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## Detection of Avian Influenza Virus Antigen in Chicken Tissues Following Intranasal Inoculation

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**Abstract:** To understanding the pathogenicity of H9N2 in broiler chickens, the tissue distribution of viral antigen following intranasal (IN) inoculation of this subtype was studied. Eighteen 3-week-old chickens were inoculated with  $10^6$  EID<sub>50</sub> per bird with H9N2 avian influenza virus. Then on days 1, 2, 4, 6, 8 and 11 post-inoculation (PI) samples of the trachea, lung, liver, pancreas, spleen, thymus, duodenum, kidney, brain and bursa of Fabricius were collected for immunofluorescence study. The AIV antigen was detected in the trachea, lung and kidney of inoculated chickens using indirect immunofluorescence technique. The results indicated that the H9N2 avian influenza virus is epitheliotropic in chicken. After IN inoculation it has tissue tropism for trachea, lung (pneumotropic) and kidney (nephrotropic).

**Key words:** Avian influenza virus, H9N2, intranasal, broiler chicken

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### INTRODUCTION

Avian influenza viruses are a diverse group of viruses in the family Orthomyxoviridae, genus influenza virus A and can be categorized into subtypes based on the two surface glycoproteins, the haemagglutinin (H) and neuraminidase (N). There are 16 different haemagglutinin (H1-16) and 9 different neuraminidase (N1-9) subtypes, which make 144 possible combinations of H and N subtypes. Avian influenza viruses can be further classified into two different pathotypes (low and high pathogenicity), based on the ability to cause disease and death in the major domestic poultry species, the chicken (*Gallus domesticus*) (Alexander, 2000; Slemons and Swayne, 1995; Swayne, 2007).

Highly pathogenic avian influenza (HPAI) is a devastating disease of poultry caused by some viruses of the H5 and H7 subtypes. These strains replicate throughout causing damage to the vital organs and multiple tissues and thus bring about the death of affected birds (Capua *et al.*, 2000; Rott, 1992). In contrast Low Pathogenic Avian Influenza (LPAI) viruses are capable of replicating only in few organs, mainly the respiratory and Gastrointestinal (GIT) and do not invade the rest of the body. However, frequent incidences of high mortality have been reported in field situation in outbreaks of low pathogenic avian influenza viruses such as H9N2 subtypes. Avian influenza disease, due to H9N2 subtype in poultry during later part of the 1990s, has been noticeably increased worldwide. The H9N2 subtype outbreaks occurred in domestic ducks, chickens and turkeys in Germany during 1995 and 1998, in chickens in Italy in 1994 and 1996, in pheasants in Ireland in 1997, ostriches in South Africa in 1995, turkeys in the USA in 1995 and 1996 and in chickens in Korea in 1996 (Bano *et al.*, 2003; Capua and Alexander, 2004; Naeem *et al.*, 1999). More recently, H9N2 viruses have been reported in Middle Eastern countries and have been responsible for widespread and serious disease problems in commercial chickens in Iran, Pakistan, Saudi Arabia and United Arab Emirates (Aamir *et al.*, 2007; Alexander, 2003; Banks *et al.*, 2000; Capua and Alexander, 2004; Naeem *et al.*, 1999, 2007; Nili and Asasi, 2002, 2003). In 1998 an outbreak of low pathogenic avian influenza virus (H9N2 subtype) has occurred in Iranian poultry industry (Nili and Asasi, 2002, 2003). Earlier pathogenesis studies revealed



that LPAI viruses are pneumotropic following intranasal inoculation (Swayne and Slemons, 1994). Data collected from recent avian influenza outbreaks indicate that LPAI virus may mutate and become HPAI, probably after infection of poultry (Garcia *et al.*, 1996), resulting in extremely complex situations that may have dramatic effects on the poultry industry. The purpose of this experiment was to determine the effect of IN inoculation of Iranian AIV H9N2 subtype on viral antigen distribution in chicken tissues.

