

Minimal Susceptibility to Highly Pathogenic Avian Influenza H5N1 Viral Infection of Pigeons (*Columba livia*) and Potential Transmission of the Virus to Comingled Domestic Chickens

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ABSTRACT

In order to elucidate the epidemic dissemination role of pigeons in the outbreak of the highly pathogenic avian influenza (HPAI) H5N1 (influenza A) virus, this study was conducted by experimental infection of the HPAI H5N1 virus in pigeons. Twenty-eight pigeons, aged 1 yr that were serologically negative for the H5N1-specific HI antibodies, were inoculated intranasally with various doses of the HPAI H5N1 virus at infectious doses of 10 to 1×10^6 median tissue culture TCID₅₀, this is the amount of dose that will produce pathological change in 50% of the cell cultures inoculated. To evaluate the virus transmission of infected pigeons to the environment, pigeons inoculated with 1×10^5 TCID₅₀ of HPAI H5N1 were determined for their transmission ability to sentinel avian influenza virus-free chickens. Viral isolation and real-time hydrolysis probe (*TaqMan*) reverse transcriptase polymerase chain reaction were performed to detect viruses from choanal cleft and cloacal swabs. Antibody responses were detected by hemagglutination inhibition and serum neutralization assay. In this study, the median infective dose (ID₅₀) and the median lethal dose (LD₅₀) of the HPAI H5N1 virus of inoculated pigeons were 1×10^5 and 1×10^6 TCID₅₀, respectively. The viruses were also consistently isolated from either cloacal or cloacal swabs of the infected groups. The sentinel chickens housed in the same cage were infected with the HPAI H5N1 virus shedding from the experimental pigeons. The sentinel chickens exhibited clinical signs with high morbidity and mortality. The results showed that the pigeons were less susceptible to HPAI H5N1 virus infection than chickens. The pigeons might be play role as distributors of avian influenza virus transmission and shedding from the infected pigeons to contacted chickens. Therefore, the good biosafety and biosecurity management in farms should be emphasized for control and prevention of the HPAI H5N1 virus.

Keywords: Avian influenza, H5N1, pigeon, *Columba livia*, lethal dose, virus shedding

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INTRODUCTION

Highly pathogenic avian influenza (HPAI) H5N1 viruses have spread in recent years across Asia, Europe and Africa (Webster and Govorkova, 2006). Many species of free-range birds have been recognized as important linkages in the HPAI H5N1 epidemiology (Newman *et al.*, 2012) while numerous species of wild birds appear to be susceptible to the HPAI H5N1 infection including wild gallinaceous birds, geese, ratites, gulls and parrots (Pantin-Jackwood and Swayne, 2009). The natural transmission between free-range birds to humans and birds in the poultry industry has made a great impact on public health and eco-socio-economic concerns. Pigeons share habitat with humans and domestic poultry in cities, towns and cultivated areas from lowland to mountains and in particular with backyard chickens and other free-range birds (Tiensin *et al.*, 2005). Pigeons are theoretically one of the avian species that can play an important role in the distribution of the H5N1 virus in endemic areas. However, the potential transmission of the HPAI H5N1 virus from infected pigeons to chickens is still unsolved. The purposes of the current study were to elucidate HPAI H5N1 transmission from experimentally infected pigeons to chickens and to determine the infective dose, lethal dose, duration of virus shedding and humoral immune response in experimentally infected pigeons.

MATERIALS AND METHODS

Virus

The HPAI A/Chicken/Thailand/vsmu-3/2004 (H5N1) virus was isolated in the laboratory of the Veterinary Science Faculty, Mahidol University from dead infected chickens in 2004. The isolate was twice propagated in Madin-Darby canine kidney (MDCK) cells. The cell culture supernatant containing the virus was harvested and the viral concentration was estimated according to the method of Reed and Muench (1938).

The prepared virus was stored at -76 °C until used. All eight genomic fragments of the virus were characterized by sequencing. The genomic sequences were submitted to GenBank (accession numbers EF593099, EF593100, EF593101, EF593102, EF593103, EF593104, EF593105 and EF593106). The HPAI H5N1 virus used in this study was genomically typed as clade 1. The experiment was conducted in the biosafety level 3 (BSL-3) facilities at the Faculty of Veterinary Science, Mahidol University. All studies were permitted by the animal welfare committee of the Faculty of Veterinary Science, Mahidol University.

