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Experimental Assessment of Houseflies as Vectors in Avian Influenza Subtype H5N1 Transmission in Chickens

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SUMMARY. In this study, laboratory-reared houseflies were experimentally exposed to the high pathogenicity avian influenza virus (HPAI) subtype H5N1 virus to evaluate the houseflies as vectors in HPAI-H5N1 virus transmission in chickens. One hundred and fifty houseflies (*Musca domestica* L.) were equally allocated into three groups. Groups 2 and 3 were exposed to the HPAI-H5N1 virus by allowing the flies to consume food containing the virus for 15 min, while the flies in group 1 were allowed to consume H5N1-free food and would serve as a negative control group. Group 2 flies were euthanatized immediately after H5N1 exposure, while group 3 were held at room temperature for 24 hr and euthanatized. The houseflies in the transmission of the HPAI-H5N1 virus were examined by challenging three groups of housefly homogenates into layer chickens via the oral drop. Morbidity and mortality were observed for 14 days, and virus shedding monitored via oropharyngeal swabs (OS) and cloacal swabs (CS), which were collected daily and determined by real-time reverse transcription-PCR and virus titration. Experimental challenge showed that all the chickens of groups 2 and 3 died within 7 days of inoculation. The OS had higher concentrations of virus than CS. Moreover, the chickens of group 2 had higher concentrations of virus shedding than the chickens of group 3. Immunohistochemistry detected the nucleoprotein of the type A influenza virus in all tissue samples collected, including the trachea, duodenum, pancreas, and brain. In summary, this study demonstrates that houseflies could serve as vectors in HPAI-H5N1 virus transmission in chickens under experimental conditions.

RESUMEN. Evaluación experimental de las moscas domésticas como vectores para la transmisión a los pollos del virus de la influenza aviar subtipo H5N1.

En este estudio, moscas domésticas criadas en laboratorio se expusieron experimentalmente al virus de la influenza aviar de alta patogenicidad subtipo H5N1 para evaluar a estos insectos como vectores para la transmisión de este virus a los pollos. Ciento cincuenta moscas domésticas (*Musca domestica* L.) se asignaron de manera igual en tres grupos. Los grupos dos y tres fueron expuestos al virus de la influenza aviar alimentándolas por 15 minutos con alimento que contenía el virus, mientras que a las moscas en el grupo uno se les permitió consimir alimento libre del virus y servir como control negativo. A las moscas del grupo dos se les practicó la eutanasia inmediatamente después de la exposición con el virus de influenza aviar H5N, mientras que el grupo tres se mantuvo a temperatura ambiente por 24 horas antes de ser sometidas a la eutanasia. Las moscas domésticas se examinaron en la transmisión del virus de influenza aviar de alta patogenicidad H5N1 mediante el desafío de aves de postura con macerados de moscas por vía oral. Se observaron la morbilidad y la mortalidad por 14 días, y la eliminación viral se determinó mediante hisopos orofaríngeos y cloacales, los cuales fueron recolectados diariamente se analizaron por transcripción reversa y PCR en tiempo real además por titulación viral. El desafío experimental mostró que todos los pollos de los grupos dos y tres murieron dentro de los siete días después de la inoculación. Los hisopos orofaríngeos mostraron las concentraciones virales más altas en comparación con los hisopos cloacales. Además, los pollos del grupo 2 mostraron concentraciones más altas de eliminación viral en comparación con el grupo tres. Mediante inmunohistoquímica, se detectó a la nucleoproteína del virus de la influenza A en todas muestras de tejidos recolectadas, incluyendo la tráquea duodeno, páncreas y cerebro. En resumen, este estudio demuestra que las moscas domésticas pueden servir como vectores en la transmisión del virus de la influenza aviar de alta patogenicidad subtipo H5N1 a los pollos bajo condiciones experimentales.

Key words: avian influenza subtype H5N1, houseflies, vectors, chickens, transmission, experimental conditions

Abbreviations: AIV = avian influenza virus; BHI = brain heart infusion broth; BSL = biosafety level; CS = cloacal swabs; DPI = days postinoculation; ELD_{50} = 50% egg lethal dose; HI = hemagglutinin inhibition; HPAI-H5N1 = high pathogenicity avian influenza virus subtype H5N1; IHC = immunohistochemistry; M = matrix; MDT = mean death time; NIAH = National Institute of Animal Health; OS = oropharyngeal swabs; PBS = phosphate-buffered saline; PRRSV = porcine reproductive and respiratory syndrome virus; RBC = red blood cell; RT-PCR = reverse transcription-PCR; RRT-PCR = real-time reverse transcription-PCR; TCV = turkey coronavirus

Avian influenza virus causes a highly contagious respiratory disease in birds and has been isolated from wild bird species, especially from waterfowl, throughout the world (8). On January 23, 2004, the H5N1 virus was first reported in Thailand in a layer chicken farm in Suphanburi province. Since that time, there have been more than 149 reported cases throughout the country, more than 62 million birds were destroyed (3), and the outbreak

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continued until 2008 (19). Risk factors of H5N1 virus spread within a geographic region, depending on the density of the avian population, human habitat, and environmental characteristics (21). Moreover, the potential role of houseflies in the transmission of H5N1 virus in poultry has been suggested (28).

