PATHOGENICITY OF A GOOSE ISOLATE OF HIGHLY PATHOGENIC H5N1 INFLUENZA A VIRUS FOR CHICKENS, MICE, AND PIGS

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Summary. – In recent years, a highly pathogenic avian influenza virus (AIV) H5N1 subtype has emerged as a pathogenic agens for a variety of species including humans. To investigate the pathogenicity of AIV, the isolate ZFE/2004 (H5N1) was used to infect chickens, mice, and pigs. The results showed that the isolate showed attributes of the highly pathogenic AIV such as high pathogenicity index (intravenous pathogenicity index (IVPI) of 2.85 and intracerebral pathogenicity index (ICPI) of 3.00) and a specified hemagglutinin (HA) cleavage site. *In vivo* experiments indicated that this isolate was highly lethal to the Balb/c mice. In addition, it was able to infect pigs, elicit specific antibodies, and cause "flu-like" clinical symptoms. Typical pathological changes were observed in the chickens as well. *In vitro* experiments showed that the isolate ZFE/2004 was able to induce apoptosis of infected MDCK cells that was detected by the DNA fragmentation analysis and electron microscopy.

Key words: apoptosis; avian Influenza virus A; H5N1 subtype; pathogenicity

Introduction

Highly contagious avian influenza, previously known as "fowl plague" is a devastating infectious disease of poultry caused by infection with AIV, subtypes H5 and H7. Many domestic and wild avian species, such as chickens, turkeys, ducks, geese, swans, and other birds are susceptible to the highly pathogenic AIV (Hulse-Post *et al*., 2005; Sims *et al*., 2005; Capua and Alexander, 2004). In the year 2004 at Lake Qinghai in western China, 1500 of wild geese were infected with highly pathogenic AIV H5N1 and died (Liu *et al*., 2005). Recently, H5N1 viruses were also found in wild birds in Europe (Salzberg, 2007), indicating that the highly pathogenic AIV H5N1 had a potential to be a global threat.

Usually, AIV does not infect human, but may be transmitted to humans directly from birds, AIV-contaminated environment or through an intermediate host, such as pig (http://www.cdc.gov/flu/avian/gen-info/transmission.htm). Firstly reported in Hong Kong in 1997, the AIV H5N1 had the ability to pass directly from poultry to humans and caused 18 human cases of respiratory disease, six of them fatal (Anonymous, 2003; Katz, 2003; Bridges *et al*., 2002; CDC, 1997). Then, it was reported that AIV H10N7, H9N2, H7N2, H7N3, H7N7, and H5N1 subtypes could be detected in humans (CDC, 2005). The outbreak of H5N1 AIV in southeastern Asia starting from 2003 has resulted in the slaughter of millions of birds posing a huge economic impact on the poultry industry and a potential public health hazard (Obenauer *et al*., 2006; Chen *et al*., 2005; Wan *et al*., 2005). From 2003 to 2007, 213 deaths occurred among 346 infected individuals (http://www.who.int/csr/disease/avian_influenza/country/cases_table_2007_12_28/en/index.html). Genetic analysis of AIV H5N1 viruses isolated from humans revealed that

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Abbreviations: AIV = avian influenza virus; EID_{50} = eggs infectious dose; HA = hemagglutinin; HE = hematoxylin and eosin; IVPI = intravenous pathogenicity index; ICPI = intracerebral pathogenicity index; p.i. = post infection

all genes were of avian origin (Wan *et al*., 2005). The pathogenicity of AIV H5N1 viruses has been studied in details (Hulse-Post *et al*., 2005; Zitzow *et al*., 2002; Lu *et al*., 1999; Collins, 1995; Razvi and Welsh, 1995). As an intermediate host or mix vessel served a swine playing an important role in the "avian-human" transmission chain (Guo *et al*., 2005). Recently, in southeastern Asian countries, especially in China, pig farming developed rapidly and as a result, the animals had more chance to get into contact with terrestrial and aquatic poultry. Therefore, it is critical to examine the virulence of these viruses and their transmissibility from avian species to swine.