Animals and experimental designs

Experiment 1: Experimental infection of highly pathogenic avian influenza H5N1 in chickens

HPAI Ck/Th/vsmu-3-BKK/04 H5N1 infection was studied in domestic Thai-native chicken (*Gallus gallus domesticus*). Thirty chickens aged 8 wk that were negative to AI virus isolation and the hemagglutination-inhibition test were allotted into six groups, with five chickens in each group. Groups 1 to 6 were intranasally inoculated with the virus at infective doses of 1×10 , 1×10^2 , 1×10^3 , 1×10^4 , 1×10^5 and 1×10^6 TCID₅₀ median tissue culture (TCID₅₀ = the amount of dose that will produce pathological change in 50% of the cell cultures inoculated), respectively. The control group was intranasally inoculated with 0.1 mL of phosphate buffered saline (PBS). The Thai-native chickens were raised in a 1.19 m² animal BSL-3 poultry isolator (Allentown Inc.; Allentown, NJ, USA), with five chickens in each cabinet. Clinical signs were observed daily. Virus shedding and antibody against the virus were monitored. Choanal and cloacal swabs were collected every day and stored in 2 mL of viral transported medium (VTM). Blood samples were collected from wing veins at 0, 3, 7, 10, 14, 17 and 21 d post infection (PI) and determined for antibody titer against the HPAI

H5N1 virus. Tissues from the vital organs of the infected birds were collected by necropsy after death and at the end of the experiment. Viruses in these tissues were detected by using the H5-specific *TaqMan* real-time reverse transcriptase polymerase chain reaction (RT-PCR).

Experiment 2: Experimental infection of highly pathogenic avian influenza H5N1 in pigeons

Twenty-eight male and female pigeons (*Columba livia*) aged 1 yr that were serologically negative for H5N1-specific hemagglutination-inhibiting antibodies and AI virus free were purchased from a commercial pigeon farm in Nakhon Pathom, Thailand. The birds were divided into seven groups, with four birds in each group. Groups 1 to 6 were intranasally inoculated with different doses of HPAI Ck/Th/vsmu-3-BKK/04 (H5N1) virus at doses of 1×10 , 1×10^2 , 1×10^3 , 1×10^4 , 1×10^5 and 1×10^6 TCID₅₀, respectively. Group 7 was the non-infected control group intranasally inoculated with 0.1 mL PBS. The pigeons were fed in an animal BSL-3 poultry isolator, with four pigeons in each cabinet. Clinical signs and death were observed daily. To study interspecies viral transmission, choanal and cloacal swabs were collected every day and stored in 2 mL of VTM. Blood samples were collected from wing veins at 0, 3, 7, 10, 14, 17, 21, 24, 27, 31 and 35 d PI of the experiment for investigation of antibody titer against HPAI H5N1. Tissues from the vital organs of infected birds were collected by necropsy after death and at the end date of experiment. Viral genomes in these tissues were detected by using H5 specific *TaqMan* real-time RT-PCR.

Experiment 3: Highly pathogenic avian influenza H5N1 virus transmission from infected pigeons to sentinel chickens

Eight chickens free from the avian influenza (AI) virus and antibody against the AI virus and eight pigeons infected with the HPAI H5N1 virus, were used in this experiment. The chickens and pigeons were divided into two groups

(Group 1 and Group 2). In individual group, four chickens and four pigeons were reared in the same BSL-3 isolator. Feed and water were provided *ad libitum*. Clinical signs and death were observed and recorded daily for 21 d PI. Choanal and cloacal swabs were collected daily and stored in 2 mL of VTM. Blood samples were collected from wing veins at 0, 3, 7, 10, 14, 17, 21, 24, 27, 31 and 35 d PI. Tissues from the vital organs of infected birds were collected by necropsy after death and at the end of experiment. Viral genomes in these tissues were detected by using H5 specific *TaqMan* real-time RT-PCR.

Virus isolation and real-time reverse transcriptase polymerase chain reaction

Cloacal and cloacal swab samples were tested for the presence of the HPAI H5N1 virus by viral isolation and real-time RT-PCR.

The swab samples were taken from experimental birds and virus isolation detected using MDCK cells according to the method described in the World Health Organisation manual (Webster *et al.*, 2002). The viral RNA of the tissue was extracted using an RNA extraction kit (Qiagen Inc.; Valencia, CA, USA) as described previously (Webster *et al.*, 2002). Briefly, 140 μ L of tissue specimens were applied in real-time *TaqMan* RT-PCR to detect the hemagglutinin, neuraminidase and matrix genes of the HPAI H5N1 viruses. The primers were designed as follows:

H5 specific primers, (H5F: 5'-ACGTATGACTACCCGCGAGTATTCAG-3' and H5R, 5'-AGACCAGCTACCATGA TTGC-3');