## MATERIALS AND METHODS

This study was conducted from February 2008 to September 2008.

### Experimental Design

Thirty six 3-week-old broiler chickens (Ross, UK) were randomly divided in two equal groups (Test and Control) and were housed in the same condition in two separate rooms. Chickens were monitored on a daily basis for general condition and the presence of clinical signs. All the birds were bled for detection of specific antibodies against H9N2 subtype of AIV. Subsequently the test group was inoculated intranasally with  $10^6$  EID<sub>50</sub> per bird of H9N2 avian influenza virus at 20 days of age. Three birds from each group (test and control) were randomly selected on days 1, 2, 4, 6, 8 and 11 post-inoculation (PI). Then they were humanly sacrificed and were subjected to throughout necropsy. Gross lesions were recorded and samples of different tissues including the trachea, lung, liver, pancreas, spleen, thymus, duodenum, kidney, brain and bursa of Fabricius were collected for immunofluorescence study.

### Fluorescent Microscopy

At necropsy the tissue samples were collected and immediately frozen for immunofluorescence. Frozen sections of each tissue were prepared with cryostat (Cryocut 1800, Leica, Reichert Jung) and gently placed onto a glass microscope slide, then sections were allowed to air dry. Sections were then fixed in acetone for 15 min at room temperature and again allowed to air dry. An appropriate dilution (1/70) of mouse anti-Influenza A monoclonal antibody (MAB8258, Chemicon, USA) was applied for each tissue sections and incubated at 37°C for 45 min. Unbound antibodies were removed by three washes in Phosphate-Buffered Saline (PBS), pH 7.2. Then appropriate dilution (1/50) of goat-anti mouse FITC conjugate antibody (AP130F, Chemicon, USA) added to each tissue sections. Then slides were incubated at 37°C for 30 min and unbound conjugate was removed by three washes in PBS. Slides were mounted with 50% buffered glycerol and examined under a fluorescent microscope.

## RESULTS

### Clinical Findings

Daily monitoring did not show any changes in clinical behaviour of the birds in control group. Infected chickens showed clinical signs such as depression, puffing, oedema of face and head, conjunctivitis and ruffled feathers on days 3 or 4 PI.

### Gross Necropsy Findings

Control chickens did not show any gross lesions. However, the most frequent gross lesions in infected birds were turbidity of the thoracic and abdominal air sacs and mild congestion of the trachea and lung and mild accumulation of fibrinous exudate on the tracheal mucosa.