The aim of the present study was to characterize the pathogenicity of AIV H5N1 isolate ZFE/2004 in mice and chickens, and to investigate the ability of this isolate to replicate and to induce the immune response in pigs.

Materials and Methods

Virus. The isolate used in this study A/goose/Hubei/ZFE/2004 (H5N1) was isolated from a goose during the avian influenza epidemics in 2004 in southeastern China. The virus was propagated in the allantoic cavities of 9–11 days old specific pathogen free (SPF) embryonated eggs at 35°C for 48 hrs. The allantoic fluid was harvested, clarified by centrifugation, and stored at -70°C.

Cells. Madin-Darby canine kidney (MDCK) cells were cultured in Dulbecco's minimal essential medium containing 10% fetal calf serum and 100 µg/ml of streptomycin and 100 U/ml penicillin (Invitrogen) at 37°C in an atmosphere containing 5% CO_2. Tissue culture infectious dose 50 ($TCID_{50}$) was determined by titration of allantoic fluid in MDCK cells.

Analysis of HA cleavage site. Viral RNA was isolated by a standard procedure and a clone of HA gene was prepared (Xu *et al*., 2007). Cleavage site of the HA from the isolate AIV ZFE/2004 was compared with that of highly pathogenic AIV (Webster *et al*., 2002).

Determination of intravenous (IVPI) and intracerebral (ICPI) pathogenicity indexes in chicken. To assess the pathogenicity of the virus, ICPI and IVPI tests were performed using SPF chickens as described (Alexander, 2004).

Animal experiments. A total of eighteen 6-week-old SPF chickens were infected intranasally with $10^{8.8}$ eggs infectious dose 50 (EID_{50}) of the virus in 0.2 ml, and 4 chickens were used as control. The infected and control groups of chicken were euthanized at 36 hrs post infection (p.i.) and the pathological changes were examined.

Six 4–6-week-old Balb/c mice were inoculated intranasally with 10^6 EID_{50} of the virus in 50 µl. The mice in control group were inoculated with the same volume 0.85% NaCl. The mortality was recorded and the virus was isolated from various tissues.

Four one-month-old, healthy, AIV antibody-negative pigs were infected intranasally with $10^{9.8}$ EID_{50} of the virus in 2 ml. Two pigs were used as a negative control and injected with 2 ml PBS. Body temperature and clinical symptoms were recorded daily. The sera were collected at day 0, 28, and 45 p.i. and the levels of antibodies against AIV were tested by ELISA method (Jin *et al.*, 2004). On the day 45 p.i., the animals in infected and negative control groups were euthanized and pathological changes recorded.

Light- and electron microscopy. For light microscope observation, uninfected and infected chicken tissues were stained with hematoxylin and eosin (HE) and examined in a standard manner. For electron microscopy, the uninfected and ZFE/2004-infected MDCK cells were harvested at 0, 6, 12, 18, 24, 30 hrs p.i. and prepared as described (Li *et al*., 2003).

Assay of cellular DNA fragmentation. Fragmentation of the cellular DNA into characteristic apoptotic ladder was assessed according to the Jin's methods with some modification (Jin *et al*., 2001). MDCK cells infected with multiplicity of infection (MOI = 0.1) were harvested at 6, 12, 18, 24, 30 hrs, respectively. The cell samples in TE 9S buffer (500 mmol/l Tris-HCl, pH 9.0, 20 mmol/l EDTA, 10 mmol/l NaCl, 10% SDS and 1 mg/ml proteinase K) were incubated at 48°C for 30 hrs. Proteins were extracted twice with 200 µl of Sevag solution (phenol:chloroform:isoamylalcohol = 25:24:1). The aqueous phase containing nucleic acids was treated with 0.1 mg/ml RNase at 56°C for 30 mins and then loaded onto a 1.5% agarose gel containing 10 µg/ml ethidium bromide to visualize DNA fragmentation under UV light.