H5 specific probe, (5' FAM-TATACAGCTACCATGATTGC-TAMRA 3');

N1 specific primers, (N1F: 5'-CAATGATGGAACAGGTAGTTGTGG-3' and N1R, 5'-AAGCCGCTCCTGGAATTAGTG-3');

N1 specific probe, (5' FAM-CTCCCGATCCAGACACCATTGCCGT-TAMRA 3');

M specific primers: (AM151F: 5'-CATCCATGGCTAAAGACAAGACC-3'

and AM397R, 5'-AAGTGCACCAGCAG AATAACTGAG-3');

M specific probe: (AM245P: 5' FAM-CTGCAGCGTAGACGCTTTGTCCAAAATG-TAMRA 3').

Real-time *Taqman* RT-PCR was used to detect the hemagglutinin gene of the HPAI H5N1 viruses using H5-specific primers and an H5-specific probe modified from Spackman *et al.* (2002). The copy numbers of the HA gene were calculated by comparing with standard concentration curves resulting from 10-fold dilution of a standard RNA concentration. Reactions were performed in the Artus 3000™ real-time PCR machine (Rotorgene, Chadstone, VIC, Australia).

Serological analysis

The collected sera from each bird were serologically tested by hemagglutination inhibition assay (HI) and microneutralization assay (Micro-NT) as described below.

The HI assay was performed following previous studies (Stephenson *et al.* 2003; Webster *et al.*, 2002). In brief, nonspecific inhibitors in the sera were inactivated by a receptor destroying enzyme (RDE) at a ratio of 1:4 and incubated at 37 °C overnight. A sample of 50 µL of each treated serum was mixed with 4 HA units of formalin-inactivated HPAI Ck/Th/vsmu-3-BKK/04 H5N1 virus that was used as the standard antigen. The mixture was incubated at room temperature for 45 s. Then, 50 µL of goose red blood cells were added into the wells, gently mixed and incubated at room temperature for 30 min. The HI titers were analyzed using Sigma Plot™ (Systat Software Inc.; Chicago, IL, USA).

The Micro-NT assay tested serum was inactivated at 56 °C for 30 min and then twofold diluted with L-1-tosylamide-2-phenylethyl chloromethyl ketone maintenance medium, starting from a dilution of 1:5 up to 1:2,560. The assay was performed by mixing 60 µL of the diluted serum with 60 µL of 2×10^2 TCID₅₀ of the

HPAI Ck/Th/vsmu-3-BKK/04 H5N1 virus for a final concentration at 1×10^2 TCID₅₀ and incubated at 37 °C for 2 hr. Then, 100 µL of the mixture were transferred onto an MDCK cell monolayer and incubated at 37 °C for 18–20 hr. In order to verify the amount of virus inoculums, virus back-titrations at doses of 0.1, 1, 10 and 1×10^2 TCID₅₀ were included in every assay plate, together with the positive control serum and native cell culture as a negative control. The test reactions were prepared in duplicate. Viral nucleoprotein produced in the infected MDCK cells was detected by indirect enzyme-linked immunosorbent assay using mouse monoclonal antibody against influenza A virus nucleoprotein (Chemicon International Inc.; Temacula, CA, USA) as the primary antibody and goat anti-mouse immunoglobulin conjugated with horseradish peroxidase (Southern Biotechnology Associates Inc.; Birmingham, AL, USA) as the secondary antibody. The tetramethylbenzidine peroxidase substrate system (Kirkegaard & Perry; Gaithersburg, MD, USA) was used as the chromogenic substrate. The color product was read for the optical density (OD) at dual wavelengths of 450 and 630 nm. A positive micro-NT result was obtained when the test serum yielded a greater than or equal to 50% reduction in the corrected OD value compared with that of the virus control. The antibody titer was defined as the reciprocal value of the highest serum dilution that gave greater than or equal to 50% neutralization of 1×10^2 TCID₅₀ of the test virus (Webster *et al.*, 2002).

Statistical analysis

Data of copies of the viral genomes were statistically analyzed by the SPSS v17.0 software (SPSS Inc.; Chicago, IL, USA). A paired *t*-test was performed to compare viral genome obtained from choanal and cloacal swab in each group. The non parametric Kruskal-Wallis and Dunn's tests were used to analyze the results of each swab. Significant differences were determined at the *P* < 0.05 level.

RESULTS

Virus isolation and real time reverse transcriptase polymerase chain reaction

The HPAI H5N1 virus was successfully isolated and multiplied from the MDCK system. The virus was kept in liquid nitrogen to preserve the viable pathogenic virus until it was used for the *in vivo* experiments.

