Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Human Pathology (2009) **40**, 735–739

ELSEVIER

Human PATHOLOGY

www.elsevier.com/locate/humpath

Case study

Systemic infection of avian influenza A virus H5N1 subtype in humans☆

Zengfeng Zhang MSc[a,b], Jinxia Zhang PhD[a], Kai Huang MSc[a], Kang-Sheng Li PhD[b], Kwok-Yung Yuen MD[a], Yi Guan PhD[a,b], Honglin Chen PhD[a,b], Wai Fu Ng MD[c,*]

[a]*State Key Laboratory for Emerging Infectious Diseases, Department of Microbiology and Research Center of Infection and Immunology, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Pokfulam, Hong Kong SAR, China*
[b]*International Institute of Infection and Immunity, Shantou University Medical College, Guangdong, China*
[c]*Department of Pathology, Princess Margaret Hospital, Hospital Authority, Lai Chi Kok, Hong Kong SAR, China*

Received 28 June 2008; revised 28 July 2008; accepted 29 August 2008

Keywords:
Avian influenza A virus;
H5N1 subtype;
Human infection;
Tissue tropism;
Immunohistochemistry

Summary The viral dissemination in a patient with avian influenza A subtype H5N1 infection was retrospectively studied by the immunohistochemical localization of viral nucleoprotein antigen. The pathology was marked by diffuse alveolar damage, lymphoid depletion, and reactive hemophagocytic syndrome. Besides the lung and the upper respiratory tract, viral antigen was detected in the small and large intestinal epithelial cells, hematopoietic cells in the bone marrow, glial cells and neurons of the brain, and lymphocytes. The results confirmed that H5N1 virus disseminated to multiple organs beyond the respiratory system. However, specific pathological changes were noted in the respiratory system only, and productive viral replication confirmed by culture was noted only in the lung. More postmortem studies are needed to elucidate the pathogenesis of this highly fatal zoonotic disease.

1. Introduction

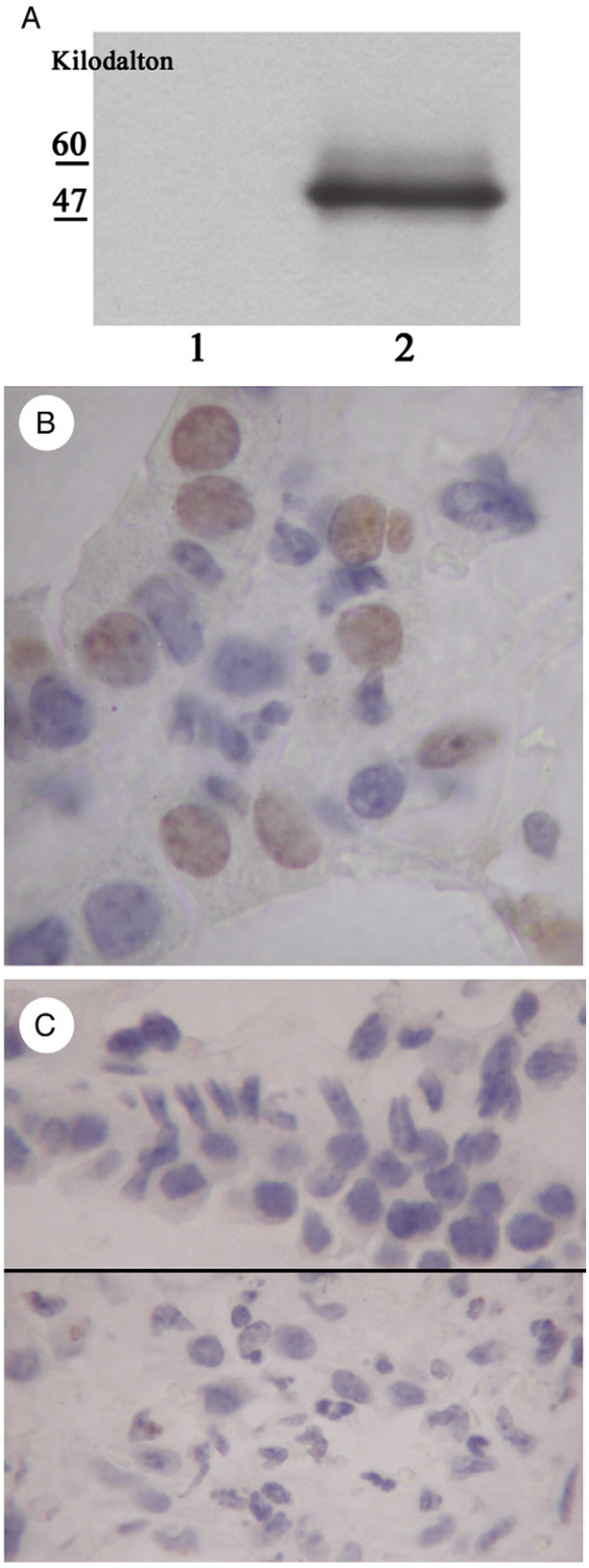
Highly pathogenic avian influenza A virus H5N1 subtype is endemic in poultry in some countries [1] and has caused more than 300 human infections in 15 countries with about 60% mortality since 2003 [2]. The pathology was marked by diffuse alveolar damage, lymphoid depletion, and reactive hemophagocytic syndrome [3,4]. Positive postmortem viral culture was limited to the lung. Recently, Gu et al [5] demonstrated extrapulmonary dissemination of H5N1 in 2 cases, including vertical transmission to a fetus. Here, we report the retrospective examination of viral dissemination in a patient by the immunohistochemical localization of viral nucleoprotein (NP) antigen. NP could be demonstrated in the cytoplasm and nuclei of infected host cells during viral replication.

