

Limited Susceptibility of Pigeons Experimentally Inoculated with H5N1 Highly Pathogenic Avian Influenza Viruses

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(Received 29 June 2011/Accepted 5 September 2011/Published online in J-STAGE 16 September 2011)

ABSTRACT. An experimental infection study was performed using pigeons reared for racing or meat production in Japan and clade 2.2 and 2.3.2 isolates of H5N1 highly pathogenic avian influenza virus to evaluate the possible role of pigeons in virus transmission to poultry. In experiment 1, when 20 pigeons were intranasally inoculated with high or low viral doses, no inoculated pigeon exhibited clinical signs for 14 days. Drinking water and almost all swab samples were negative for virus isolation. Virus isolation was positive in 3 oral swab samples from 2 pigeons from day 2 through 4 postinoculation, but viral titers of positive samples were extremely low. Immunohistochemical analysis for virus detection was negative in all tissue samples. Along with seroconversion in a limited number of pigeons postinoculation, these results suggest that pigeons have limited susceptibility to the virus used for experimental infection. In experiment 2, when uninoculated chickens were housed with virus-inoculated pigeons, all pigeons and contact chickens survived for 14 days without exhibiting any clinical signs. According to serological analysis, the chickens did not exhibit seroconversion after close contact with inoculated pigeons. Our data suggest that the risk posed by pigeons with respect to the transmission of the H5N1 highly pathogenic avian influenza virus to poultry would be less than that for other susceptible avian species.

KEY WORDS: H5N1 subtype, highly pathogenic avian influenza, pigeons.

doi: 10.1292/jvms.11-0312; *J. Vet. Med. Sci.* 74(2): 205–208, 2012

Since its emergence in 1996, the Asian lineage H5N1 subtype highly pathogenic avian influenza (HPAI) virus, a highly virulent pathogen for poultry, has become a serious threat to the industry [1]. Among the several candidates such as wild birds, small mammals, vehicle, and people including farmers, terrestrial birds living around poultry farms are a possible pathway of the virus to susceptible poultry [18, 23]. Small terrestrial birds that could carry the H5N1 HPAI virus may introduce this pathogen biologically or mechanically into the farms with incomplete measures against small birds attempting to enter these farms [18]. Therefore, measures against virus introduction to poultry farms are valuable to prevent the disease.

According to an outbreak report and the database Influenza Virus Resource [6, 10], H5N1 HPAI viruses were isolated from pigeons in some countries including Indonesia, Thailand, the People's Republic of China, Laos, Russia, Turkey, and Nigeria, demonstrating that pigeons can be infected with this virus. Moreover, in Thailand, a pigeon was considered to be the possible source of infection for a domestic cat that died after eating its carcass [22]. These events raise concerns that pigeons infected with the H5N1 HPAI virus may be sources of the virus to poultry. In the epidemiological report after HPAI outbreaks in Japan, the terrestrial birds have been included in the possible routes of virus introduction to poultry farms [18]. Pigeons can share its habitat with chickens around the farms [5, 7]. Therefore,

examining the pathogenesis and virus excretion of Japanese pigeons would be valuable to analyze the possible epidemiological involvement of this bird species. In this study, we conducted an experiment using pigeons and the H5N1 HPAI virus; we discussed the potential role of pigeons in virus transmission.

Twenty seven healthy pigeons (*Columba livia domestica*) reared for racing or meat production (age, 3 months or more) in Japan were kindly provided by the Nippon Racing Pigeon Union Incorporation and a local breeder for 2 experimental infection studies. Five 3-week-old specific-pathogen-free White Leghorn chickens (*Gallus gallus domesticus*) were purchased from the Nippon Institute for Biological Science. All birds, excluding 2 pigeons as uninoculated control, were kept in a bio-safety-level-3 approved laboratory during the experiment. All experimental procedures were approved by the Ethics Committee of the National Institute of Animal Health, Japan (authorization number 08–139).