Viral shedding in Thai-native chickens

In experiment 1, the infected Thai-native chickens were sensitive to avian influenza infection and most of them died after a short duration at varied concentration dosages of the virus. All native chickens with 1×10 , 1×10^2 , 1×10^3 , 1×10^4 , 1×10^5 and 1×10^6 TCID₅₀ dosages of inoculum died. The endpoint median lethal dose (LD₅₀) of these native chickens was calculated to be 1×10 TCID₅₀ and the median infectious dose (ID₅₀) was the same value. Most virus-inoculated chickens had a short duration of sickness prior to death (range: 1–13 d). After inoculation with 1×10^2 to 1×10^6 TCID₅₀ all inoculated chickens died at 3–13 d PI. Some chickens that were inoculated with high virus doses (1×10^5 TCID₅₀) showed clinical signs, including depressed weakness, diarrhea and apnea with serous nasal discharge. Nervous signs were seen such as head tremors, seizures and paralysis prior to death. The viral particles were horizontally shed by both choanal and cloacal routes correlating with the dose response. The high virus doses appeared to shed from the cloacal route more rapidly than the lower doses from 1 and 3 d PI, respectively. The cloacal route shedding in the infected chickens was found as early as the first day PI in the high doses but could be extended to 2 to 4 d PI in the low dose groups. The viral shedding duration of low dose inoculation was longer than with the high doses. Moreover, the choanal route had a higher viral concentration of shedding viruses than did the cloacal route. Differences of viral genome obtained from the choanal and cloacal swabs were

found in all groups and each route of swab showed a significant difference among chicken groups as well (Table 1).

Real-time RT-PCR was conducted with specific primers of the hemagglutinin gene for viral genome quantitation which revealed the viral genome at levels from 10 to 1×10^6 TCID₅₀ in inoculated chicken tissue including the brain, trachea, heart, lung, liver, spleen, intestine and the bursa of Fabricius approximately in a range 1×10^5 to 1×10^9 copies.g⁻¹.

Since these native chickens died during a short period after inoculation (mostly within 7 d), seroconversion data were not available.

Virus shedding in pigeons

In experiment 2, pigeons inoculated with a dosage of 10, 1×10^2 , 1×10^3 and 1×10^4 TCID₅₀ of the HPAI H5N1 virus could survive with asymptomatic infection for 35 d PI. Only the infected groups subjected to high dosage virus inoculations (1×10^4 to 1×10^6 TCID₅₀) showed clinical signs whereas the low dosages of virus inoculum could cause infection and seroconversion without any clinical signs. The duration of virus shedding via the choanal route started from day 2 PI and was prolonged until day 21 PI and was similar to that of the cloacal route (Table 2). Two out of four inoculated birds with 1×10^5 TCID₅₀ HPAI H5N1 virus reflected only non-specific signs including depression, ruffled feathers, sneezing and anorexia after 17 d PI but were still alive at the end of the experiment (35 d). In addition, two inoculated birds with 1×10^6 TCID₅₀ HPAI H5N1 inoculum became moribund and died with systemic infection after 7 and 16 d PI, respectively (Table 2).

In order to quantitate the viral load, real-time RT-PCR was conducted with specific primers of the hemagglutinin gene which revealed the viral genome only from 1×10^4 and 1×10^5 TCID₅₀ inoculated pigeon tissue including the brain, trachea, liver, spleen, thymus and intestine approximately in the range 1×10^4 to

1×10^6 copies.g⁻¹. Groups 3 to 6 had significant differences in the level of viral genome detected from the choanal and cloacal swabs. Both the choanal and cloacal routes had significant differences of viral genome among groups (Table 2). Gross lesions were mostly detected in the high dose infected group, but the most consistent

lesions were splenomegaly, pulmonary edema and congestion.

The seroconversion was detected in surviving birds until 35 d PI. For most inoculated birds (1×10^3 to 1×10^6 TCID₅₀ HPAI H5N1 virus), seroconversion could be detected by the HI and micro-NT tests (Figure1). Furthermore,

Table 1 HPAI H5N1 of Ck/Th/vsmu-3-BKK/04 infection in test groups of Thai-native chickens with different inoculation doses.