2. Materials and methods

The clinical and brief pathological features of the patient were reported previously [6]. The patient was a 33-year-old

☆ Research Grant Council (HKU 74888/05M and 7500/06M) and Research Fund for the Control of Infectious Diseases from Health, Welfare and Food Bureau, Government of Hong Kong SAR, Li Ka Shing Foundation and the Providence Foundation Limited in memory of the late Dr Liu Hac Minh.
* Corresponding author.
E-mail address: ngwf2@ha.org.hk (W. F. Ng).

0046-8177/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.humpath.2008.08.015



man who died of respiratory failure on day 11 of disease in 2003 in Hong Kong. He was admitted for symptoms of influenza for 4 days. Mild lymphopenia and radiological evidence of right lower lobe consolidation were noted. He was diagnosed with atypical community acquired pneumonia and was started on intravenous cefotaxime, oral clarithromycin, and sultamicillin after microbiological tests, which turned out to be negative. He continued to deteriorate with increased dyspnea, bilateral chest consolidation, and hypoxemic respiratory failure requiring intensive care on day 3 of admission. Oral oseltamivir was added. His oxygen saturation was at 93.3% with a 50% oxygen mask. Mechanical respiration was needed on day 4 when oxygen saturation dropped to 89.3% with a100% oxygen mask. He died of refractory respiratory failure on day 7. Corticosteroid was not given throughout the course. Influenza A subtype H5N1 was subsequently cultured from the nasopharyngeal aspirate. The present study included a complete histological review and immunohistochemical study of representative organs. The monoclonal antibody against NP was developed in the Department of Microbiology, the University of Hong Kong by hybridoma derived from mice infected with H5N1 strain CK/YU22/02 (clone 17H4). The derived antibody was immunoglobulin IgG2a class. Its specificity for NP was verified by Western blot using lysate from cells transfected with NP plasmid and control cells without transfection (cell line 293). For the immunohistochemical technique, formalin-fixed paraffin-embedded sections were incubated with the monoclonal antibody at 1:5000 dilution at 4°C overnight, then incubated with goat antimouse IgG, heavy-chain–specific and light-chain–specific biotin conjugate (Calbiochem) at 1:2000 dilution for 30 minutes at room temperature. After incubation with streptavidin/peroxidase complex reagent (Vector Laboratories) for 30 minutes at room temperature, color was developed using 3,3′-diaminobenzidine (Vector Laboratories) according to the manufacturer's instructions. The immunohistochemical technique was verified by using lung sections of mouse killed 48 hours after infection with H5N1 virus. To evaluate for false-positive reactions due to endogenous biotin, we used controls with addition of antihuman papillomavirus (anti-HPV) antibody (monoclonal antibody of IgG2a class developed locally against L1 antigen of HPV serotype 18, dilution 1:5000) and phosphate-buffered saline (PBS) instead of the anti-NP antibody. In addition, sections of similar organs of 2

