Iranian Journal of Veterinary Research, Shiraz University, Vol. 9, No. 4, Ser. No. 25, 2008

Pathological changes in turkeys experimentally infected with different doses of A/ostrich/Italy/984/2000 H7N1 avian influenza virus

Nili, H.[1, 3*]; McNally, A.[2]; Aldous, E.[3]; Nunez, A.[4]; Banks, J.[3] and Brown, I. H.[3]

[1]Department of Avian Medicine, School of Veterinary Medicine, Shiraz University, Shiraz, Iran; [2]School of Biomedical and Natural Science, Nottingham Trent University, Clifton Lane, Nottingham, NG11 8NS, UK; [3]Department of Avian Virology, Veterinary Laboratory Agency, Weybridge, Surrey, KT15, 3NB, UK; [4]Department of Histopatholog, Veterinary Laboratory Agency, Weybridge, Surrey, KT15, 3NB, UK

[*]**Correspondence:** H. Nili, Department of Avian Medicine, School of Veterinary Medicine, Shiraz University, Shiraz, Iran. E-mail: nili@shirazu.ac.ir

(Received 24 Sept 2007; revised version 3 Feb 2008; accepted 24 May 2008)

Summary

Following experimental inoculation of 3-week-old turkeys with different titres (10^6, 10^4, 10^3, 10^2 and 10^1 egg infectious dose (EID_{50})) of A/ostrich/Italy/984/2000 H7N1 highly pathogenic avian influenza virus (HPAIV), the selected tissues and organs were examined for pathological changes. Tissue samples from different organs that obtained from dead and sacrificed birds were fixed in 10% neutral buffer formaldehyde. Mortality of turkeys which inoculated with different doses of EID_{50} at different times post inoculation (PI) is as follows: 1) at 48 h PI (HPI): one, two and four turkeys inoculated with 10^3, 10^4 and 10^6 EID_{50}, respectively 2) at 72 HPI: two, two and one turkeys inoculated with 10^2, 10^3 and 10^6 EID_{50}, respectively 3) at 96 HPI: one and two turkeys inoculated with 10^2 and 10^4 EID_{50}, respectively and 4) at 120 HPI: just one turkey inoculated with 10^4 EID_{50}. Birds inoculated with 10^1 EID_{50} did not show any mortality. Seven days PI (DPI) the remaining birds were sacrificed. Postmortem examination of birds that died 48 HPI showed very severe hyperaemia and haemorrhage of the lung, slight swelling of kidneys and splenomegaly. Moderate to slight hyperaemia of the lung was observed in the birds sacrificed on day 7. Histopathology showed very severe haemorrhage and vasculitis in the lung, multifocal areas of degeneration and necrosis in the pancreas of birds inoculated with 10^6 EID_{50}. Hyperaemia, haemorrhage, degeneration and vasculitis were also observed in the lung of birds from the other groups; however the severity of lesions correlated positively with the viral dose. The spleen, caecal tonsils and thymus showed extensive necrosis and lymphoid depletion, even in birds inoculated with 10^2 and 10^1 EID_{50} that were sacrificed 7 DPI, and some repopulation of the spleen was observed 7 DPI. Other organs including the kidneys and adrenal gland showed moderate to slight hyperaemia and necrosis. In conclusion, the lung vascular damage, lymphoid tissue destruction and necrosis were notable even with low viral doses.

Key words: Avian influenza virus H7N1, Viral dose, Pathological changes, Turkey

Introduction

In recent years, frequent outbreaks of highly pathogenic avian influenza viruses (HPAI) in different parts of the world have resulted in real concerns, not only because of the great economic losses in the poultry industry, but also because of possible cross-species transmission of the virus, leading to a potentially fatal human pandemic (Capua and Alexander, 2006; OIE, 2006).