Parameter	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
Inoculation dose (TCID ₅₀)	1×10	1×10^2	1×10^3	1×10^4	1×10^5	1×10^6
Birds in group (n)	5	5	5	5	5	5
Seroconvalescence (%)	28.57	100	100	100	100	ND
Virus shedding in choanal (%)	100	100	75	80	60	ND
Time of virus shedding in choanal (days PI)	3–21	1–12	1–4	1–2	1–2	ND
Average copy number of virus genome in choanal swabs (copies.mL ⁻¹ × 10 ⁶)	2.39±0.32 ^{*,a}	84.42±10.76 ^{*,b}	9.19±0.64 ^{*,c}	6.42±32.14 ^{*,d}	14.8±2.46 ^{*,e}	ND
Virus shedding in cloaca (%)	100	28.57	50	100	100	ND
Time of virus shedding in cloaca (days PI)	4–21	2–4	2–4	1–2	1–2	ND
Average copy number of virus genome in cloaca swabs (copies.mL ⁻¹ × 10 ⁶)	2.88±0.65 ^{**,a}	4.86±0.70 ^{**,b}	0.28±0.05 ^{**,c}	0.18±0.05 ^{**,d}	0.52±0.06 ^{**,e}	ND
Number of birds showing clinical signs (%)	40	100	100	100	100	100
Time of illness (days PI)	1–12	1–13	2–5	1–2	1–2	ND
Number of death birds (%)	100	100	100	100	100	100
Time of death (days PI)	3–13	3–13	2–5	2	2	Within 1 day PI

TCID₅₀ = the amount of dose that will produce pathological change in 50% of the cell cultures inoculated; PI = Post infection;

ND = Not done since all the chickens died rapidly; Values are shown as mean ± SD.

a, b, c, d, e = Values with different lowercase superscripts in the same row are significantly different ($P < 0.05$).

*, ** = Values with different asterisk superscripts in the same column are significantly different ($P < 0.05$).

1×10^3 TCID₅₀ infected pigeons showed the highest immune titers against viral surface and nucleoprotein antigens (1:160–1:320) by the HI and micro-NT tests which sharply increased from 14–21 d PI. The specific antibody titers appeared to slightly decrease until the end of the experiment (Figures 1a and 1b). The seroconversion seemed to drop in the 1×10^4 and 1×10^5 TCID₅₀ groups.

The 1×10^6 TCID₅₀ HPAI H5N1 virus inoculated pigeons recorded the lowest titers of immune response with short duration (14 d).

In experiment 3, the HPAI H5N1 transmission with high dosage (1×10^5 to 1×10^6 TCID₅₀), resulted in the HPAI H5N1 virus being shed from the pigeons to sentinel chickens. Pigeons deaths were recorded at 7, 8 and 10 d

Table 2 HPAI H5N1 Ck/Th/vsmu-3-BKK/04 infection in test groups of pigeons with different inoculation doses.

Parameter	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
Inoculation dose (TCID ₅₀)	1×10	1×10^2	1×10^3	1×10^4	1×10^5	1×10^6
Birds in group (n)	4	4	4	4	4	4
Seroconvalescence (%)	0	0	25	100	100	100
Virus shedding in choanal (%)	0	0	25	25	25	100
Day of virus shedding in choanal (days PI)	NA	NA	21	4–14	2–7	8–18
Average number of virus genome in choanal swabs (copies.mL ⁻¹ $\times 10^6$)	NA	NA	$0.08 \pm 0.16^{*,a}$	$0.02 \pm 0.40^{*,a}$	$3.4 \pm 0.46^{*,a}$	$8.5 \pm 0.88^{*,b}$
Virus shedding in cloaca (%)	NA	NA	25	50	25	100
Days of virus shedding in cloaca (days PI)	NA	NA	21	3–15	2–3	8–15
Average copy number of virus genome in cloaca swabs (copies.mL ⁻¹ $\times 10^6$)	NA	NA	$0.65 \pm 0.13^{**,a}$	$0.40 \pm 0.46^{**,a}$	$0.68 \pm 0.78^{**,a}$	$6.97 \pm 1.82^{**,b}$
Number of birds showing clinical signs (%)	0	0	0	0	25	50
Time of illness (days PI)	NA	NA	NA	NA	>17	9
Number of death birds (%)	0	0	0	0	0	50

TCID₅₀ = the amount of dose that will produce pathological change in 50% of the cell cultures inoculated; PI = Post infection;

NA = Not applicable; Values are shown as mean \pm SD.

^{a, b} = Values with different lowercase superscripts in the same row are significantly different ($P < 0.05$).

^{*, **} = Values with different asterisk superscripts in the same column are significantly different ($P < 0.05$).