Fig. 1 Characterization of the monoclonal anti-NP antibody. A, Western blot with anti-NP in lysates of (1) cultured 293 cells and (2) cultured 293 cells transfected with NP plasmid. The band showed localization of NP at about 50 kd. B-C, Immunohistochemistry of lung sections of mouse infected with H5N1 virus. Positive reaction was demonstrated by brown reaction of the nuclei. B, Positive epithelial cells in bronchiolar epithelium. C, Negative control with anti-HPV. Upper part bronchiolar epithelium and lower part alveolar septum (original magnifications: B ×1000, C ×630).

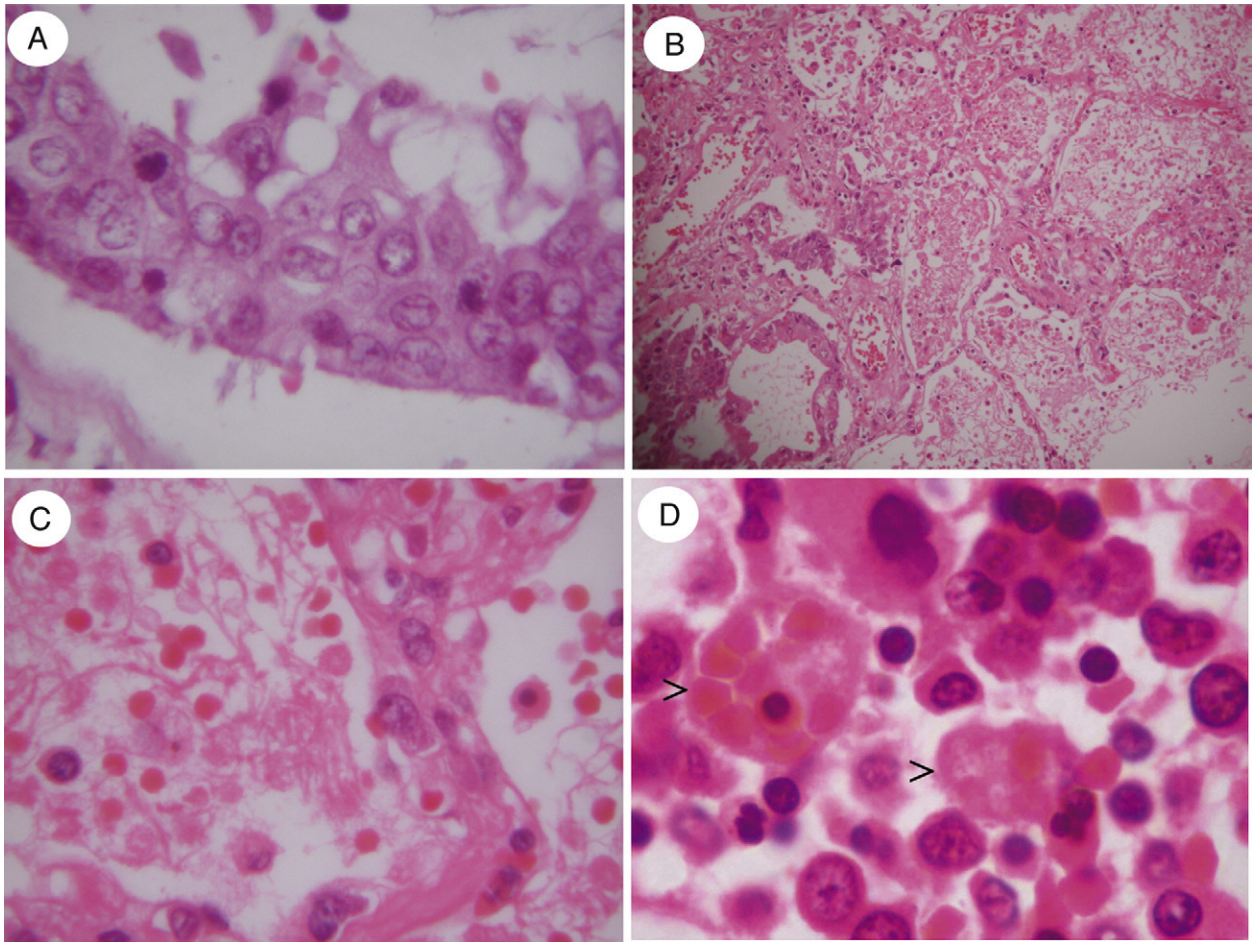


Fig. 2 Pathological changes. A, Tracheal epithelium with regenerative hyperplasia. B, Lung with diffuse alveolar damage and bronchiolar epithelial hyperplasia in left lower corner. C, High-power view of alveoli with fibrin, red cell and inflammatory cells in alveolar lumen, and plump pneumocytes in the septa. D, Bone marrow with hemophagocytosis of red cells and an erythroblast (>) (hematoxylin and eosin stain, original magnifications: A and C ×630, B ×100, D ×1000).

control cases were also tested. One was a patient dying of severe acute respiratory syndrome (SARS), and the other was a patient dying due to a traffic accident.

3. Results

Western blot confirmed the specificity of the anti-NP antibody with demonstration of the NP at a band about 50 kd (Fig. 1). Immunohistochemistry showed infected bronchiolar epithelium (Fig. 1) in the mouse evident by brown nuclear reaction, whereas the controls with anti-HPV (Fig. 1) and PBS were negative.

