

Pathogenicity of Chinese H5N1 highly pathogenic avian influenza viruses in pigeons

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Abstract It has long been thought that pigeons are resistant against H5 highly pathogenic avian influenza (HPAI) viruses. Recently, however, highly pathogenic H5N1 avian influenza viruses have demonstrated distinct biological properties that may be capable of causing disease in pigeons. To examine the susceptibility of domestic pigeons to recent H5N1 viruses, we inoculated pigeons using H5N1 viruses isolated in China from 2002 to 2004. Within 21 days following inoculation, all pigeons had survived and fully recovered from temporary clinical signs. However, seroconversion assays demonstrated that several viruses did in fact establish infection in pigeons and caused

a certain amount of viral shedding in the oropharynx and cloaca. There was not, however, a definitive relationship between viral shedding and viral origin. Viruses were also inconsistently isolated from various organs of pigeons in infected groups. Pathological examination revealed that the infection had started as respiratory inflammation and caused the most severe lesions in the brain in later stages. These results indicate that pigeons are susceptible to the more recent Asian H5N1 HPAI and could be a source of infection to other animals, including humans.

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Introduction

Influenza A viruses are likely to have originated from wild waterfowl, which act as a natural reservoir [5, 9]. Given the long period of coexistence, these viruses are thought to be evolutionarily stable and non-pathogenic to their waterfowl hosts [11, 13]. There are several subtypes of the viruses according to the antigenicity of surface glycoproteins, but only certain subtypes, generally subtypes H5 and H7, are associated with acute highly pathogenic avian influenza (HPAI) disease in birds. The acute HPAI disease outbreaks usually become a matter of public health concern only among populations of domestic land-based poultry, because the natural hosts, waterfowl, are believed to be resistant to HPAI disease. Since late 2003, however, several endemics of highly pathogenic H5N1 avian influenza viruses have also demonstrated pathogenicity among waterfowl and wild birds, indicating that the viruses have distinct biological properties compared to previously endemic HPAI viruses [1, 2].

Pigeons, often sold in live-bird markets in East Asian countries, are believed to exhibit resistance against HPAI

viruses [3, 4, 7, 8]. Therefore, the general consensus had been that pigeons play a minimal role in spreading H5 viruses. Following reports starting in 2002 that described pigeons potentially involved in and perishing during H5N1 outbreaks of domestic and wild birds, the general consensus has begun to change on the possible susceptibility of pigeons to HPAI [2, 12]. The susceptibility of pigeons to H5N1 virus and the role of pigeons in the transmission of avian influenza viruses to domestic birds and humans remain controversial topics. Further study is therefore required to elucidate the susceptibility of pigeons to recent H5N1 HPAI A viruses.

This study was thus undertaken to compare the susceptibility of pigeons to a panel of ten strains of H5N1 HPAI viruses isolated from several different species of infected birds during the outbreaks of 2004 H5N1 HPAI in China. We examined the extent to which these viruses are able to replicate and be shed in pigeons.

Materials and methods

Viruses

A total of ten H5N1 HPAI viruses (four chicken-derived, three duck-derived, and three pigeon-derived) were selected: A/Chicken/Anhui/85/2004 (Ck/Ah/04), A/Chicken/Guangxi/12/2004 (Ck/Gx/04), A/Chicken/Hubei/14/2004 (Ck/Hb/04), A/Chicken/Tianjin/65/2004 (Ck/Tj/04), A/Duck/Guangdong/23/2004 (Dk/Gd/04), A/Duck/Guangxi/13/2004 (Dk/Gx/04), A/Duck/Hunan/15/2004 (Dk/Hn/04), A/Pigeon/Hunan/39/2002 (Pg/Hn/02), A/Pigeon/Jilin/30/2004 (Pg/Jl/04), and A/Rock Pigeon/Shanxi/47/2004 (Pg/Sx/04). Nine viruses were isolated from tissue samples collected from birds with significant disease signs during the H5N1 HPAI outbreaks in China in 2004, and one virus was isolated from a dead pigeon in 2002 (Table 1). All H5N1 viruses had been grown in the allantoic cavities of

10-day-old embryonated chicken eggs, and harvested virus fluid titers ranged from 7.5 to 8.9 log₁₀ EID₅₀/ml.

Pigeons

Seven-week-old pigeons, from a local farm in Harbin, China, with no antibody against H5, H7 and H9 subtypes of influenza viruses were used in these experiments. All pigeons were housed separately in each isolation unit, ventilated under negative pressure with HEPA-filtered air. Feed and water were provided *ad libitum*. All experiments using H5N1 viruses were performed in a biological safety level 3 facility at the Harbin Veterinary Research Institute of CAAS and were authorized by the Chinese Ministry of Agriculture.

