples was extracted using the RNX TM (-Plus) kit (CinnaGen Inc.) according to the manufacturer's protocol. 50 to 100 mg of tissue or 100 µl of blood sample was mixed with 1ml RNX and incubated at room temperature for 5 minutes. After addition of 200 µl chloroform and mixing, the liquid was clarified by centrifugation at 12,000 rpm at 4° for 15 min. The supernatant was transferred into a new tube and mixed with an equal volume of isopropanol followed by centrifugation at 12000 rpm at 4° for 15 min. The pellet was washed with 1ml of 75% ethanol. Finally, the pellet was dissolved in 50 µl of DEPC treated water.

## RT-PCR

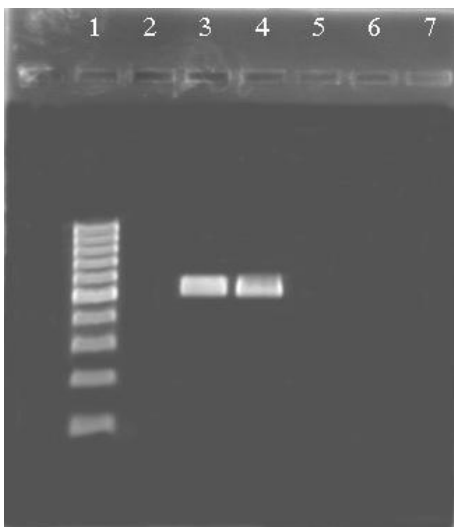
The cDNA was synthesized using AccuPower RT-Premix kit (BioNeer corporation, South Korea) according to the manufacturer's protocol. The primer sequences are shown in Table 1. 1 µg of total RNA and 20 pmol of each primer were used for cDNA preparation. PCR was performed to amplify 510 bp fragment of matrix protein gene of influenza virus using the AccuPower PCR PreMix kit (BioNeer Corporation, South Korea). The reaction mixture contained 5 µl cDNA in a final volume 20 µl was subjected to 94°C for 5 min a 35 cycles of 94°C for 30 s, 49°C for 30 s, 72°C for 40 s and followed by final extension at 72°C for 5 min. The PCR products were separated by electrophoresis using a 1.5% agarose gel in 1xTBE buffer.

**Table 1.** RT-PCR Primer Sequences.

Specificity	primers	sequences	size
Influenza A virus	MF	GGC TCT CAT GGA ATG GCT AA	510
Influenza A virus	MR	CTG GCC TGA CTA GCA ACC TC	510

**Table 2.** H9N2 serum antibody titration (Mean titer) of the test and control groups of chickens experimentally infected with H9N2 AI virus.

DPI	Day 0	Day 1	Day 3	Day 5	Day 7	Day 9	Day 10	Day 13	Day 15
test Group	0	0	0.4	1.4	2.4	3.6	4	6.6	7.4
control Group	0	0	0	0	0	0	0	0	0

**Figure 1.** Results of the RT-PCR assay. Amplifying 510-bp segment of M gene of AIV. Lane 1: DNA marker (100-bp), Lane 2: negative control, lane 5,6 and 7: negative samples, lane 3: positive control, lane 4: positive sample.

Amplified products were visualized under ultraviolet light after staining with 0.1 µg /ml ethidium bromide. A 100 base pair ladder was used as a molecular weight marker.

## RESULTS

### Clinical signs

Daily monitoring did not show any sign of illness in the chickens from control group. The clinical signs observed in the inoculated chickens were depression, facial edema, conjunctivitis, ruffled feather, decrease feed consumption and diarrhoea. Clinical signs were observed

from third day post inoculation. On day 7 PI the number of chickens showing clinical signs reduced. The clinical signs disappeared at 12 DPI. No mortality was recorded from each isolate.

### Gross Lesions

No gross lesions were observed in the uninfected control group. In experimental chickens the lesions such as mild congestion of the trachea and lungs, hemorrhage in small intestine and pancreas and swollen kidneys were observed.

### HI test

There was no evidence of any change in specific antibodies against AIV in pre and post inoculation of control chickens. As shown in Table 2 the mean of antibody titer was increased at 5 DPI and reached to  $2^{-8}$  at 15 DPI in the experimental group.

### RT-PCR

The presence of the virus in various organs obtained from the inoculated and control birds at different DPI was determined by RT-PCR test. Tissue samples from 5 different birds that had been taken before inoculation and samples from control group were all negative for virus detection. First positive samples were seen on day 3 PI and the last positive sample was detected on day 9 PI (Figure 1). The virus was detected in the trachea, lungs, pancreas, cloaca and kidney of infected birds during the experiment course. The results of the virus detection are shown in Table 3. The results show that most positive samples were detected on days 5 PI. All brain, blood, thymus, spleen, proventriculus and bursa of fabricius,

**Table 3.** The results of virus detection from various organs of SPF chickens at different days post inoculation with H9N2 AI virus.

Day PI	Group	Tr	L	Th	Sp	Pr	Cl	Bu	P	K	Br	Bl
1	T	0/5*	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
	C	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1
3	T	2/5	3/5	0/5	0/5	0/5	0/5	0/5	1/5	2/5	0/5	0/5
	C	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1
5	T	2/5	4/5	0/5	0/5	0/5	2/5	0/5	4/5	3/5	0/5	0/5
	C	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1
7	T	0/5	2/5	0/5	0/5	0/5	0/5	0/5	0/5	1/5	0/5	0/5
	C	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1
9	T	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	1/5	0/5	0/5
	C	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1
10	T	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
	C	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1

T= test group C= control group Tr= Trachea, L= Lung, Th= Thymus, Sp= Spleen, Pr= Proventriculus, Cl= Cloaca, Bu= Bursa of Fabricius, P= Pancreas, K= Kidney, Br= Brain, Bl= Blood. \*= No. of positive samples/total samples taken.

samples were negative for virus detection.