Results and Discussion

Pathogenicity of the isolate ZFE/2004 was examined in the chicken. Tissues from the infected animals were collected at 24 hrs p.i. and stained with HE. As expected, the magnitude of pathological changes induced by the infection, such as presence of necrotic cells with swollen cytoplasm, were observed in liver, brain, spleen, and pancreas (Fig. 1A–H). The observed clinical symptoms and pathological changes recalled the symptoms of typical highly pathogenic AIV. Values of IVPI and ICPI were up to 2.85 (228/80) and 3.00 (240/80), respectively. The value of EID_{50} reached $10^{-10.5}$/ml of allantoic fluid. These results were consistent with the previous reports (Alexander, 2004; Webster *et al*., 2002) and indicated that the isolate ZFE/2004 was highly virulent and pathogenic for chicken. Others experiments showed that the tested isolate could not cause death of domestic ducks, although it could be released to the environment from their cloacae (data no shown).

Table 1. Tissue tropism of the isolate ZFE/2004 in mice

Tissue	Mouse No.						Virus presence[a] (%)
	1	2	3	4	5	6	
Heart	–	–	–	–	–	–	0
Liver	–	–	–	–	–	+	16.7
Spleen	–	-	–	–	-	+	16.7
Lungs	+	+	+	+	+	+	100.0
Kidneys	–	+	+	–	–	–	33.3
Brain	–	+	+	–	–	+	50.0

[a]The virus was detected by 3 serial passages in embryonated eggs.