In experiment 1, we established 4 inoculation groups (5 pigeons per group) for low-dose or high-dose inoculation of 2 H5N1 HPAI viruses, clade 2.2 isolate A/chicken/Miyazaki/K11/2007 (Ck/Miya/K11/07) or clade 2.3.2 isolate A/whooper swan/Akita/1/2008 (Ws/Akita/1/08) [24]. One negative-pressure isolator (120 × 65 × 80 cm) was prepared for each group during the study. Pigeons in each group were inoculated intranasally with 10³ or 10⁶ 50% egg infectious dose (EID₅₀) of the corresponding virus. Drinking water and food were replaced daily. Two birds in each group were randomly selected for euthanasia on day 3 postinoculation (PI). Other birds were monitored for 14 days. Oral and cloacal swabs from all birds and 1 ml of drinking

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water from each group were sampled daily for virus isolation. The visceral organs of euthanized birds on days 3 and 14 PI were examined by virus isolation and histopathological analysis. Pre and postinoculation sera from pigeons were tested by 2 serological tests, i.e., hemagglutination inhibition (HI) antibody and virus neutralization (VN) antibody.

Virus titers in the drinking water, oral and cloacal swabs, and visceral organs comprising the brain, trachea, lung, pancreas, and rectum were determined using 10-day-old embryonated chicken eggs and expressed as EID₅₀/ml. Swabs were collected in 1 ml of phosphate-buffered saline supplemented with antibiotics. For the organs, a supernatant of 10% (w/v) homogenate of organs was titrated. A virus titer of less than 10^{1.6} EID₅₀/ml was considered negative for virus isolation.

For histopathological analysis, the systemic organs of pigeons were fixed in 10% neutral-buffered formalin. Samples were embedded in paraffin, sectioned in 3 µm slices, and stained with hematoxylin and eosin. Immunohistochemical testing was performed to detect influenza viral antigens using a Histofine Simple Stain MAX PO (M) kit (Nichirei Inc., Tokyo, Japan) and the mouse monoclonal antibody specific for type A influenza virus matrix protein (dilution 1:500; clone GA2B; AbD Serotec, Kidlington, U.K.), as described previously [28].

Preinoculation sera and the sera of birds on day 14 PI were examined by HI tests and by VN assay to measure the antibody response to the virus. All sera examined were pre-treated at 56°C for 30 min. Serum-positive controls used were derived from a domestic duck in our previous study [28]. Serum samples from 2 uninoculated pigeons were used as negative controls. The HI test was performed using 4 hemagglutination units of each virus as antigen and 0.5% chicken red blood cell suspension [27]. The sera were absorbed with packed chicken red blood cells at room temperature for 1 hr to reduce the nonspecific hemagglutination factors in pigeon serum. After centrifugation to remove chicken red blood cells, the sera were tested by the standard method [27]. An HI antibody titer of less than 1:16 was considered negative for antibody production [27].

In the VN assay, serum samples were diluted 2-fold with a starting dilution of 1:4. The VN antibody titer of samples was calculated by the Reed–Muench method using homologous virus (200 median tissue culture infective doses in 0.1 ml) for inoculation and Madin–Darby canine kidney cells in 96-well microtiter plates [20, 26]. The results of the preinoculation sera from all pigeons and chickens were negative (<1:16) in case of HI tests and less than 1:4 in case of the VN assay.

During experiment 1, no pigeon exhibited any clinical signs. Virus isolation was positive in the 3 oral swab samples from 2 pigeons inoculated with 10⁶ EID₅₀ of Ck/Miya/K11/07. One pigeon was positive for isolation on day 3 PI, the day of euthanasia, with a viral titer of 10^{2.5} EID₅₀/ml. The other pigeon was positive on days 2 and 4 PI, with a virus titer of 10^{1.8} EID₅₀/ml. Other swabs, drinking water,

and visceral organs were negative for isolation. Histopathologically, there was no significant finding associated with viral infection in pigeons on days 3 and 14 PI. Intestinal capillariasis and coccidiosis were observed in many pigeons including control birds, and these were considered incidental and unrelated to the virus infection. Immunohistochemical analysis for virus detection was negative in all samples. HI antibody seroconversion was observed in the high-dose inoculation groups of each virus, i.e., 2 birds inoculated with 10⁶ EID₅₀ of Ck/Miya/K11/07 and 1 bird inoculated with 10⁶ EID₅₀ of Ws/Akita/1/08 (Table 1). Moreover, VN antibody seroconversion was observed in 2 birds that were seroconverted in HI tests (Table 1).