PI. All chickens had clinical signs and lesions in both the respiratory and nervous systems and died within 8-14 d PI. The HPAI H5N1 virus was detected solely from the choanal swab samples of the infected pigeons from days 2–10 PI, except with the dead birds where the virus could be detected from both the choanal cleft and cloaca. On the

other hand, the virus could be isolated from both routes from all chickens as well as being detected in the experimental pigeons (Table 3). There was a significant difference in the transmission of the HPAI H5N1 virus shedding from the pigeons to the sentinel chickens between the choanal and cloacal swabs in groups 1 and 2. Copies of the

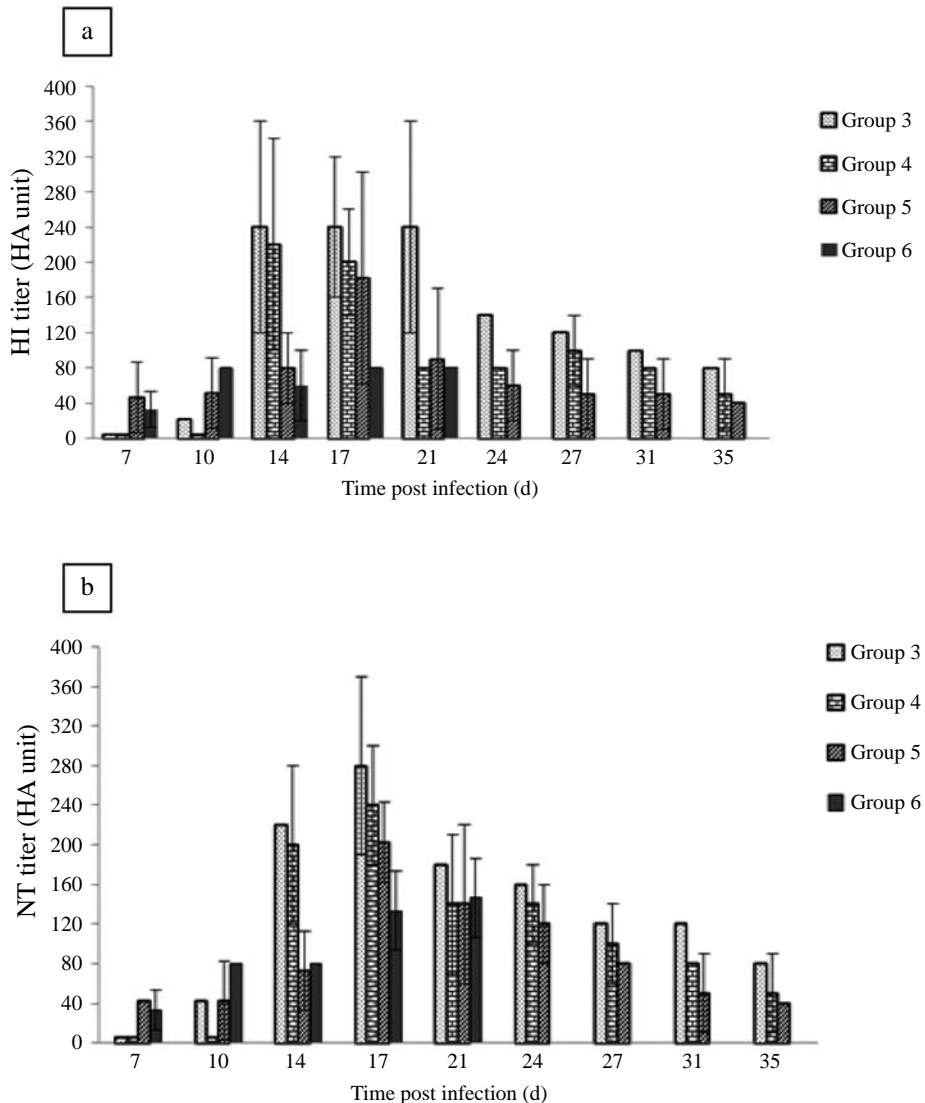


Figure 1 (a) Hemagglutination inhibition (HI) titer of inoculated pigeons; (b) Neutralization titer (NT) of inoculated pigeons. (Group 3 = Inoculum dosage at 1×10^3 TCID₅₀ [TCID₅₀ = the amount of dose that will produce pathological change in 50% of the cell cultures inoculated]; Group 4 = Inoculum dosage at 1×10^4 TCID₅₀; Group 5 = Inoculum dosage at 1×10^5 TCID₅₀; Group 6 = Inoculum dosage at 1×10^6 TCID₅₀; four pigeons per group. Vertical error bars show \pm SD.)

viral genome of both the choanal and cloacal routes were significantly different among groups (Table 2).