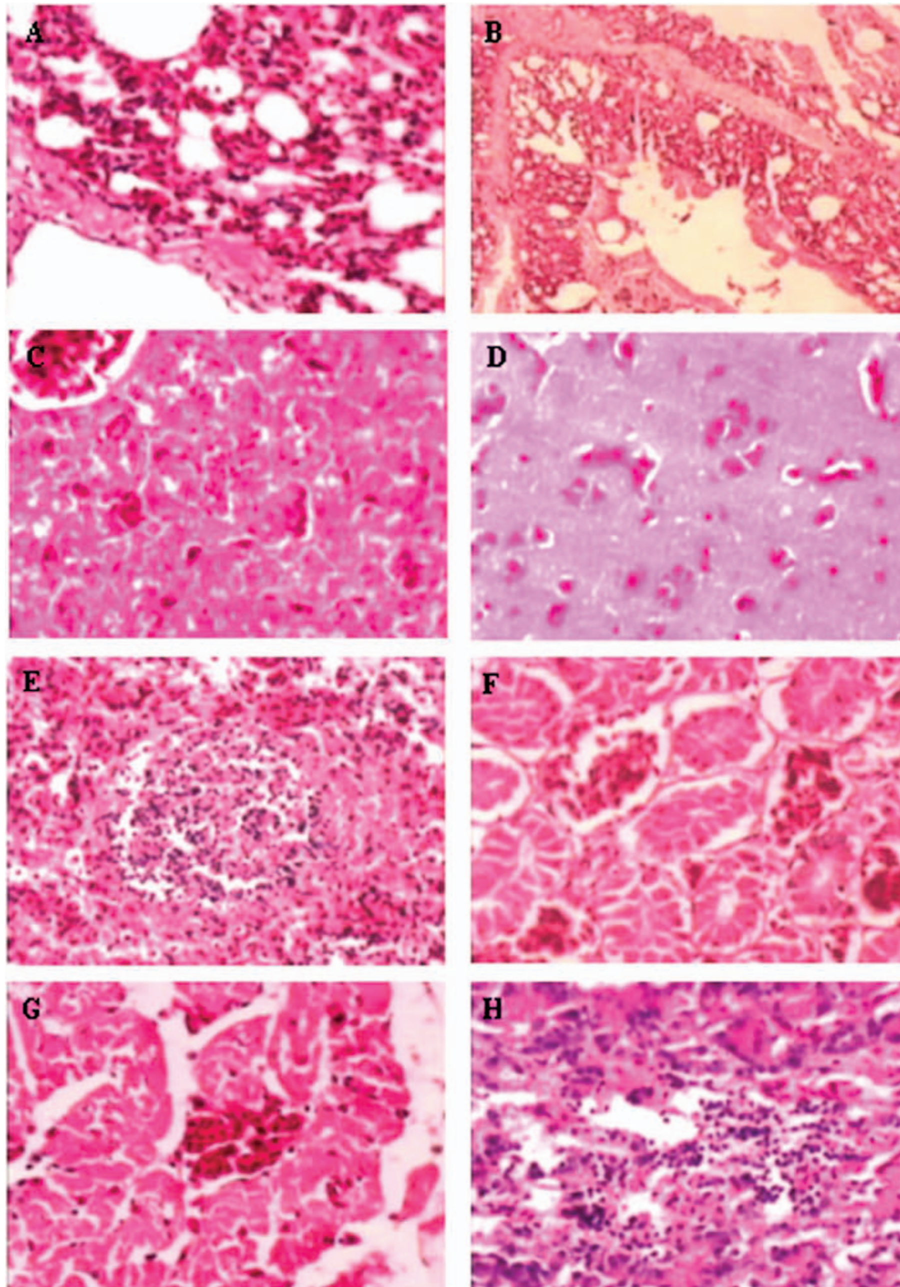

Fig. 1

Histochemical analysis of various organs from chicken infected with the isolate ZFE/2004

Hemorrhage (A) and hyperplasia (B) of connective tissue in lungs. Destruction of the cells in liver (C), nerves (D), and epithelial cells in kidneys (F). Hemorrhage and cell destruction in the heart (G) and pancreas (H). Stained with HE, magnification 10–20´.

Pathogenicity of the isolate ZFE/2004 was examined also in mice. The infected mice began to die at day 7 p.i. and all animals died at day 12 p.i. (Fig. 2). The infectious virus was isolated from various organs with highest score (100%) in the lungs and with lowest (0%) in the heart (Table 1). These results were consistent with the results obtained in chicken experiment. It indicated that the isolate was a highly pathogenic AIV that had the ability to cross the barrier between species (Capua and Alexander, 2004).

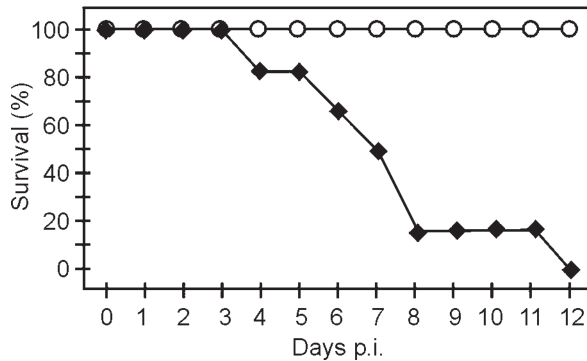

Fig. 2

Survival of mice infected with the isolate ZFE/2004

Infected (○) and control mice (♦).

A pig plays a critical role in the "avian-human" virus-transmission chain. To investigate whether the tested isolate had a capacity to infect pigs, each of the four pigs was infected intranasally with $10^{9.8}$ EID_{50} of the virus. The animals developed severe respiratory symptoms within 5 days p.i. and a continuous fever over 40°C for 5–7 days (Fig. 3). Apart from the signs of cough and snivel, the animals also displayed anorexia, conjunctivitis, and a green water-like discharge. Antibodies against the virus could be detected at day 28 p.i. (values of $OD_{630} = 1.45 \pm 0.43$) and at day 45 p.i. ($OD_{630} = 0.58 \pm 0.06$). The major pathological symptom observed was viral pneumonia.

It was reported that AIV H5N1 could infect also humans, tigers, horses, and cats. In this study, we found that the isolate ZFE/2004 could replicate in pigs and cause a development of the flu-like symptoms. Recent research also suggested that currently circulating strains of H5 viruses are becoming more pathogenic for mammals than previously circulating H5 viruses (Webster *et al*., 2002).