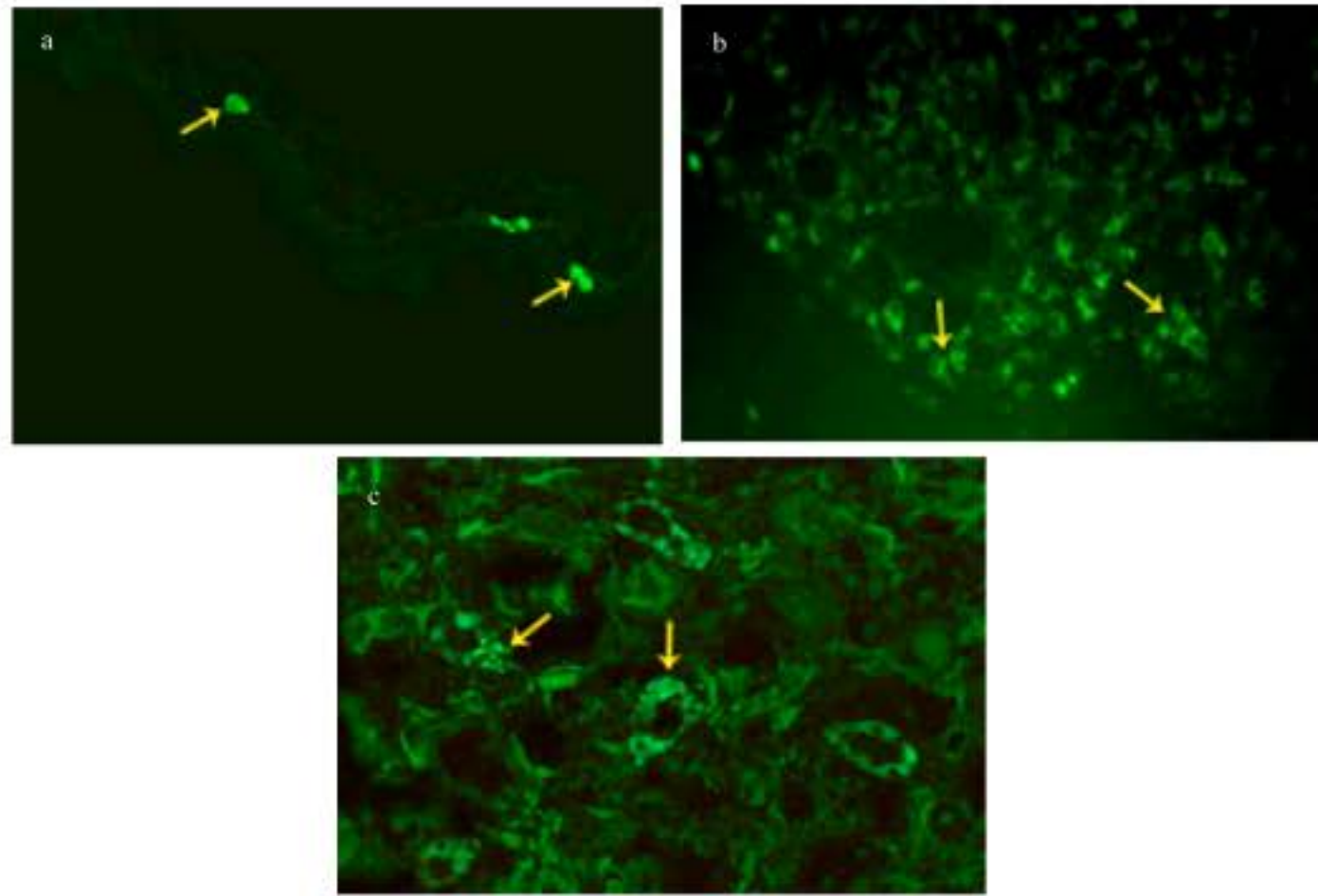


Fig. 1: (a) Indirect immunofluorescence technique shows the clear localization of AIV antigen in the trachea frozen section (arrows) on day 4 PI (IIF x100), (b) distribution of AIV antigen 6 days PI in the lung frozen section can be seen (arrows), using indirect immunofluorescence technique (IIF x250) and (c) distribution of AIV antigen 8 days PI in the kidney tubules can be seen (arrows), using indirect immunofluorescence technique (IIF x250)

#### Serological Findings

The specific antibody titer against H9N2 avian influenza virus at days 1, 2, 4, 6, 8 and 11 post-inoculation in treatment group was 3, 2.4, 1.7, 3.3, 4.2 and 5.6 and in control group was 3, 2.7, 2.34, 1.8, 1.24 and 0, respectively.

#### Viral Antigen Distribution

In the control chickens (18 birds), there was no detectable H9N2 virus antigen in all of the examined organs.

In inoculated chickens, using indirect immunofluorescence technique, influenza virus nucleoprotein was demonstrated in the trachea on days 2, 4, 6, 8 (Fig. 1a) and 11 PI, in the lung on days 2, 4, 6, 8 (Fig. 1b) and 11 PI and in the kidney on days 6, 8 and 11 PI (Fig. 1c).

### DISCUSSION

Although experimental study of low pathogenic AI viruses in SPF chicken cause no or low mortality, frequent high mortality rates have been reported in the field cases (Bano *et al.*, 2003; Naeem *et al.*, 1999, 2003; Nili and Asasi, 2002, 2003).

