Histopathological Study of Intratracheally Inoculated A/Chicken/Iran/259/1998 (H9N2) Influenza Virus in Chicken

Hablolvarid*[1], M.H., Sohraby Haghdost, I.,[2] Pourbakhsh, S.A.[3] and Gholami, M.R.[1]

1. Pathology Dept., Razi Vaccine & Serum Research Institute, P.O.Box 11365-1558, Tehran, Iran

2. Pathology Dept, Faculty of Veterinary Medicine, Tehran University, Tehran, Iran

3. Avian Diseases Research & Diagnosis, Razi Institute

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Summary

The type, severity and frequency of gross lesion, histopatholgic change, and tissue tropism of A/Chicken/Iran/259/1998(H9N2) avian influenza virus following intratrachealy (IT) inoculation were studied in chickens. Twenty, 5-week-old chickens (hatched from SPF eggs) were inoculated with this virus. Another twenty chickens were inoculated with sterile chorio allantoic fluid. Tracheitis, pneumonia and tubulointerstital nephritis were the most frequent specific histopathologic changes. Influenza nucleoproteins were demonstrated in epithelium of trachea, secondary bronchi and cecal tonsile of an inoculated chicken. Common non-specific histopathologic changes were lymphoid and reticuloendothelial cell hyperplasia in spleen, cecal tonsil and leukocyte cell infiltration in myocardium. These results indicate that the low-pathogenic avian influenza virus is epithliotropic in chicken. In IT route of inoculation, it has tissue tropism and pathogenicity for trachea, lung (pneumotropic) and kidney (nephrotropic).

***Key words*:** influenza virus, low-pathogenic, intratracheal, histopathology, chicken

Introduction

Avian influenza (AI) viruses may cause two different diseases on the basis of the severity of clinical signs, which induce in susceptible species. Highly pathogenic avian influenza (HPAI) is a devastating disease of poultry caused by some viruses of

*Author for correspondence. E-mail:hablolvarid@yahoo.com

the H5 and H7 subtypes. These strains replicate throughout the host, damage vital organs and tissues, and thus brings about the death of the bird (Capua *et al* 2000, Rott 1992). In contrast, low pathogenicity avian influenza (LPAI) viruses are capable of replicating only in limited tissues and organs, mainly the respiratory and digestive tracts, and do not invade the rest of the body. In 1998 an AI outbreak was occurred in Iran, caused severe economic losses in poultry industry and a non-highly pathogenic (n-HPAI) subtype H9N2 AIV was isolated as causative agent (Pourbakhsh *et al* 2000). Outbreaks due to H9N2 subtype have been markedly common during 1994-1999 in different parts of the world (Vasfi Marandi *et al* 2002, Naeem *et al* 1999). Earlier pathogenesis studies revealed that LPAI viruses are nephrotropic following intravenous inoculation while; following intratracheal or intranasal inoculation they are pneumotropic (Swayne *et al* 1994a). Data collected from recent influenza outbreaks indicates that LPAI may mutate and become HPAI, probably after introduction to poultry (Garcia *et al* 1996, Perdue *et al* 1997), resulting in extremely complex situations that may have dramatic effects on the poultry industry. The purposes of this experiment were 1) to determine the effect of IT inoculation of Iranian AIV H9N2 isolate on pathogenesis in chicken, 2) to demonstrate the type, severity and frequency of gross lesion and histopathologic changes produced in chickens following IT inoculation of the isolate and 3) to show H9N2 AI viral antigen in chicken tissues, distribution and tissue tropism of the virus.

Materials and Methods

Experimental design. Forty 5-week-old chickens hatched from SPF eggs (Valo, Lohman, Germany) were randomly divided in two equal groups. Both groups were housed in same condition in two separate places. All chickens were provided feed and water *ad libitum.* All the birds were weighed and bled for detection of specific antibodies against the H9 subtype of AIV, Newcastle disease virus (NDV),

infectious bronchitis virus (IBV) and infectious bursal disease virus (IBDV). Subsequently, the treated group was inoculated intratracheally with 0.2ml of the first passage of A/Chicken/Iran/259/1998(H9N2) of 1:10 dilution ($10^{9.1}EID_{50}$) in chorioallantoic fluid (CAF). The control group was received sterile CAF with the same manner. Five chickens from each group were randomly sampled on days 1, 3, 6 and 10-post inoculation (PI). They were weighed and bled again. Then, they were humanly sacrificed and necropsy was performed. Gross lesions were recorded and samples of different tissues including lung, trachea, heart, liver, pancreas, duodenum, jejunum, thymus, spleen, brain, cecal tonsil and bursa of fabricius were collected for virus isolation, histopathology and immunohistochemistry studies. They were done according to the previous work (Hablolvaride *et al* 2003).