In experiment 2, 5 pigeons were inoculated intranasally with 10⁶ EID₅₀ of Ck/Miya/K11/07 and were placed in a negative-pressure isolator (120 × 65 × 80 cm). Twenty-four hours later, 5 uninoculated chickens as contact birds were housed together in the same isolator. Pigeons and chickens shared the same living space, air, and drinking water. Commercial food was prepared for each species. Drinking water and food were replaced daily. Birds were observed for clinical signs and mortality for 14 days. One milliliter of drinking water was sampled daily for virus isolation. All birds were examined by histopathological analysis and HI antibody tests.

As a result of experiment 2, all pigeons and contact chickens survived for 14 days without exhibiting any clinical signs. Drinking water samples were negative for virus isolation. Lymphoplasmacytic encephalitis with occasional virus antigens in neurons and glial cells was observed in 3 pigeons on day 14 PI (Fig. 1). Seroconversion of the HI antibody was observed in 2 out of 5 pigeons (1:32 and 1:64) that had encephalitis. All chickens on day 14 PI were negative for HI tests.

We examined Japanese pigeons experimentally infected with Asian lineage H5N1 HPAI viruses to evaluate their possible role in virus transmission to poultry. In experiment

Table 1. HI and VN antibody titer of pigeons intranasally inoculated with H5N1 HPAI viruses on day 14 PI in experiment 1^{a)}

Virus	Inoculation dose (EID ₅₀)	Pigeon No.	HI	VN
Ck/Miya/K11/07	10 ³	1	–	<4
		2	–	<4
		3	–	<4
	10 ⁶	4	32	6
		5 ^{b)}	16	<4
		6	–	<4
Ws/Akita/1/08	10 ³	7	–	<4
		8	–	<4
		9	–	<4
	10 ⁶	10	–	<4
		11	–	<4
		12	16	52

a) Preinoculation sera of all pigeons were negative for HI tests and were less than 1:4 in VN assay. b) Pigeon positive for isolation on days 2 and 4 PI.

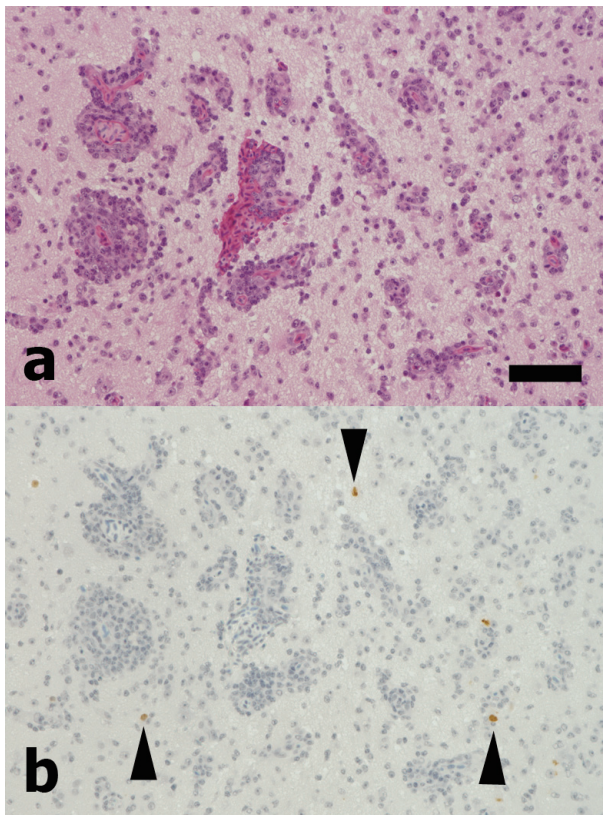


Fig. 1. Histopathology of the serial sections of the pigeon's cerebellum on day 14 PI in experiment 2. (a) Lymphoplasmacytic encephalitis. Hematoxylin and eosin staining (bar=30 μ m). (b) Viral antigens (arrowheads) occasionally detected in neurons and glial cells. Immunohistochemistry.