Experimental design

One hundred sixty pigeons were divided into ten groups (16 birds/group) and intranasally inoculated with 0.1 ml of virus fluid containing 10⁶ EID₅₀ of an H5N1 virus. All pigeons were monitored daily for clinical signs. Oropharyngeal and cloacal swabs were taken from live pigeons at 3, 5, 7, 10, and 14 days postinfection (dpi) and were collected in PBS with antibiotics for virus isolation and titration. Blood samples were collected from live pigeons at 21 dpi for analysis of specific serum antibody. Two pigeons in each infected group were necropsied at 3, 5, 7, 10 and 14 dpi for virus titration. Tissues of the brain, heart, liver, spleen, lungs, kidneys, pancreas, trachea, small intestine and bursa of fabricius were collected from each bird.

Virus isolation

Oropharyngeal and cloacal swabs and all tissues collected from infected and control pigeons were stored at -70°C until virus titrations were performed. Standard procedures

Table 1 H5N1 highly pathogenic avian influenza viruses used in this study

Virus strain	Description of the virus	Notation in the text
A/Chicken/Anhui/85/2004	Isolated from sick chicken in Juchao in Anhui Province	Ck/Ah/04
A/Chicken/Guangxi/12/2004	Isolated from sick chicken in Longan in Guangxi Province	Ck/Gx/04
A/Chicken/Hubei/14/2004	Isolated from sick chicken in Wuxue in Hubei Province	Ck/Hb/04
A/Chicken/Tianjin/65/2004	Isolated from sick chicken in Jinnan in Tianjin Province	Ck/Tj/04
A/Duck/Guangdong/23/2004	Isolated from sick duck in Jiedong in Guangdong Province	Dk/Gd/04
A/Duck /Guangxi/13/2004	Isolated from sick duck in Longan in Guangxi Province	Dk/Gx/04
A/Duck /Hunan/15/2004	Isolated from sick duck in Wugang in Hunan Province	Dk/Hn/04
A/Pigeon/Hunan/39/2002	Isolated from dead pigeon in Hunan Province	Pg/Hn/02
A/Pigeon/Jilin/30/2004	Isolated from sick pigeon in Changchun in Jilin Province	Pg/Jl/04
A/Pigeon/Shanxi/47/2004	Isolated from sick pigeon in Xianyang in Shanxi Province	Pg/Sx/04

were applied for the titration using 10-day-old embryonated chicken eggs.

Serological analysis

Sera collected from pigeons in the infected and control groups were pretreated by receptor destroying enzyme (RDE; Sigma, USA) at 37°C overnight and inactivated for 30 min at 56°C. They were then processed for detection of H5-specific antibodies by hemmagglutinin-inhibition test (HI test) using chicken red blood cells and an inactivated homologous H5N1 influenza viruses as antigen.

Pathological examination

Pigeons in the Dk/Gd/04- or Pg/Sx/04-infected groups were selected for pathological examination. Excised tissues of the liver, kidneys, heart, lungs, trachea, pancreas, intestine, brain, spleen and bursa of fabricius from pigeons were preserved in 10% neutralized buffered formalin. Tissues were then processed for paraffin embedding and cut into 5- μ m-thick sections. One of the sections was stained with routine hematoxylin-and-eosin, and the other was processed for immunohistological staining using rabbit anti-H5N1 influenza virus polyclonal antibody (anti-A/Vietnam/1203/04[H5N1]). Specific antigen-antibody reactions were visualized by 3,3'-diaminobenzidine tetrahydrochloride treatment using the Dako EnVision system (Dako Co. Ltd., Tokyo, Japan).

Results

Clinical findings of pigeons

No deaths were observed in any of the infected birds during the observation period. Among the ten different viruses examined, only two duck-derived strains (Dk/Gd/04 and Dk/Gx/04) and two pigeon-derived strains (Pg/Hn/02 and Pg/Sx/04) caused observable clinical symptoms such as decreased activity and neurological signs (Table 2). All of these clinical signs resolved within 3 dpi.