Results

Clinical findings. Control chicken didn't show any sign of illness. In infected chickens the sign of disease including depression, decrease feed consumption, reduction in weight gain, dysponea (rales), sneezing, edema of face and head in some cases and ruffled feathers became apparent on days 2 or 3 PI. On day 4 PI one infected chickens died.

Gross necropsy findings. Control chickens lacked any gross lesions. The most frequent gross lesions in infected chickens were congestion of trachea (30%), abnormal kidney (20%), congestion of lung (10%), splenomegaly (10%), decrease size of the bursa of fabricius (5%) and congestion of brain (5%).

Serological findings. There was no evidence of specific antibodies against AIV, IBDV and NDV in pre and post inoculation of control chickens. The presence of specific antibody against AIV, but not others, was detected in treated group on days 6 and 10 PI.

Virus isolation. After three passages no AIV was isolated from pooled visceral and respiratory organs of the control chickens while virus was easily recovered from

pooled samples of infected chickens at first passage on days 1, 3 and 6 PI. AIV was recovered from respiratory organs after two passages on day 10 PI while no virus was recovered from visceral organs after three passages.

Histopathology and viral antigen distribution. In the control chickens, all of the examined organs were histologically normal and there was no detectable lesion. Data related to infected chickens were summarized in tables 1 and 2.

Table 1. *Organ histopathology and immunohistochemistry data in chickens inoculated IT with AIV H9N2*

Tissue	Frequency of changes (%)	Predominant lesions type	Category	Nucleo-protein
Trachea	89	Necrosis, tracheitis	Specific[1]	+
Lung	65	Necrosis, pneumonia	Specific	+
Heart	36.8	Lymphocyte infiltration	Non-specific[2]	-
Spleen	30	Lymphoid and RE hyperplasia	Non-specific	-
C.tonsile	22.2	Lymphoid hyperplasia	Non-specific	+
Kidney	21	Necrosis, nephritis	Specific	-
Thymus	18.8	Lymphoid atrophy	Non-specific	-
Duodenum	10	Lymphoid hyperplasia	Non-specific	-
Bursa	5.3	Lymphoid atrophy	Non-specific	-
Liver	5.3	Lymphocyte infiltration	Non-specific	-
Pancreas	5.3	Lymphocyte infiltration	Non-specific	-

1:necrosis and inflammation, 2:lymphoid hyperplasia, lymphoid atrophy, lymphocyte depletion and lymphocytic cuffing

Spleen had the highest frequency of histopathologic changes among lymphoid tissues. Necrosis and inflammation were not seen but mild to moderate (few to more obvious changes) reticuloendothelial cell hyperplasia and/or increased number of lymphoid follicles on days 3, 6 and 10 PI were found. Cecal tonsils of some chickens showed mild increase in number of lymphoid follicles on days 3 and 10 PI. However, on day 6 PI influenza viral nucleoprotein was found in epithelium of cecal tonsile of a chicken by applying IHC technique. In bursa of fabricius of some samples there was only a little edema, not scored, on days 3 and 6 PI. On day 6 PI, there was a case of lymphoid athrophy. Thymus of some samples showed depletion of lymphocytes in cortical region on days 1 and 10 PI.