AI viruses are classified into two pathotypes, low-pathogenicity (LPAI) and HPAI viruses based on virulence for chickens using an intravenous pathgenecity test. The HPAI viruses are defined as follows: 1) any AI virus that produces ≥75% mortality or has an intravenous pathogenicity index of >1.2 or 2) any H5 and H7 AI viruses that have a hemagglutinin proteolytic cleavage site comparable with a HPAI virus (OIE, 2004).

During 1999-2000, an epidemic of highly pathogenic avian influenza virus

H7N1 occurred in Italy and various species of Anseriformes and Gallinaformes were infected (Capua and Mutinelli, 2001). The outbreak started with LPAIV which mutated to HPAIV that resulted in death or culling of over 13 million birds (Capua *et al*., 2003).

Understanding host pathogen interactions is crucial in defining the disease process in susceptible hosts. Since gross and microscopic lesions of HPAI H7N1 virus circulating in Italy during 1999-2000 has been described in natural outbreaks, this study was conducted to determine gross and microscopic lesions in different organs of commercial turkeys (confirmed AI free by serology and virus isolation), which were experimentally inoculated with different doses of A/ostrich/Italy/984/2000 H7N1 HPAI.

More information and scientific data are required to understand the host-virus interaction in different species of animals. Such information could be used to implement more efficient control measures both at national and international level (Perdue and Swayne, 2005).

Materials and Methods

Three-week-old commercial turkeys, confirmed AI free by virus isolation and serology (n = 25) were divided into 5 separate groups and allowed free access to food and water. Each group was inoculated with one of the following viral doses: 10^6, 10^4, 10^3, 10^2 and $10^1 EID_{50}$ of A/ostrich/Italy/984/2000 H7N1 HPAI virus via a combined intranasal/intraocular route. Mortalities were recorded, dead birds were subjected to necropsy, the remaining birds were sacrificed after 7 days PI (DPI) and then necropsied to determine pathological changes. Gross lesions were recorded and tissue samples of the lung, liver, trachea, spleen, pancreas, intestine, caecal tonsils, and adrenal gland were fixed in 10% neutral buffered formaldehyde. Tissues were routinely processed and embedded in paraffin wax. Five to seven micrometer sections were cut and stained with haematoxylin and eosin, then the prepared tissues were studied under light microscope. All experimental studies with viable virus were performed in certified biosafety level 3 facilities.

Results

Mortality

Mortality of birds inoculated with different doses of A/ostrich/Italy/984/2000 H7N1 is presented in Fig. 1.

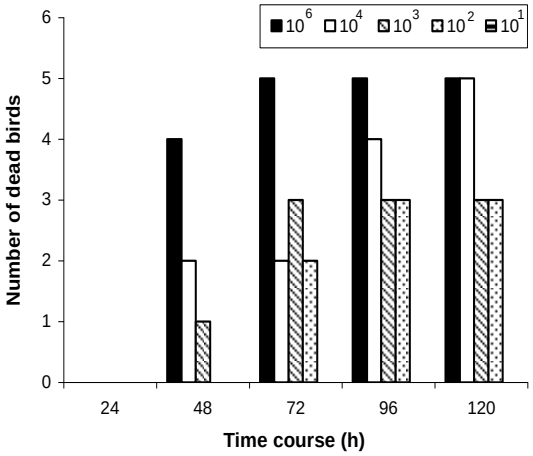

Fig. 1: Graph showing cumulative mortality of turkeys infected with different doses of H7N1 at different time course. Numbers in legend indicate the viral doses given to that group of birds

Birds showed predominantly depression and ruffled feathers prior to death. Four birds inoculated with 10^6 EID_{50}, two birds in the 10^4 group and one in the 10^3 group died 48 h PI. No mortality was observed in birds inoculated with $10^1 EID_{50}$.