Viral titers in oral/cloacal swabs of infected pigeons

Viruses were inconsistently re-isolated from oral and cloacal swabs, with titers ranging from $10^{0.98}$ EID₅₀/ml to $10^{2.25}$ EID₅₀/ml from pigeons infected with viruses derived from chickens, ducks and pigeons. Although there was a definitive association between viral shedding and viral origin, it is noteworthy that many viral strains (Ck/Ah/04, Ck/Gx/04, Ck/Hb/04, Dk/Gd/04, Dk/Hn/04, Pg/Jl/04, and Pg/Sx/04) infected pigeons and caused a certain amount of

Table 2 Clinical manifestations observed in pigeons after intranasal inoculation with 10^6 EID₅₀ of H5N1 avian influenza viruses

Viruses	Manifestation in pigeons		
	Clinical signs ^a <i>n</i> = 18	Death <i>n</i> = 18	Seroconversion ^b <i>n</i> = 6
Ck/Ah/04	0	0	1
Ck/Gx/04	0	0	1
Ck/Hb/04	0	0	1
Ck/Tj/04	0	0	1
Dk/Gd/04	1	0	2
Dk/Gx/04	6	0	3
Dk/Hn/04	0	0	3
Pg/Hn/02	6	0	3
Pg/Jl/04	0	0	2
Pg/Sx/04	8	0	4

^a Birds that experienced clinical signs, such as depressed activity or energy, ruffled feathers, or neurological signs but recovered at the end of the observation were included

^b Results shown are from 21 days post-inoculation

viral shedding from pigeon cloaca even at 5 and 7 dpi (Fig. 1).

Isolation of H5N1 viruses from the organs of infected pigeons

Viruses were inconsistently isolated from various organs of pigeons in infected groups (Table 1). Isolation patterns were: (1) systemic, mostly including the brain; (2) respiratory tropic; (3) brain and respiratory tropic; and (4) sporadic isolation from the other organs. Systemic infection through 7 dpi was confirmed in some of the pigeons infected with Dk/Gx/04 or Pg/Sx/04. The virus titers ranged from $10^{0.98}$ EID₅₀/ml to $10^{4.5}$ EID₅₀/ml. However, all infections terminated in a self-limited manner, as indicated by negative virus isolation results at 10 and 14 dpi (data not shown) (Table 3).

Histopathology

Pigeons in the Dk/Gd/04- or Pg/Sx/04-inoculated groups had respiratory inflammation, such as tracheitis, bronchitis and airsacitis at 3 dpi. Pigeons necropsied at 5 dpi showed an apparent immunologic reaction such as hyperplastic lymph follicles in the spleen and Bursa of Fabricius. In pigeons necropsied at 7 dpi, the most severe lesions were observed in the brain in both the Dk/Gd/04- and Pg/Sx/04-infected groups. Brain lesions included malacia with perivascular cuffing of mononuclear cells, degeneration and necrosis of nerve cells, activation of glial cells and meningitis (Fig. 2a). Many nerve cells and glial cells were

Fig. 1 Viral titers in oral (blue) and cloacal (dark red) swabs from infected pigeons. Several of the viral strains tested (Ck/Ah/04, Ck/Gx/04, Ck/Hb/04, Dk/Gd/04, Dk/Hn/04, Pg/Jl/04, and Pg/Sx/04) led to some viral shedding