Table 2. *Histopathologic lesions on different days in chickens inoculated IT with AIV H9N2*

Tissue	Day 1 PI	Day 3 PI	Day 6 PI	Day 10 PI
Trachea	(6/6)** At, IHC$^+$	(5/5) Lt, IHC$^+$	(4/4) Lt	(2/4) Lt
Lung	(4/6) Ils, Ap	(3/5) Ils, P, IHC$^+$	(4/5) Ils, P	(2/4) Ils, P
Heart	(0/5)	(2/5) Il	(4/5) Il	(1/4) Il
Spleen	(0/6)	(3/5) Rh	(1/5) Ilf	(2/4) Rh, Ilf
C.tonsile	((0/6)	(3/5) Ilf	(0/3) IHC$^+$	(1/4) Ilf
Kidney	(0/6)	(1/5) Ltn	(¾)) Ltn	(0/4)
Thymus	(2/6) La	(0/3)	(0/3)	(1/4) La
Duodenum	(0/6)	(0/5)	(1/5) Lh	(1/4) Lh
Bursa	(0/6)	(0/4)	(1/5) La	(0/4)
Pancreas	(0/6)	(0/4)	(1/5) Il	(0/4)
Liver	(0/6)	(0/5)	(1/5) Il	(0/4)

**The number of chickens with lesions to total number of euthanatized. Ap=Acute pneumonia, At=Acute tracheitis, IHC$^+$=Positive immunohistochemical reaction, Il=Infiltration of lymphocytes, Ilf=Increased lymphoid follicles, Ils=Infiltration of lymphocytes under secondary bronchi, La=Lymphoid atrophy, Lh=Lymphoid hyperplasia, Lt=Lymphocytic tracheitis, Ltn=Lymphocytic tubulointerstitial nephritis, P=Pnemonia, Rh=Reticuloendothlial hyperplasia

Acute tracheitis with congestion, edema, deciliation, epithelial desquamation, infiltration of heterophils and epithelial cell necrosis were found in most samples on day 1 PI (Figure 1). However, on days 3, 6 and 10 PI, the sign of lymphocytic tracheitis with, one or more sign of; infiltration of lymphocytes, deciliation, and hyperplasia of epithelium were seen. But, on day 10 PI the number of infected chickens and the severity of the lesions had been obviously reduced. By applying IHC technique influenza viral nucleoproteins were demonstrated in epithelium of trachea on days 1 and 3 PI (Figure 2). On day 4 PI one chicken died and the sign of

tracheitis with necrosis, deciliation, infiltration of heterophils and lymphocytes and presence of fibrino-heterophilic exudates inside the lumen were seen.

Figure 1. *Acute tracheitis on day 1 PI. Congestion, edema and infiltration of heterophils (arrows) are seen (H&E×400)*

Figure 2. *Arrows points to localization sites of AIV in epithelium of trachea on day 1 PI (IHC×400)*

Sign of lymphocyte infiltration under submucosa of secondary bronchi and associated pneumonia were the most prominent histopathologic changes in the lung, on days 1-10 PI (Figure 3). Based on the course of the disease, acute or chronic, one or more lesions including congestion, hemorrhage, edema, necrosis and infiltration

of heterophils/lymphocytes were apparent. By applying IHC technique, on day 3 PI, a viral localization site in mucosa of secondary bronchi was confirmed (Figure 4). The histopathologic changes in dead chickens on day 4 PI were pneumonia with necrosis, fibrin deposition, infiltration of lymphocytes and a few heterophils.

Figure 3. *Chicken lung on day 6 PI. Infiltration of lymphocytes (black arrow) under submucosa of secondary bronchus, exudates in lumen and associated pneumonia (light arrows) are seen (H&E×100)*

Figure 4. *Mucosa of secondary bronchus on day 3 PI. Red spots are viral, AIV, localization sites (IHC×400)*

There were only a little infiltration of lymphocyte in parenchyma of pancreas and liver of a chicken on day 6 PI, and lymphoid hyperplasia in duodenum of two chickens on days 6 and 10 PI. No lesion was seen in jejunum. Diffused and/or multifocal lymphocytic tubulointerstitial nephritis with necrosis and infiltration of lymphocytes were observed on days 3 and 6 PI. There was some degeneration and/or leukocyte infiltration in myocardial cells of some samples on days 3, 6, and 10 PI. A little congestion, not scored, was observed in brain of a few chickens.