Real-time RT-PCR was performed for quantitation of the viral RNA from the infected tissues of the experimental chickens including the brain, trachea, heart, lung, liver, spleen, intestine and the bursa of Fabricius in the range 1×10^5 to 1×10^{10} copies.g⁻¹. The virus was detected from samples of infected pigeons with nervous signs but the virus was not detected from either the lung or heart samples of the pigeons.

Based on the clinical results, these chickens died in a short period after inoculation (most had died within 7 d). Therefore no seroconversion was available from the infected chickens.

Pathology of highly pathogenic avian influenza H5N1 infection in experimental pigeons.

In this study, the dead pigeons showed lesions in many organs including the brain, liver, pancreas and kidney. Non-suppurative encephalitis and perivascular cuffing were found in the brain. Moreover, multifocal lymphocytic myocarditis, multifocal lymphocytic periportal hepatitis and necrotizing pancreatitis were found in the dead pigeons.

DISCUSSION

In this study, the HPAI H5N1 virus could be detected from 1×10^2 to 1×10^6 TCID₅₀ of the HPAI H5N1 virus from both the trachea and cloaca of infected Thai-native chickens at 1 to 12 d PI. The infected chickens showed high susceptibility

Table 3 Transmission of HPAI Ck/Th/vsmu-3-BKK/04 H5N1 virus with high doses (1×10^5 TCID₅₀) in infected pigeons to sentinel chickens.

Avian species	Group 1		Group 2	
	Pigeon	Sentinel chicken	Pigeon	Sentinel chicken
Birds in group (n)	4	4	4	4
Seroconvalescence (%)	100	100	100	100
Virus shedding in choanal (%)	50	100	50	100
Day of virus shedding in choanal (days PI)	2–7	3–7	4–10	6–14
Average copy number of virus genome in choanal swabs (copies.mL ⁻¹ ; mean±SD × 10 ⁶)	4.30±5.00 ^{a, x}	5.20±0.60 ^{a, x}	3.90±4.50 ^{a, x}	0.14±0.03 ^{b, x}
Virus shedding in cloaca (%)	25	75	50	100
Days of virus shedding in cloaca (days PI)	4–5	5–7	4–7	5–7
Average copy number of virus genome in cloacal swabs (copies.mL ⁻¹ ; mean±SD × 10 ⁶)	0.94±1.80 ^{a, x}	59.50±39.80 ^{a, x}	0.24±0.062 ^{a, x}	0.15±0.02 ^{a, x}
Number of birds showing clinical signs (%)	50	100	25	100
Time of illness (days PI)	4–10	3–10	5–14	6–14
Number of dead birds (%)	25	100	25	100

TCID₅₀ = the amount of dose that will produce pathological change in 50% of the cell cultures inoculated; PI = Post infection.

^{a, b} = Values with different lowercase superscripts in the same row are significantly different ($P < 0.05$).

^{*, **} = Values with different lowercase superscripts in the same column are significantly different ($P < 0.05$).

^x = Correlation in the group show statistical difference (normal = 0–1).

to HPAI H5N1 virus infection with very low ID₅₀ and LD₅₀ values whereas both these parameters of HPAI H5N1 virus in inoculated pigeons were 1×10^2 to 1×10^5 TCID₅₀ and were observed in 20–25% of infected birds without death. All pigeons inoculated with 1×10^6 TCID₅₀ were infected and showed 50% mortality. Although the virus could be shed from the trachea and cloaca of pigeons and Thai-native chickens, less of the virus was shed from the pigeons compared to the Thai-native chickens. As was reported in other studies (Webster *et al.*, 2002; Connie *et al.*, 2007), chickens are highly susceptible to the HPAI H5N1 virus. Viruses were also inconsistently isolated from various organs of infected groups. Compared to previous reports (Liu *et al.*, 2009; Yamamoto *et al.*, 2012), the current data suggested that the pigeons were susceptible to high doses of the HPAI H5N1 virus but showed a higher tolerance to the virus infection than did the chickens. Based on the study by Liu *et al.* (2009), the epithelial surfaces of the larynx, trachea, bronchus and bronchiole of pigeons contained abundant SAα2, 6 Gal receptors. Thus, the scarcity of SAα2, 3 Gal in the pigeon airway may partly contribute to the resistance of pigeons to the Asian lineage of the HPAI subtype H5N1 virus (Liu *et al.*, 2009). Brown *et al.*, (2009) reported lesions and viral antigen in the brain, liver and ovary, but lesions were not found in the lung and heart.