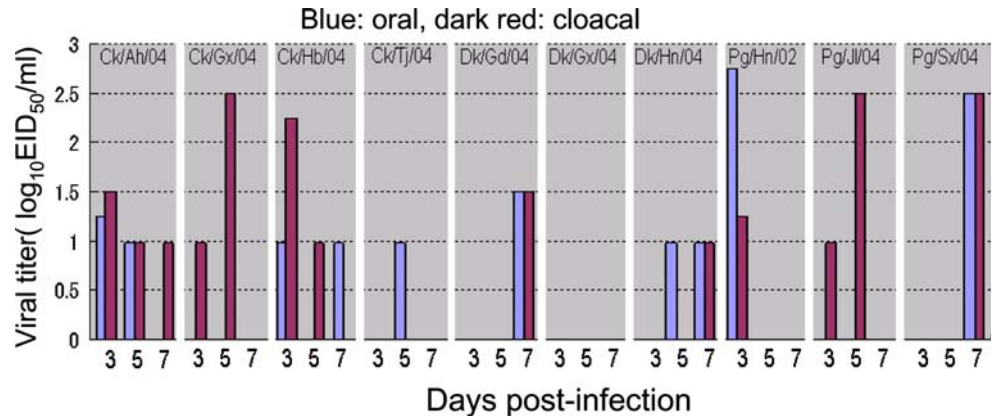


Table 3 Replication of H5N1 viruses in pigeons following intranasal inoculation

Virus strain	Days post-infection	Virus replication on days 3, 5 and 7 (Log ₁₀ EID ₅₀ /g)								
		Brain	Trachea	Lung	Liver	Spleen	Kidney	Heart	Pancreas	Bursa
Ck/Ah/04	3	–	–	0.98	–	–	–	–	–	–
	5	–	1.25	1.50	–	–	2.75	–	–	–
	7	–	–	–	–	–	–	–	–	–
Ck/Gx/04	3	–	–	–	–	–	–	–	–	–
	5	0.98	–	2.50	0.98	–	1.50	1.25	0.98	–
	7	–	–	2.25	–	0.98	1.50	–	–	–
Ck/Hb/04	3	–	2.50	1.75	1.50	–	0.98	2.5	0.98	–
	5	–	–	–	–	–	–	–	–	–
	7	–	–	–	–	–	–	–	–	–
Ck/Tj/04	3	–	–	–	–	–	–	–	–	–
	5	0.98	3.5	2.75	1.25	1.25	2.5	–	1.25	–
	7	–	0.98	–	–	–	–	–	–	–
Dk/Gd/04	3	–	2.5	2.50	2.5	2.5	1.75	2.5	–	–
	5	–	–	–	–	–	–	–	–	–
	7	4.25	0.98	2.75	1.50	1.50	4.25	2.25	–	0.98
Dk/Gx/04	3	–	–	–	–	–	–	–	–	–
	5	–	–	–	–	–	–	–	–	–
	7	1.50	–	0.98	–	–	–	–	–	–
Dk/Hn/04	3	–	–	–	–	–	–	–	–	–
	5	–	–	–	–	–	–	0.98	–	–
	7	–	–	–	–	–	–	0.98	0.98	–
Pg/Hn/02	3	–	–	–	–	–	–	–	–	–
	5	3.5	1.75	2.25	–	–	2.5	–	–	–
	7	–	–	3.25	–	–	3.5	–	–	2.25
Pg/Hn/04	3	–	–	–	–	–	–	–	–	–
	5	–	3.50	0.98	1.25	1.50	2.50	1.5	2.75	–
	7	–	–	–	–	–	–	–	–	–
Pg/Sx/04	3	–	–	2.75	2.25	2.5	2.5	2.5	0.98	–
	5	3.75	–	–	–	–	–	1.75	–	–
	7	3.5	2.5	4.5	2.25	1.25	3.25	2.5	–	0.98

positive for immunohistological staining for viral antigens (Fig. 2b). Viral antigens were detectable only within the brain. Developed lymph follicles with atrophic appearance

and a vascular fibrin thrombus were detected in the lungs. In pigeons in the Dk/Gd/04-infected group, severe atrophy of lymph follicles in the lymphatic organs, such as the spleen