Postmortem examination

Postmortem lesions were mainly observed in dead birds rather than sacrificed ones (Figs. 2 and 3). The main gross lesions that observed at necropsy are shown in Table 1. Very severe pancreatic necrosis with parenchymal mottling was more prominent in 10^6 EID_{50}. The pancreas showed red discoloration in 10^4 and 10^6 EID_{50} inoculated birds and patchy discoloration in 10^3 EID_{50} inoculated birds. Slight renomegaly and hyperaemia with parenchymal pallor and accentuated lobular surface architecture were also observed in this group. The trachea did not show any abnormality, however the nasal concha showed slight to moderate

hyperaemia and exudation. Generalized hyperaemia of the breast muscles was observed only in 10^4 and 10^6 EID_{50} groups. The liver was normal in colour, size and texture almost in all examined birds.

Table 1: Severity of postmortem lesions observed in turkeys inoculated with different viral doses of A/ostrich/Italy/984/2000 H7N1 AIV

Type of lesion	EID_{50}				
	10^6	10^4	10^3	10^2	10^1
Hyperaemia and congestion of the lung	+++++	+++	++	-	-
Necrosis of the pancreas	++++	+++	++	-	-
Muscular congestion	+++	+++	+	-	-
Subcutaneous and serosal heamorrhage	-	-	-	-	-

+ thgils, ++ thgils ot etaredom, +++ severe, ++++ very severe and +++++ extensive

Histopathology

Lung: The lung was one of the most consistently affected organs with hyperaemia, haemorrhage, heterophil infiltration and vasculitis and necrosis of the wall of blood vessels. The wall of blood vessels did not show continuity and rupture sites in medium and large sized blood vessels were frequently seen (Figs. 4 and 5). The wall of blood vessels appeared extremely thin and disintegrated in some areas with little oedema either around blood vessels or in interparabronchial septa. The lung damage was moderate to severe in birds inoculated with 10^2 EID_{50}. Bronchial associated lymphoid tissues (BALT) showed severe hypertrophy, hyperplasia, hyperaemia and haemorrhage in 10^3 EID_{50} group died 72 HPI (Figs. 6 and 7).

Nasal concha: There were severe hyperaemia and moderate to severe heterophil infiltration and necrosis of nasal concha in the 10^6 EID_{50} group. This was accompanied by mucoid exudates containing sloughed epithelial cells and thrombosis in small blood vessels in 10^3 and 10^4 EID_{50} groups. More subtle reactions were observed in the 10^1 EID_{50} group. Some minimal apoptotic figures could also be seen in the10^1 EID_{50} group.

Trachea: The trachea was generally normal though there was slight hyperaemia and patchy deciliation in some birds received 10^3 or more EID_{50}. In one bird from the 10^4 EID_{50} group which died 72 HPI, there was very severe necrotizing tracheaitis. The tracheal mucosa was considerably thicker than in control birds and showing coagulative necrosis.

Ovary: In one of the turkeys from the 10^3 EID_{50} inoculated group, the ovary showed severe hyperaemia and a couple of necrotic foci.

Pancreas: Severe multifocal necrosis and degeneration were observed in the pancreas of birds from 10^4 and 10^6 EID_{50} groups. The pancreas of birds from 10^3 EID_{50} and 10^2 EID_{50} groups also showed some necrosis and degeneration, however the necrotic foci were more scattered and limited. There was no inflammatory reaction to necrotic tissues. The necrotic foci were surrounded with vacuolar degeneration of pancreatic cells. Very slight pancreatic degeneration was observed in birds inoculated with 10^1 EID_{50}.

Thymus: The histopathology study of the thymus showed the most variable results. Hyperaemia and necrosis of the medulary region with variable severity were observed in birds inoculated with different viral doses. In one of the birds inoculated with 10^1 EID_{50}, the thymus showed extensive necrosis and very severe heterophil infiltration which affected both the cortical and medulary areas. There was complete destruction of thymus architecture. Cortical areas were ruptured in several places with necrotic masses escaped to the adjacent soft tissues.