The swine serves as an intermediate host and plays an important role in the "swine-avian-human" interspecies transmission (Guo *et al*., 2005; Capua and Alexander, 2004). H1N2, H1N1, and H3N2 viruses had already caused outbreaks in pigs. However, these subtypes were also pandemic human influenza viruses in the current period. In Asian countries, the pig production has increased dramatically over the past decade and the number of raised pigs was huge. Over 40% of the meat products were made of pork and over 65% of the pork products were produced in China. In this study, we confirmed that the AIV isolate ZFE/2004 could infect pigs under experimental condition. In the case, a pig is infected with a human influenza virus and a high-pathogenic AIV H5N1 at the same time, a genetic reassortment could occur with the production of a new influenza virus. The resulting new virus might be able to infect humans and spread from person to person, ultimately causing a human influenza pandemic. Therefore, we should pay more attention to the presence of AIV H5N1 in swine.

In addition, we examined the cytopathological changes of MDCK cells infected with isolate ZFE/2004. The cells were infected with MOI = 0.1 and harvested at 6, 12, 18, 24, 30 hrs p.i., respectively. Apoptosis was analyzed by the

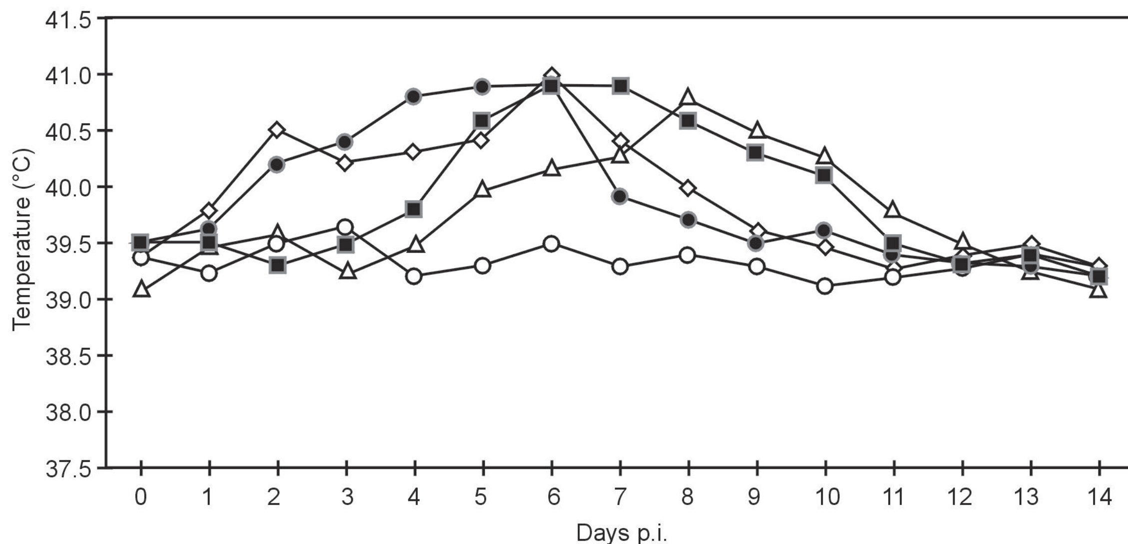

Fig. 3

Body temperature of pigs infected with the isolate ZFE/2004

Infected pig No. 1 (◇), No. 2 (■), No. 3 (△), No. 4 (●), and control pigs (○).

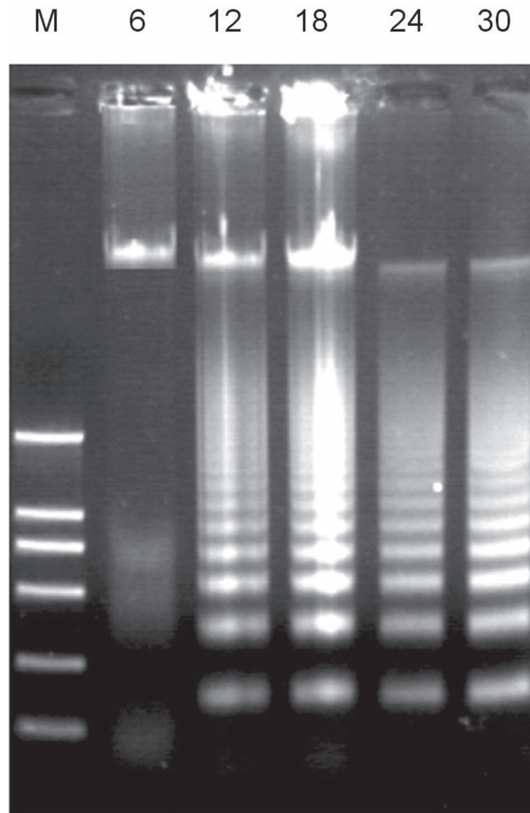

Fig. 4

Fragmentation of cellular DNA of MDCK cells infected with the isolate ZFE/2004 at different time p.i.