Discussion

Histopathologic study of experimental intravenous infection of chickens with Iranian AIV H9N2 isolate revealed that it is epithliotropic in chicken and tubulointerstitial nephritis and pancreatitis were the most frequent specific histopathologic changes (Hablolvarid *et al* 2003). In this study the clinical sign and prominent lesions at postmortem examination of intratracheally infected chickens were almost similar to lesions produced in naturally infected chickens during recently AI, H9N2, outbreaks in Iran and in Pakistan (Pourbakhsh *et al* 2000, Naeem *et al* 1999).

According to guidelines published by the Office International des Epizooties (OIE 1992) isolate that did not produce disease or that killed only 1 to 5 of 8 chickens, in pathogenicity test, was classified as low pathogenic. In some researches (Slemons 1990a,b, Swayne 1994a,b) as well as present study, inoculation of chickens with intranasal (IN) and IT routs with low-virulence chicken or duck-origin influenza virus isolates has produced mortality and kidney lesions in 1-day-old chickens and adult hens. However, in other studies absence of mortality was reported (Adel *et al* 1994, Swayne *et al* 1994b). In the current study, presence of necrosis and inflammation and the recovery of AIV from pooled respiratory and visceral organs, along with demonstration of influenza nucleoprotein in epithelium of trachea and lung, indicate that there is a direct pathogenic mechanism in the respiratory/urinary

failure of IT inoculated chickens. However, AIV nucleoprotein could not be demonstrated in kidney of IT inoculated chickens. These data indicate that the Iranian AIV H9N2 isolate has tissue tropism and pathophysiologic effects for respiratory system and kidney of chickens. Tracheitis, pneumonia and tubulointerstitial nephritis were the most specific histopathologic lesions. The lesions were obvious on days 1-6 PI but on day 10 PI a reduction on severity was seen, which concurrent with improvement in general condition of virus inoculated chickens. Swayne and Slemons (1994a) inoculated three strains of AIV with chicken/duck origins to 5-week-old chickens by intravenous (IV), intranasal and IT routes of inoculation. Chickens inoculated by IT and IN routes had mild to severe tracheitis, bronchitis and pneumonia associated with secondary bronchi but lacked renal tubule necrosis and nephritis. Presumably, isolation of virus from pooled visceral organs and presence of nephritis foci in kidneys during the days 1 and 3 PI were resulted from a localized infection of the respiratory tract and was not the result of systemic or parenchymal influenza infection. Birds have large specialized diverticula radiating from the lungs and the air sacs, which permeate the coelomic cavity. This unique anatomic feature can allow communication and transportation of infectious agents from outside the body into the coelomic cavity via the respiratory tract and within the confines of the air sacs (Shalaby *et al* 1994). This finding indicate that renal failure resulted from kidney lesion may be encountered in H9N2 AIV infection in chicken. In current experiment most histopathologic changes were lymphoid and reticuloendothelial hyperplasia in spleen, cecal tonsils and duodenum. According to Swayne and Slemons (1995) these changes could be an immune response of B and T-lymphocytes to foreign antigens. Because such histopathlogic changes indicate a host-initiated immune response to contain, destroy, and/or remove an agent, they are not specific to the AIVs and do not indicate any direct or indirect pathologic effects of the virus. Mild positive IHC reaction in epithelium of cecal tonsile of a chicken on day 6 PI, might be as the result of virus replication in

alimentary system, but lack of specific histopathologic lesions and minimum non-specific lesions did not support it.

Lymphoid atrophy in thymus and bursa of fabricus was most compatible with non-specific endogenous glucocorticoide response and infiltration of lymphocytes in myocardium and with lesser degree in liver and pancreas were similar to mild non-specific immunologic reaction (Khodakaram Tafti & Mrjanmehr 1997). Probably, congestion of the brain of some samples was resulted during euthanasia. Absence of lymphocyte necrosis and viral antigens in primary and secondary lymphatic organs indicated that AIV H9N2 is neither lymphotrop nor lymphocide. Presence of foci of viral nucleoprotein localization in necrotic sites of lung and trachea, and in epithelium of cecal tonsile of a chicken, concurrent with histopathological changes indicate direct pathologic effects of AIV H9N2 and that A/Chicken/Iran/259/1998 (H9N2) isolate is epitheliotropic, which in IT rout of inoculation has tissue tropism and pathogenicity for trachea, lung, pneumotropic, and kidney, nephrotropic, (Swayne & Slemons 1984a).

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