Spleen: Extensive necrosis was observed in the spleen of birds inoculated with 10^6 and 10^4 EID_{50} that had died 72 and 48 HPI, respectively (Fig. 8). Surviving birds in groups inoculated with 10^3 and 10^2 EID_{50} were sacrificed 7DPI which showed severe necrosis of the spleen. However, some repopulation of the spleen with lymphocytes was observed in birds inoculated with 10^2 EID_{50} and 10^3 EID_{50}, killed 7 DPI (Fig. 9).

Caecal tonsils: Severe necrosis, lymphoid depletion and heterophil infiltration were observed in birds inoculated with more than 10^2 EID_{50}. Some degeneration in lymphoid tissues was observed in birds inoculated with 10^1 EID_{50}.

Liver: Variable severity of lesions was observed in the liver. One of the birds in the 10^6 EID_{50} inoculated group which died 48

HPI, showed severe hyperaemia of the liver, but no necrosis. Multifocal hepatocellular necrosis and degeneration were observed in birds inoculated with 10^2 and 10^3 EID_{50}. Also, the liver in one turkey inoculated with 10^2 EID_{50} and two inoculated with 10^3 EID_{50} showed hydropic degeneration. Generally, the severity of lesions in liver was less than pancreas and lymphoid tissues.

Kidney: Degenerative changes ranging from the presence of circular eosinophilic materials in proximal and collecting tubules and necrosis of the proximal tubules in 10^4 EID_{50} inoculated group to severe hyperaemia and necrosis in 10^6 EID_{50} inoculated group were observed.

Discussion

During 1999, northeastern Italy was affected by an epidemic of LPAI due to a H7N1 virus. In December 1999 the H7N1 LPAI virus mutated to a HPAI virus that spread rapidly, affected 413 farms of different species and caused great economic loss with the death of over 14 million birds Gross and microscopic findings in field cases affected by the virus has been reported (Mutinelli *et al*., 2003). This study was conducted to evaluate gross and microspcopic lesions in turkeys following experimental inoculation of different doses of H7N1 avian influenza virus.

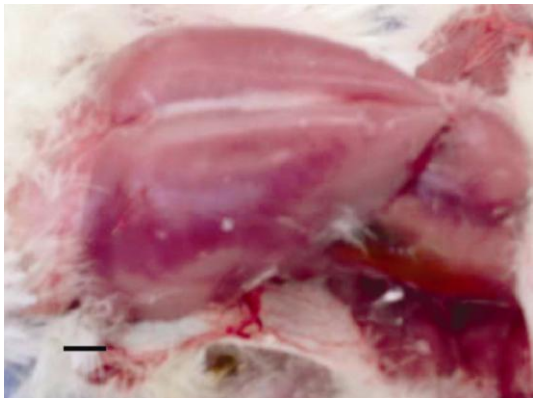

Fig. 2: Breast muscle from a 3-week-old turkey inoculated with 10^4 EID_{50} of H7N1 viruse, died 72 h post inoculation, showing very severe muscular hyperaemia

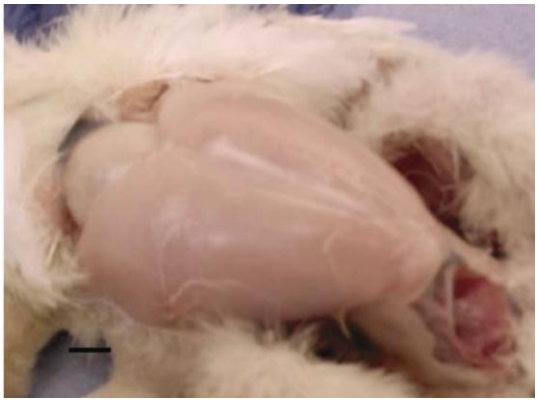

Fig. 3: breast muscles from a turkey in 10^2 EID_{50} inoculated group that sacrificed 7 days post inoculation