Intranasal inoculation of chickens with Iranian AIV H9N2 subtype revealed that the virus is epitheliotropic in chicken and tracheitis, pneumonia and tubulointerstitial nephritis were the most frequent histologic changes.

In some researches (Slemons and Swayne, 1990; Slemons *et al.*, 1990; Swayne and Slemons, 1992, 1994, 1995; Swayne *et al.*, 1994; Swayne and Pantin-Jackwood, 2006), inoculation of chickens by



intranasal (IN) and intratracheal (IT) route with low virulence chicken- or duck-origin influenza virus isolates produced mortality and kidney lesions in 1-day-old chickens and adult hens. However, in other studies absence of mortality has been reported by Shalaby *et al.* (1994) and Swayne *et al.* (1994). In the current study, presence of necrosis and inflammation in the trachea, lung and kidney, along with demonstration of influenza nucleoprotein using IFA in the trachea, lung and kidney indicate that the Iranian AIV H9N2 subtype has tissue tropism for respiratory system and kidney of chickens.

In this experiment, tracheitis, pneumonia and tubulointerstitial nephritis were the most frequent histologic lesions. The lesions were obvious on days 2-8 PI but on day 11 PI the severity of lesions was reduced. Clinically this was associated with improvement in general condition of inoculated birds.

Inoculation of an isolate of H9N2 avian influenza virus to chickens using different routes and subsequently challenged with other infectious agents. The AIV antigen was detected in the trachea, lung, kidney and cloacal bursa among infected birds (Bano *et al.*, 2003).

In chickens intratracheally inoculated with avian influenza virus isolates of either low or high pathogenicity the low pathogenicity isolates produce histologic lesions most frequently in the trachea and lung or lacked histologic lesions. Viral antigen was present only and infrequently in the trachea and lung of inoculated chickens (Mo *et al.*, 1997). In another study after intravenous inoculation of avirulent H4N4, H6N2 and H3N8 viruses into chickens, specific lesions and immunoperoxidase staining were noted in the kidney only (Hooper *et al.*, 1995).

Inoculation of two low pathogenicity and two high pathogenicity avian influenza viruses into chickens by intranasal route showed that the LPAI viruses caused localized virus infections in the respiratory and gastrointestinal tracts (Swayne and Beck, 2005). Inoculation of three strains of AIV with chicken/duck origins to 5-week-old chickens by intravenous (IV), intranasal and IT routes revealed that the 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 (Swayne and Slemons, 1994). As abdominal air sacs are next to kidneys, presumably, presence of nephritis foci in the kidneys during 6-11 days PI could be resulted from infection of the respiratory tract (air sacs). Regarding kidney lesions, the result obtained from the present study is in agreement with finding by Slemons and Swayne (1990) and Slemons *et al.* (1990).

This finding indicate that renal failure resulted from kidney lesion could be encountered in H9N2 AIV infection in chicken. In current experiment common non-specific changes were lymphoid and reticuloendothelial hyperplasia in duodenum and spleen. These changes could be an immune response of B and T-lymphocytes to foreign antigens. Lymphoid atrophy in thymus and bursa of Fabricius was most compatible with non-specific endogenous glucocorticoid response and mild infiltration of lymphocytes in pancreas was similar to mild non-specific immunologic reaction (Swayne and Slemons, 1995). Absence of lymphocyte necrosis and viral antigens in primary and secondary lymphatic organs show that lymphoid tissues are not target of AIV H9N2.

## CONCLUSION

Presence of viral antigen in the trachea, lung and kidney of chickens, concurrent with histologic lesions indicate that the H9N2 avian influenza virus is epitheliotropic, which in IN route of inoculation has tissue tropism and pathogenicity for the trachea and lung (pneumotropic) and kidney (nephrotropic).

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