On histological review, the respiratory epithelium from the trachea to the bronchioles showed reactive hyperplasia (Fig. 2). The lungs showed diffuse alveolar damage in the acute phase, with alveolar hemorrhage, edema, fibrin, and inflammatory cell exudate (Fig. 2C). Reactive hemophagocytosis and activated lymphocytes were noted in the reticuloendothelial system including the hilar lymph nodes and bone marrow (Fig. 2). The spleen showed lymphoid depletion. The heart and adrenal glands showed focal ischemic necrosis without inflammatory changes. The skeletal muscle showed focal fiber necrosis with sparse lymphohistiocytic infiltration. The liver showed mild centrilobular macrovesicular steatosis. Other organs including the stomach, intestines, pancreas, kidneys, brain, thyroid, and pituitary showed no remarkable pathology. Viral culture of the postmortem lung was positive for H5N1, but cultures on the liver, kidney, bone marrow, and the brain were negative.

Immunohistochemistry showed a positive reaction in the upper and lower respiratory epithelium (Fig. 3) and pneumocytes (Fig. 3). The intensity of reactive pneumocytes was generally low with a maximal intensity of about 5 to 10 reactive cells per high power field (Nikon Eclipse 80i microscope, ×400, field diameter 400 μm). Occasional lymphocytes in the alveolar exudate and in the parenchymal or intravascular location of a few organs such as the tonsil, spleen, and heart were reactive. The small and large intestinal epithelium (Fig. 3); hematopoietic cells of the bone marrow