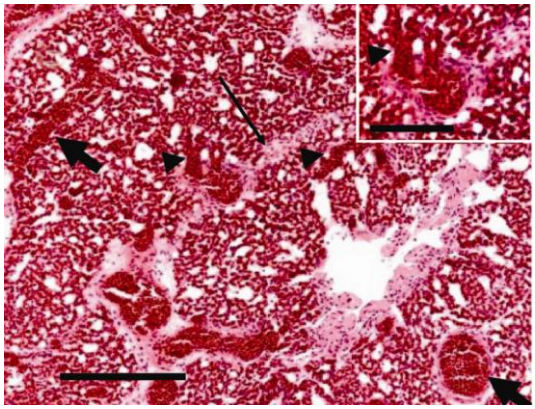

Fig. 4: Lung section from a turkey in 10^6 EID_{50} inoculated group showing very severe hyperaemia (thick arrows) and obliteration of air capillaries due to hyperaemia and haemorrhage, frequent breakdown of blood vessel walls (arrow heads) also minimal damage to interparabronchial wall could be seen (thin arrow), scale bars = 200 µm

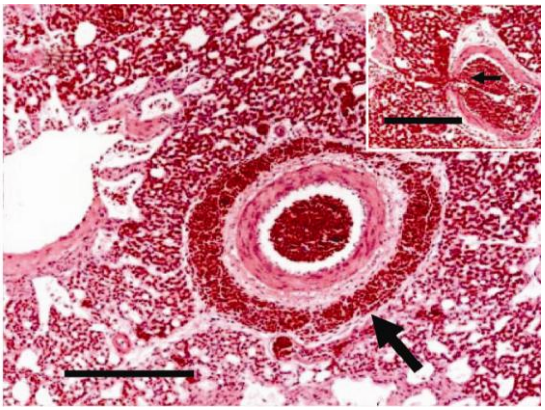

Fig. 5: Lung section from a turkey in 10^3 EID_{50} inoculated group survived 7 days post inoculation, showing haemorrhage around large blood vessel (big arrow) and a rupture site of a blood vessel (*inset*, small arrow). The haemorrhage was not wide spread and the rupture site of blood vessels showed less frequency, scale bars = 200 µm

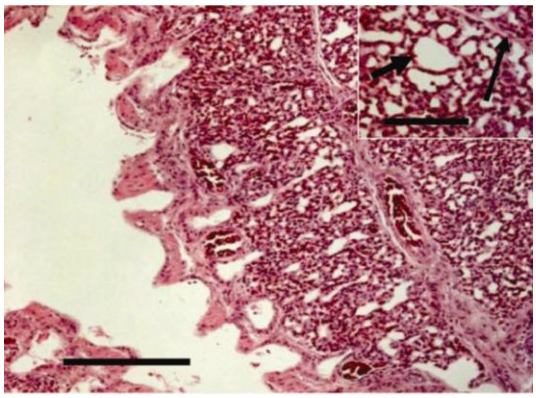

Fig. 6: Lung section from a bird in control group showing normal vascularity, air capillary (thick arrow) and interparabronchial septa (thin arrow), scale bars = 100 µm

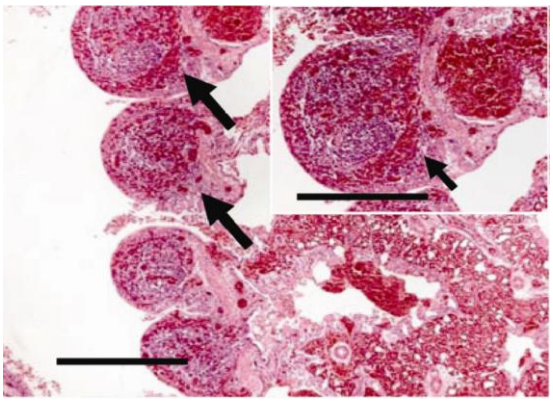

Fig. 7: Bronchial associated lymphoid tissues from a bird in 10^3 EID_{50} group died 72 h post inoculation showing hypertrophy and haemorrhage (arrows), scale bar = 500 µm (*inset*, scale bar = 625 µm)