The current results are in accordance with other studies (Perkins and Swayne, 2002; Boon *et al.*, 2007; Smietanka *et al.*, 2011), where pigeons showed limited susceptibility to HPAI H5N1 virus infection. Contrary to the results from this study, however, there is a high potential for the transmission of the virus shedding from the experimentally infected pigeons to the contact chickens, since the contact chickens clearly exhibited clinical signs and death. This indicates that the shedding ability of the infected pigeons with low titers can provide a sufficiently lethal dose for a susceptible host such as domestic chickens. However, failure of disease induction in sentinel

chickens with the HPAI A/Chicken/Indonesia/2003 H5N1 virus by direct contact with the feces of experimentally infected pigeons has been reported. The reasonable explanation for this phenomenon may be insufficient time with close contact and the inability to come in contact with the contaminated secretion shed through the choanal and cloaca might have substantially reduced the effectiveness of interspecies infectivity (Werner *et al.*, 2007). A study conducted in China, reported that the experimental pigeons were resistant to infection with five different HPAI H5N1 virus isolates and did not transmit to the contacted chickens (Liu *et al.*, 2007). In that study, the pigeons could be infected with a high concentration of the H5N1 HPAI (A/Whooper swan/Mongolia/244/05) virus although the pigeons were tolerant to infection.

Remarkably, the results of the serological response against HPAI infected pigeons, the antibody titers, were sharply increased in a short period from days 14 to 21 PI, and then slightly decreased. The results of this study conflicted with a previous report which failed to raise the seroconversion from high dose HPAI infection of pigeons (Yamamoto *et al.*, 2012). The proper clarifications might be associated with a limitation in the viral susceptibility and the narrowing spectrum of the expression of host cytokines (Hayashi *et al.*, 2011). Moreover, a study reported unsuccessful induction of the disease in the sentinel chickens by indirect contact (separate feeding of food and water) from the infected pigeons (Werner *et al.*, 2007). The direct contact and aerosol transmission were estimated for the major route of transmission between pigeons and sentinel chickens when compared with contaminated water ingestion. This study indicated that the prolonged virus shedding from pigeons could be observed in contaminated feed and water which was similar to the results of Brown *et al.* (2009). Although at no time in the current study were pigeons observed bathing in the water basin, fecal droppings were presented in the water, indicating that the pigeons were either perching on the water basin or standing

in the water. Feeding the food and water might cause transmission of shedding the HPAI H5N1 virus among poultry. Interestingly, the amount of viral genome detected from the choanal cleft of pigeons subjected to a high dose of infection was significantly more than that of cloaca. However, in low dose infected pigeons, the viral genome from the choanal cleft was less when compared to that of the cloacal route. This may imply that the virus mostly sheds via the cloaca in nonclinical or subclinical pigeons. In fact, the gastro-intestinal tract is also a target of avian influenza infection and the virus can be transmitted by the oral-fecal route (Webster *et al.*, 1978; Connie *et al.*, 2007). This may be supported by a study where the pharynx and intestinal tracts of pigeons (duodenum, ileum, rectum) had abundant SA α 2, 3 Gal receptors (Lui *et al.*, 2009). Probably, sample collections for viral detection by active surveillance in pigeons roaming at low risk of the HPAI H5N1 infection should be mostly done by cloacal swabbing.

The infected and dead pigeons in this study had lesions in many organs including the brain, liver, pancreas and kidney. Contrary to Brown *et al.* (2009), the infected pigeons only had main lesions in the brain, liver and ovary. Nonsuppurative encephalitis with lymphocytic infiltration and perivascular cuffing were found in the brain of infected pigeons in this study, and this was similar to a previous study (Perkins and Swayne, 2002).

These findings present a possible connection among land-based wild birds such as pigeons and poultry in which the pigeons may probably play a role in epidemiological dissemination, in the case of a high dosage of the HPAI H5N1 infection of pigeons.

CONCLUSION

This study provided reasonably assurance that the experimental pigeons were resistance to the HPAI Ck/Th/vsmu-3-BKK/04 H5N1 virus. Only when the birds were inoculated with a high

concentration of the virus were there clinical signs, seroconversion and interspecies transmission. The shed virus can contaminate feed and fecal samples which extend its survival and play an important role in the virus interspecies transmission in birds between poultry and pigeons.

Transmission of the disease among wild bird species and domestic poultry is occasionally found and is difficult to control in a practical manner. The relationship between pigeons and the raising of backyard poultry is the key condition. There should be greater concern regarding open system raising in poultry farms to avoid direct and indirect contact with the natural avian carriers of the virus, including pigeons. Another result stemming from these experiments is that a surveillance program would be useful to investigate the transmission of other influenza A subtypes. Further studies are needed to determine the role of direct contact between poultry and naturally infected migratory terrestrial wild avian species.

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