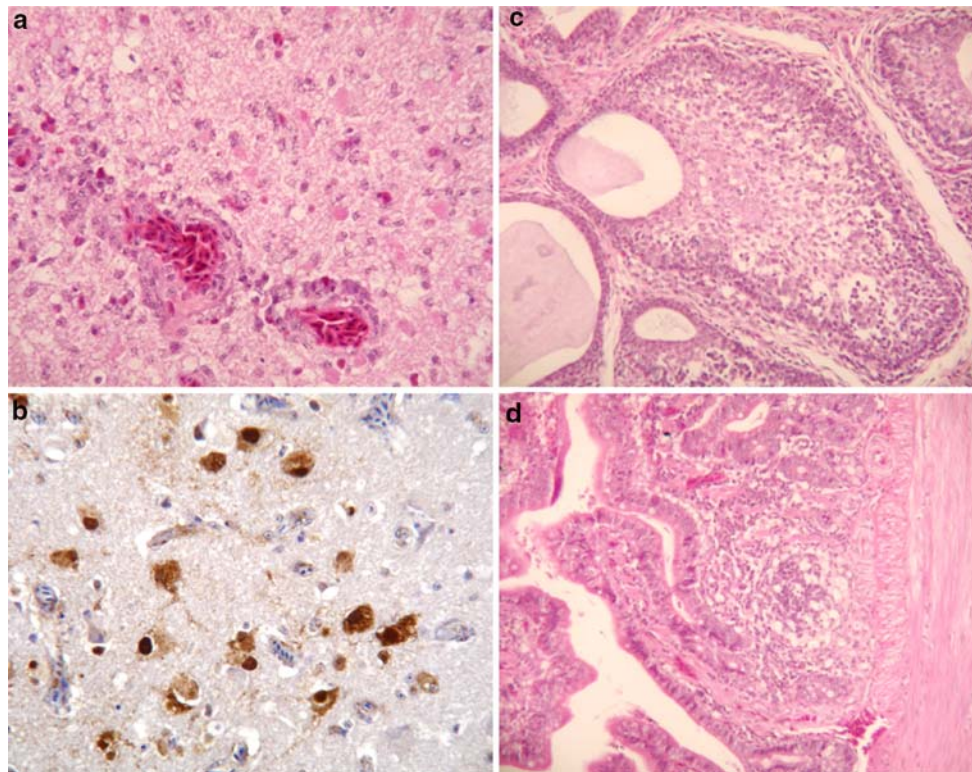


Fig. 2 Pathological changes observed in infected pigeons. **a** Malacia with perivascular cuffing of mononuclear cells, degeneration and necrosis of nerve cells and activation of glial cells were observed in the brain. Pigeons were infected with Pg/Sx/04, 7 dpi. **b** Nerve cells and glial cells were positive for viral antigens (*brown pigment*).

Pigeons were infected with Pg/Sx/04, 7 dpi. **c** Severe atrophy of lymph follicles in the bursa of fabricius. Pigeons were infected with Dk/Gd/04, 7 dpi. **d** Lymphocyte depletion in Peyer's patch follicles. Pigeons were infected with Pg/Sx/04, 7 dpi

and bursa of fabricius (Fig. 2c), and perivascular infiltration of mononuclear cells in intestinal serosa were prominent. Small infiltrative foci of pseudoeosinophils in kidneys and lymphocyte depletion in Peyer's patch follicles (Fig. 2d) were also observed in pigeons infected with Pg/Sx/04.

Serology

Among the six pigeons in each group that were inoculated with one of the chicken-derived viruses, one had H5-specific HI antibody. Among the pigeons in the group inoculated with duck-derived viruses, two or three of the six pigeons in the group had HI antibody, while two or four of the six pigeons in the pigeon-derived virus group showed a positive reaction (Table 2). These H5-specific titers were detected in the sera collected at 21 dpi and ranged from 1:32 to 1:64.

Discussion

Previous research had indicated that pigeons were completely resistant or minimally susceptible to infection with HPAI viruses and even with low-pathogenic avian

influenza viruses [3, 4, 10]. This notion was supported by findings that groups of pigeons inoculated with either HPAI virus (A/chicken/Pennsylvania/1370/83 or A/chicken/Australia32972/85) or non-pathogenic virus (A/chicken/Pennsylvania/13609/93 or A/emu/Texas/42499/93) remained healthy during the 21-day observation period, did not shed virus, and did not even develop antibodies to these pathogens [7]. Further research had suggested that pigeons were mostly resistant and probably played a minimal epidemiological role in the dissemination of the H5N1 Hong Kong-origin influenza viruses compared with chickens, quail and ducks [8].

Contrary to these findings, we found that the H5N1 viruses used in this study can successfully infect and cause disease in pigeons. Although infection efficiency varies among birds, the results of seroconversion (antibody production) suggest that infection is established in pigeons even in temporal or non-efficient infection cycles. These findings support several reports that described some susceptibility of pigeons to recent H5N1 viruses. Namely, during the outbreak of H5N1 avian influenza infection in Hong Kong in 2002, one pigeon was found dead [2]. Furthermore, a recent H5N1 virus from Indonesia (A/chicken/Indonesia/2003) showed significant pathogenicity in experimentally infected

pigeons [6]. Therefore, it is likely that recent prevalent viruses with worldwide distribution including China, possess different properties of susceptibility in pigeons than previous H5N1 strains before the early part of 2002.

Pathological examination revealed that some H5N1 influenza viruses caused pathological changes in respiratory organs in early stages and invaded the brain in later stages, accompanying apparent lymphatic atrophy. Although no pigeons died and indeed only showed mild clinical signs in the 48 h after infection, the virus replicated in the brains of pigeons, reaching titers of $10^{4.25}$ EID₅₀ at 7 dpi. The clinical signs and mortality were not the same as those of the pigeons infected with A/chicken/Indonesia/2003 [6]; however, neurotropism is a noteworthy characteristic for the recent H5N1 viruses. Moreover, our data showed that pigeons with neuronal infections also discharged infectious viruses in oral or cloacal secretions. We did not conduct transmission experiments, but several reports have indicated low transmission efficiency from infected pigeons to sentinel animals. However, during the second wave of avian influenza in Indonesia in 2006, at least one fatal human case from infection by H5N1 HPAI virus was reported in which the victim had a history of contact with pigeons in his house. Our findings demonstrate that pigeons can indeed be susceptible to the more recent Asian H5N1 HPAI viruses and suggest that pigeons could be a source of infection to other animals including humans.

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