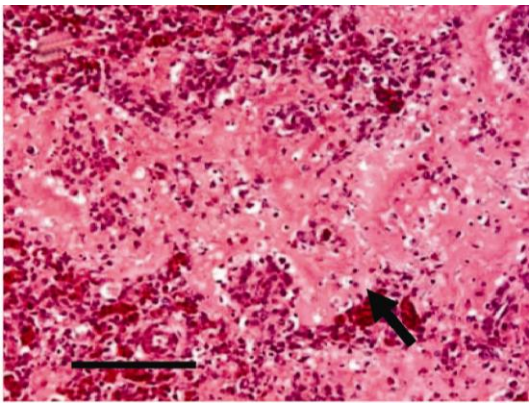

Fig. 8: Spleen section from a turkey in 10^4 EID_{50} inoculated group died 72 h post inoculation, showing very severe necrosis and degeneration (arrow), scale bar = 75 µm

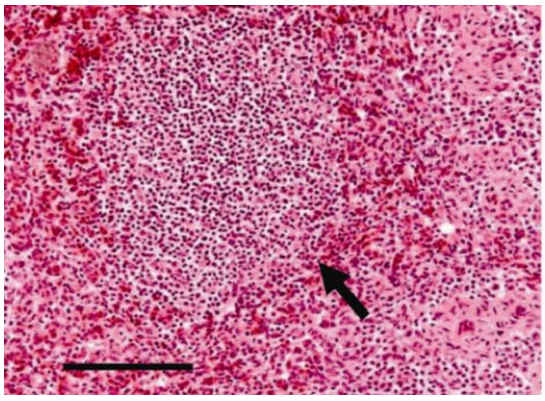

Fig. 9: Spleen section from a turkey in 10^3 EID_{50} survived 7 days post inoculation, showing repopulation of lymphoid cells (arrow), scale bar = 100 µm

The mortality of turkeys in 10^2-10^6 EID_{50} inoculated groups demonstrates that the virus is highly pathogenic and well adapted to turkeys.

During 1999-2000 in Italy, mortality ranging from 5% to 97% and 100% were observed in turkeys in field outbreaks of LP and HP H7N1, respectively (Mutinelli *et al*., 2003). In the field some of the mortality could be attributed to combined effects of other pathogens such as *Escherichia coli* and *Pasteurella multocida* which were also isolated from birds submitted during the outbreak (Muinelli *et al*., 2003). However, the results of this study showed that even in a well controlled environment in confinement facility, the virus is able to cause similar mortality in 10^4 EID_{50} inoculated group and also produce severe tissue damages in several organs in a group infected with as little as 10^2 EID_{50} of virus (Kobayashi *et al*., 1996). This finding is in agreement with those of previous studies suggesting species adaptation and maximal pathogenecity of AIV isolates for their corresponding gallinaceous host of origin (Perkins and Swayne, 2001). The severity of postmortem lesions such as lung hyperaemia and heamorrhage, pancreatitis, and muscular congestion were dose dependent, however they were not present in birds inoculated with 10^1 and 10^2 EID_{50}.

Severe necrotizing tracheaitis that observed in one of the birds inoculated with 10^4 EID_{50} could not be directly attributed to pathogenecity of the virus, because this lesion was not observed in the trachea of other birds inoculated with higher and lower

viral doses. However slight damage to mucociliary apparatus in the trachea might be responsible for facilitating secondary bacterial invasion of an opportunistic commensal organism.