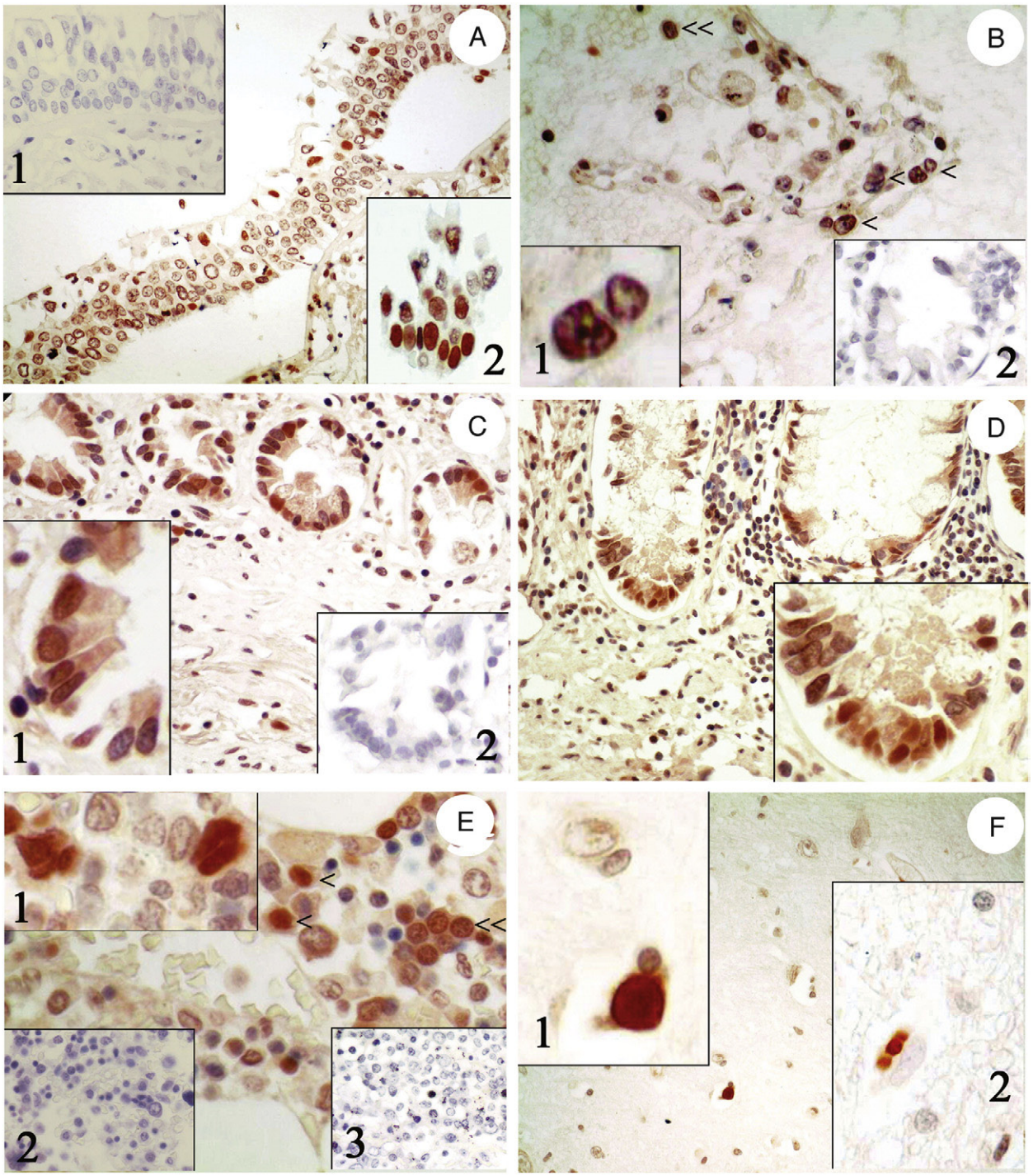


Fig. 3 Immunohistochemical demonstration of viral NP. Positive reaction was demonstrated by brown reaction of the nuclei. A, Reactive tracheal epithelium with high-power view in inset 2. Inset 1 showed control section with anti-HPV. B, Lung alveolus with positive pneumocytes (<) along the septum and 1 positive lymphocyte in the lumen (<). Inset 1 showed high-power view of the reactive pneumocytes, and inset 2 showed control alveolus of a patient with SARS. C, Small intestine with positive epithelial cells and high-power view in inset 1. Inset 2 showed small intestine of a patient with SARS. D, Large intestine with positive epithelial cells and high-power view in inset. E, Bone marrow with reactive myelocytes (<) and erythroblasts (<<) and high-power view in inset 1 showed 2 reactive megakaryocytes. Inset 2 showed control section with anti-HPV. Inset 3 showed bone marrow of a patient with SARS. F, Midbrain with a reactive neuron. Inset 1 showed a high-power view of the reactive neuron with an adjacent nonreactive astrocyte and another pair of negative neurons and astrocytes above. Inset 2 showed the cerebral cortex with an elongated reactive astrocyte, 3 negative small neurons with round nuclei, and another negative astrocyte with slightly elongated nuclei (original magnifications ×400, except B1, E1, F1 ×1000; C1, D inset, E, F2 ×800).