## DISCUSSION

In this study tissue tropism and dissemination of H9N2 virus throughout the various organs were evaluated following intranasal/intraoral inoculation. The clinical signs and gross lesions found at post mortem examination were alike and milder than lesions reported in naturally infected birds with H9N2 (Pourbakhsh et al., 2000). Although In Some researches (Shalaby et al., 1994; Swayne et al., 1994) as well as present study, inoculation of low pathogenicity AIVs in SPF or broiler chickens have produced absence of mortality, but mortality between 20 and 60% in natural outbreaks have been reported (Naeem et al., 2003; Nili et al., 2003; Vasfi Marandi and Bozorgmehrifard, 2002). Previous studies have shown that the M gene RT-PCR is sensitive and specific method for the detection of influenza A viruses of human and avian origin (Saberfar et al., 2009).

In this study predominant infection in the respiratory organs was observed between days 3 and 7 PI. Detection of the virus from the trachea and lungs indicates that H9N2 AI virus is pneumotropic following intranasal/intraoral inoculation. Repetition of virus recovery in respiratory system was mostly higher for lung tissues. Viral RNA was identified in lung tissue on days 3, 5 and 7 PI. Previous studies (Kwon et al., 2008) have reported that H9N2 viral antigen were detected in the trachea, lungs, thymus, spleen, bursa, cecal tonsils and

kidneys of SPF chickens on day 5 PI. We observed infections that are localized to the GI tract at days 3 and 5 PI. The H9N2 virus were detected in the cloaca at day 5 PI and in the pancreas at day 3 and 5 PI. Detect of the H9N2 virus in cloaca only on day 5 PI perhaps originated in temporary replication of the virus in GI tract. Swayne and Halverson reported that LPAI viruses produce infections in respiratory and GI tracts of chickens. AIV nucleoprotein has been identified in epithelial cells of the trachea, lungs, and intestine (Shalaby et al., 1994; Slemons and Swayne, 1995; Swayne et al., 1994). Hablolvarid et al. (2004) detected nucleoproteins of the H9N2 virus in the trachea, lungs and cecal tonsils of experimentally infected 5-week-old SPF chickens using immunoperoxidase assay. We detected H9N2 virus in the pancreas on day 3 and 5 post inoculation. Shinya et al. (1995) reported positive immunoreaction to H5N3 virus antigen in the pancreas of inoculated chicks. Hablolvarid et al. (2003) detected H9N2 virus nucleoproteins in the pancreas of experimentally infected 5-week-old chickens using immunoperoxidase assay following intravenous inoculation. Sometimes LPAIV can spread further than the respiratory and GI tracts, replicate and cause lesions in primarily visceral organs including epithelial cells such as pancreas and kidney (Shalaby et al., 1994; Vasfi Marandi and Bozorgmehrifard, 2002).

Mosleh et al. (2009) showed that A/Chicken/Iran/772/1998 (H9N2) had tissue tropism and pathogenicity for the respiratory system (lung and trachea), immune system (spleen), urinary system (kidneys) and digestive system of commercial broiler

chicks following IN inoculation. We observed predominant infection in the kidney at days 3, 5, 7, and 9 PI. Virus detection in the kidney could indicate virus tropism for the urinary system as previously reported (Shalaby et al., 1994; Swayne and Slemons, 1995; Vasfi Marandi and Bozorgmehrifard, 2002). Swayne and Slemons (1994) reported that LPAIVs were nephrotropic following IV inoculation and pneumotropic following intranasal inoculation, but they did not detect the virus antigens in kidney using immunohistochemistry assay. In other study LPAI virus was not detected in parenchymal cells of the kidneys following IN inoculation (Swayne and Beck, 2005). In previous studies the H9N2 AIV detected from trachea, kidney and lung tissues using indirect immunoperoxidase test (Shamsedini et al., 2002). H9N2 viral antigen was detected from different tissues of experimental infected three-week-old SPF chickens such as spleen, kidney, lung, trachea, thymus, bursa and cecal tonsil (Kwon et al., 2008). However In this study we did not detect the viral RNA in the lymphoid tissues. Viral RNA was not detected in the blood of the chickens in experimental group. Mosleh et al, (2009) did not also detect the virus from blood samples.

## Conclusion

Most of the Non Highly Pathogenic AIVs have two basic amino acids at the proteolytic cleavage site of the hemagglutinin protein (Wood et al., 1993) and require cleavage by a trypsin-like enzyme to be infectious and perform multiple virus replication cycles (Klenk et al., 1975). Respiratory and gastrointestinal tracts have this enzyme activity in some cells or lumenal contents and it is responsible for the GI and respiratory tracts being primary sites for LPAI virus replication and lesion production. Therefore more studies must be investigated to realise the ability of replication of the virus in the organs with no trypsin-like enzyme such as lymphoid tissues. The virus was not detected from any organs on day 10 PI. It might be resulted from increasing of the specific AIV antibody titer in chicken's blood. Current study showed that H9N2 AIV has tropism for the respiratory organs, urinary system and digestive system.

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