The severity of postmortem and histopathological findings in some organs correlated with viral dose. For example, 10^2 EID_{50} viral dose caused moderate to severe tissue reaction in lymphoid organs, nasal concha and the lung in related birds. The thymus showed extensive inflammatory reaction, tissue destruction and necrosis in one of the birds inoculated with 10^1 EID_{50} which indicate that the lymphoid tissues and especially the thymus might be the primary target organs for the virus. Of particular interest is the pattern of lung reaction in all inoculated groups which followed break down of vascular integrity and caused very severe pulmonary haemorrhage in all inoculated groups except in 10^1 EID_{50}. No such severe lung damage has been reported before (Perkin and Swayne, 2001, 2003). Fibrinoid necrosis of vascular wall has been reported in field cases during 1999-2000 Italian outbreaks. Similar studies in SPF chickens using different doses of H5N1 A/turkey/Turkey (Türkiye)/1/05 H5N1 avian influenza virus resulted in severe pulmonary oedema in chicken with no pulmonary haemorrhage (Nili *et al*., 2006).

We expected to see more necrosis and degeneration in the pancreas because it contains endogenous trypsin which can facilitate exposure of cleavage sites of haemagglutinin antigen. However, lymphoid organs such as spleen, thymus and caecal tonsils showed more severe necrosis and tissue damage even with a low viral titre (Nakatani *et al*., 2005).

References

Capua, I; Marangon, S; Pozza, MDl; Terreginon, C and Gattoli, G (2003). Avian influneza in Italy 1997-2001. Avian Pathol., 10: 281-293.

Capua, I and Mutinelli, F (2001). Mortality of Muscovy ducks (*Cairina moschata*) and domestic geese (*Anser anser var. domestica*) associated with natural infection with a highly pathogenic avian influenza virus of H7N1 subtype. Avian Pathol., 30: 179-183.

Capua, I and Alexander, DJ (2006). The challenge of avian influenza to the veterinary community. Avian Pathol., 35: 189-205.

Kobayashi, Y; Horimoto, T; Kawaoka, Y; Alexander, DJ and Itakura, C (1996). Pathological studies of chickens experimentally infected with two highly pathogenic avian influenza viruses. Avian Pathol., 25: 285-304.

Mutinelli, FI; Capua, C; Terregino, C and Cattoli, G (2003). Clinical, gross, and microscopic findings in different avian species naturally infected during the H7N1 low- and high-pathogenecity avian influenza epidemics in Italy during 1999-2000. Avian Dis., 47: 844-848.

Nakatani, H; Nakamura, K; Yamamoto, Y; Yamada, M and Yamamoto, Y (2005). Epidemiology, pathology, and immunohistochemistry of layer hens naturally affected with H5N1 highly pathogenic avian influenza in Japan. Avian Dis., 49: 3, 436-441.

Nili, H; Aldous, E; McNally, A; Banks, J and Brown, IH (2006). Pathological changes in chickens infected experimentally with different doses of A/turkey/Turkey (Türkiye)/1/05 H5N1 avian influenza virus. 6th international symposium on avian influenza. Camberidge University, UK. P: 49.

Office of International des Epizooties (OIE) (2004). Manual of standards for diagnosis tests and vaccines, 2004. Office International des Epizooties, Paris, France, PP: 957.

Office International des Epizooties (OIE). Update on avian influenza A in animals (Type H5). Available at: http://www.oie.int/downld/avian%20influenza/A-AI-Asia.htm. Accessed: March 24, 2006.

Perdue, ML and Swayne, DE (2005). Public health risk from avian influenza viruses. Avian Dis., 49: 317-327.

Perkins, LEL and Swayne, DE (2001). Pathobiology of A/Chicken/Hong Kong/220/97 (H5N1) Avian influenza virus in seven Gallinaceous species. Vet. Pathol., 38: 149-164.

Perkins, LEL and Swayne, DE (2003). Comparative susceptibility of selected avian and mammalian species to a Hong Kong-origin H5N1 Avian Influenza Virus. Avian Dis., 47: 951-955.