DNA ladder (2000, 1000, 750, 500, 250 and 100 bp) (lane M). Different time p.i. in hrs (lanes 6, 12, 18, 24, 30).

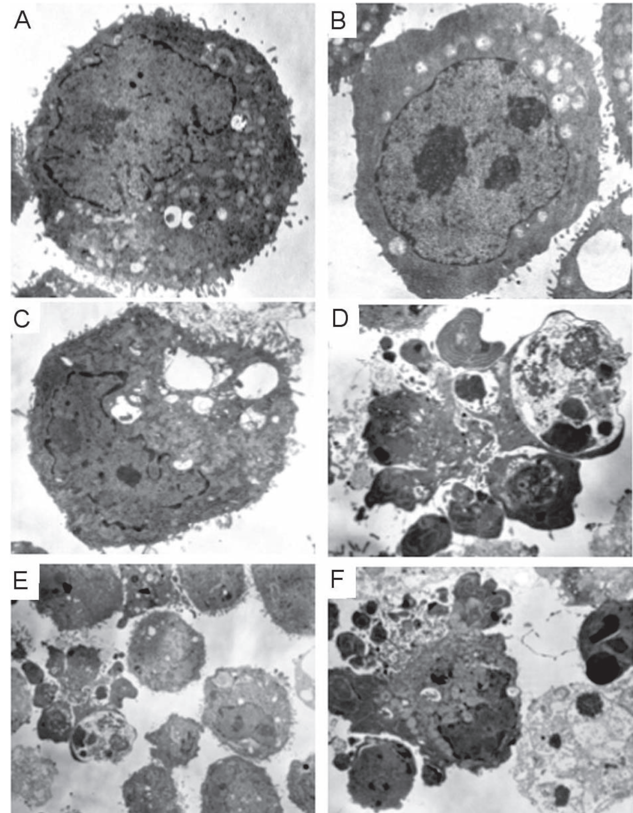

Fig. 5

Apoptosis of MDCK cells infected with the isolate ZFE/2004 detected by electron microscopy

Non-infected cells (A). Cells infected for 6, 12, 18, 24, and 30 hrs (B, C, D, E, F, respectively). Magnification 2500–4000´.

fragmentation of cellular DNA and electron microscopy. Apoptosis began to appear at 12 hrs p.i, and DNA ladder formation of approximately 200 bp multiples could be clearly observed in the cells infected for 18, 24, 30 hrs p.i. (Fig. 4). Typical ultra-structural features of apoptosis were present in cells at 12, 18, 24, 30 hrs p.i. (Fig. 5). A chromatin began to condense at the nuclear membrane in a shape of half moon (Fig. 5C). The membrane engulfed the apoptotic bodies reminding horse hoofs (Fig. 5D) and the infected cells began to show a necrotic appearance at 30 hrs p.i. (Fig. 5F).

To characterize further the pathogenicity of the virus isolate, the HA gene was cloned and a cleavage site was deduced. The cleavage site had typical feature of a highly pathogenic AIV H5 subtype isolate, 341-RRRKKRŻG-347. This result was similar to the previous findings (Collins, 1995; Hinshaw *et al*., 1994; Razvi *et al*., Takizawa *et al*., 1993).

In conclusion, the ZFE/2004 (H5N1) isolate belonged to the group of highly pathogenic AIV and was lethal to mice and chicken. The chicken showed typical pathological changes. Most importantly, the virus could infect pigs and elicit the production of antibodies against AIV. The highly pathogenic isolate ZFE/2004 was able to induce apoptosis in MDCK cells, what is an important mechanism in the pathogenesis of AIV. The isolate is a significant public health threat and should be under constant surveillance.

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