1, when healthy pigeons were inoculated with 2 different HPAI viruses at 2 different inoculation doses, no inoculated birds exhibited any clinical signs or mortality, indicating that pigeons are basically resistant to H5N1 HPAI virus infection. Seroconversion in a limited number of pigeons postinoculation suggests that pigeons have limited susceptibility to the Asian lineage H5N1 HPAI virus. Some species of birds such as ducks are often problematic when infected birds excrete the virus asymptotically, leading to silent spread of the virus [13]. In this respect, the negative results for virus isolation from drinking water and almost all swabs indicate that virus excretion from infected pigeons did not occur, and virus excretion, if any, was minimal and below the detection limit. Therefore, our data suggest that when healthy pigeons contact the H5N1 HPAI virus in fields, the risk of virus transmission to poultry would be small.

Positive results for isolation from oral swabs in 2 pigeons in experiment 1 cannot eliminate the possibility of virus excretion at a very low level from pigeons infected with H5N1 HPAI virus. Similar to our results, infectious virus or viral genes were detected in oropharyngeal and cloacal swabs of asymptomatic pigeons in experimental studies [8, 9, 12, 21, 25]. In experiment 2, however, no chickens

housed with inoculated pigeons were infected where the same inoculation conditions as for pigeons were applied, as in experiment 1. These results show that virus transmission did not occur from pigeons to chickens under conditions of close contact although the infection in pigeons was confirmed by HI antibody seroconversion and histopathology. Virus transmission studies using virus-inoculated pigeons and contact chickens have been performed, but none have succeeded in infecting chickens [14, 16, 21, 25]. In the field surveillance studies targeting pigeons in Egypt, Germany, Poland, and Thailand, all pigeons examined were negative for the H5N1 HPAI virus [2, 4, 11, 15].

Reported virus detection from field pigeons might be attributed to several factors with respect to infection in both viruses and hosts. Although no pigeons exhibited any clinical signs in our experimental infection, the inoculation dose has been related to the severity of disease in pigeons in another study [9, 14]. Brown *et al.* [9] reported that 10^6 EID₅₀ of the clade 2.2 H5N1 HPAI virus caused mortality in 2 out of 5 pigeons in an experimental infection using inoculation doses ranging from 10^2 to 10^6 EID₅₀. Klopfeisch *et al.* [14] reported that the higher viral dose of 10^8 EID₅₀ caused mortality with neurological signs in 5 out of 14 racing pigeons inoculated with the clade 2.1 H5N1 HPAI virus. To our knowledge, in other related experimental studies using 10^6 EID₅₀ or less of the virus, no mortality was reported in inoculated pigeons [8, 12, 16, 19, 21]. The genetic lineage of the H5N1 HPAI virus appears less involved with the severity of disease in pigeons compared to the inoculation dose. In our experiment 1 using clade 2.2 and 2.3.2 isolates, the significant difference between the 2 viruses did not exist in clinical signs, histopathological findings and antibody response, although the virus shedding was observed for a short period only in pigeons inoculated with clade 2.2 virus. Similar results were reported by Smietanka *et al.* [21], where the clade 1 virus replicated relatively stronger in pigeons than clade 2.2 virus, but neither of the 2 viruses caused clinical signs. Possible host factors that may affect H5N1 HPAI virus infection in pigeons, such as health and immune status, remain largely unclear. Smietanka *et al.* [21] mentioned that there were no age-related differences in susceptibility between 4-week-old and 1-year-old pigeons after experimental infection. Liu *et al.* [17] reported that virus receptor distribution in pigeons may explain their limited susceptibility to the H5N1 HPAI virus.

As observed in experiment 2, nonsuppurative encephalitis is a characteristic finding in pigeons infected with the H5N1 HPAI virus [9, 12, 14, 21], suggesting that the central nervous system of pigeons is frequently affected by this virus, even with asymptomatic infection. In addition to paramyxovirus infection [3], H5N1 HPAI virus infection should be included in the differential diagnosis when encephalitis is observed in pigeons.

ACKNOWLEDGMENTS. The authors thank Masaru Kobayashi and Megumi Shimada for their technical assistance. This study was supported by Grants-in-Aid for scien-

tific research from the Zoonoses Control Project of the Ministry of Agriculture, Forestry and Fisheries of Japan.

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