including myelocytes, erythroblasts, and megakaryocytes (Fig. 3); and neurons and astrocytes of the brain (Fig. 3) were also positive. The brain regions studied included cerebral cortex, basal ganglia, midbrain, pons, medulla, and cerebellum. Scattered immunopositive neurons and astrocytes were noted in all these regions except the medulla. All the positive reactions were largely confined to the nuclei. Reactions of the myocardium, stomach, pancreas, kidney, liver, adrenal, and skeletal muscle were negative. The negative controls, including anti-HPV, PBS, and the 2 control postmortem cases, were all negative except for very occasional weak nuclear or cytoplasmic reaction ascribed to endogenous biotin reactions (inset of Fig. 3).

4. Discussion

We confirmed the results of Gu et al that H5N1 infected the trachea, bronchi, pneumocytes, lymphocytes, and neurons [5]. However, we demonstrated a positive reaction also in the small intestine and astrocytes (negative by Gu) and colon and bone marrow (not done by Gu). Thus, our case has shown the widest tissue tropism of H5N1 virus in human cases so far studied. These results differed from the negative findings by To et al [7] and the positive reaction confined to the pneumocytes by Uiprasertkul et al [8]. These differences could be related to the duration of disease (1 month in To's cases), technical issues such as sensitivity of methodology, and other factors. The possibility that different H5N1 strains may have different tissue tropism in humans was not excluded. Similar to Gu, we demonstrated a low intensity of positive pneumocytes in the lung despite severe diffuse alveolar damage (DAD). One possibility was that the high intensity of infection had already been eradicated by the patient's immune system, correlating with the severe DAD. Alternatively, the low intensity of viral infection did not play a major role in the pathogenesis of the lung injury. Some studies suggested that dysregulation of immunoresponses led to the respiratory and multiorgan failure [7,8]. Despite viral infection in multiple extrapulmonary organs, they did not show any specific pathological change, apart from necrosis likely related to hypoxia. Furthermore, postmortem viral culture was positive only in the lung and negative in the bone marrow and brain. These observations indicated ineffective or very minimal viral replication and/or minimal viral antigenic expression in the infected cells. If substantial viral expression was present in the infected host cells, the inmate and cell-mediated immunity arm including cytotoxic T lymphocytes would be expected to mount an immune reaction against them and result in tissue injury.

In conclusion, we have demonstrated a widespread systemic infection of H5N1 virus in human. However, specific pathological changes were present only in the respiratory system. The results and available information support that H5N1 virus does not replicate efficiently in human tissue. More postmortem studies are needed to elucidate the pathogenesis of the highly fatal H5N1 virus infection in humans.

References

[1] Chen H, Smith GJ, Li KS, et al. Establishment of multiple sublineages of H5N1 influenza virus in Asia: implications for pandemic control. Proc Natl Acad Sci U S A 2006;103:2845-50.
[2] Cumulative number of confirmed human cases of avian influenza A/(H5N1) reported to WHO. May 28, 2008. http://www.who.int/csr/disease/avian_influenza/country/cases_table_2008_05_28/en/index.html [accessed June 19, 2008].
[3] Ng WF, To KF, Lam WW, Ng TK, Lee KC. The comparative pathology of severe acute respiratory syndrome and avian influenza A subtype H5N1—a review. Hum Pathol 2006;37:381-90.
[4] Ng WF, To KF. Pathology of human H5N1 infection: new findings [comment]. Lancet 2007;370:1106-8.
[5] Gu J, Xie Z, Gao Z, et al. H5N1 infection of the respiratory tract and beyond: a molecular pathology study. Lancet 2007;370:1137-45.
[6] Peiris JSM, Yu WC, Leung CW, et al. Re-emergence of fatal human influenza A subtype H5N1 disease. Lancet 2004;363:617-9.
[7] To KF, Chan PKS, Chan KF, et al. Pathology of fatal human infection associated with avian influenza A H5N1 virus. J Med Virol 2001;63:242-6.
[8] Uiprasertkul M, Puthavathana P, Sangsiriwut K, et al. Influenza A H5N1 replication sites in humans. Emerg Infect Dis 2005;11:1036-41.