The common housefly, *Musca domestica* L., belongs to the order Diptera, can be found in nonhygienic human and animal environments, and causes nuisance problems to humans and animals (12). Houseflies have a complete metamorphosis, comprising four distinct stages of development; egg, larva or maggot, pupa, and adults. Their life cycle will complete in approximately 10 days depending on temperature (13). Moreover, houseflies are considered to play an important role as mechanic and/or biologic vectors for several pathogens such as *Shigella* spp. (11), *Salmonella* spp. (14), *Escherichia coli* (5), Newcastle disease virus (34), turkey coronavirus (2) and metazoan parasites (9). This may be due to the structure of houseflies that allows the collection of pathogens via mouthparts through vomit droplets and via exoskeleton body surfaces (12). The rate of crop empty is regulated by the blood osmotic level and concentration of sugar solution in a housefly's meal. If the sugar concentration is low, the food will be directly transferred into the midgut. Conversely, if the sugar concentration is high, the food will be stored in the crop for several days (11). Moreover, the midgut of houseflies have an approximate acidic pH 3 (11), which possibly inactivated the avian influenza virus (H5N1) (29), suggesting that avian influenza virus apparently localizes and remains viable in the upper digestive tract including crop. Moreover, Nielsen *et al.* (17) also demonstrated that low pathogenic avian influenza H5N7 and H7N1 subtypes remained viable in the digestive tract of infected houseflies. The possible methods of transmission for the avian influenza virus of the housefly are via the vomit droplet, from internal organs and dislodgement from the exoskeleton (33). Furthermore, houseflies are able to disperse contaminated pathogens within their flying distance of approximately 1 to 3 km per day (13). During the outbreak period in March 2004 in Japan, Sawabe *et al.* (28) found the HPAI-H5N1 virus in blowflies and suggested that blowflies possibly carry the HPAI-H5N1 virus to poultry farms. The objective of this study was to determine the potential role of the houseflies as vectors for HPAI-H5N1 virus transmission in chickens under experimental conditions.

MATERIALS AND METHODS

Houseflies. The houseflies were reared and colonized under laboratory conditions and fed *ad libitum* using cotton-wool pads saturated with water and food (a mixture of the ultra-high-temperature processing milk and 10% sucrose solution) (33). Prior to beginning the experiment, the houseflies were randomly selected and confirmed to be free of HPAI-H5N1 virus by real-time reverse transcription (RRT-PCR) assay using specific primers and probes to influenza A matrix (M) gene. At the age of 2 days, the houseflies were held for 12 hr at room temperature without food and water prior to use.

HPAI-H5N1 virus. The highly pathogenic avian influenza (HPAI) H5N1 virus A/Chicken/Thailand/CU-K2/04 (H5N1) was used in this study. The virus was prepared as the third passage in embryonated chicken eggs following the standard protocol (18). The virus was confirmed as HPAI-H5N1 by reverset transcription (RT-PCR) as previously described (22). Virus infectivity titer was determined as $10^{9.5}$ 50% egg lethal dose (ELD_{50})/ml, and the virus was stored at −80 C until use.

Fly exposure by consuming food containing HPAI-H5N1 virus. One hundred and fifty adult houseflies were randomly and equally allocated into three groups by using an aspirator. Each group was placed into a sterile plastic box covered with a net. The flies were infected, allowing them to feed for 15 min on cotton-wool pads saturated with 1 ml of HPAI-H5N1 virus containing $5 \times 10^{8.55}$ ELD_{50} mixed with housefly food, except for the flies in group 1 that were allowed to feed on H5N1 virus-free food to serve as a negative control group. After a 15 min exposure period, the infected cotton-wool pads were removed. Group 2 flies were euthanatized immediately after H5N1 exposure by placing them into a dry ice box for 2 min and then collecting. Thereafter, the group 3 flies were held at room temperature for 24 hr and provided with H5N1 virus-free food as previously described (33). At 24 hr postexposure, the group 3 flies were euthanatized. Five exposed flies were pooled into one sample. Then, each pooled sample was homogenized with 1 ml of sterile brain heart infusion broth (BHI) by using sterile plastic pestles and centrifuged at 3000 × *g*, 4 C for 15 min and stored at −80 C.

Chickens. Thirty 1-day-old commercial layer chickens were obtained from a H5N1 virus-free farm in Chachoengsao province in Thailand. The chickens were randomly divided into three groups of 10 birds each and housed in separate rooms. Feed and water were provided *ad libitum*. Prior to inoculation, the chickens were tested for avian influenza virus subtype H5 antibodies using hemagglutinin inhibition (HI) assay. Moreover, oropharyngeal swabs (OS) and cloacal swabs (CS) were collected and tested for the M gene by RRT-PCR assay.

Experimental designs. At 32 days of age, each chicken of group 1 was orally inoculated with 1 ml of avian influenza virus- (AIV-) free housefly homogenate serving as a negative control, whereas each chicken in groups 2 and 3 was orally inoculated with 1 ml of contaminated housefly homogenate prepared from each plastic box of groups 2 and 3, respectively. All groups were separately placed in three biosafety level 3 (BSL-3) isolators (Ingenia, Paris, France) at the National Institute of Animal Health (NIAH), Thailand. OS and CS were collected daily and placed in viral transport media, consisting of BHI and antibiotics, and then stored at −80 C until virus isolation and titration were performed. The clinical signs and mortality were observed daily for 14 days, and blood samples were collected at 7 and 14 days postinoculation (DPI). On 14 DPI, all remaining chickens were euthanatized and necropsied. Visceral organs, including the trachea, lung, small intestine, pancreas, and brain were collected and fixed in 10% neutral buffered formalin for histopathology and IHC. All steps were performed under BSL-3 conditions.

Virus titration. The OS and cloacal CS contents were used to inoculate 10-day-old embryonated chicken eggs according to standard procedure (18). Briefly, the 10-fold, serially diluted swab solution was inoculated into embryonated chicken eggs. Inoculated eggs were incubated and were candled twice a day for 5 days. Virus titers were determined as ELD_{50} per milliliter according to the Reed and Muench method (24).

Serology. The HI test was performed according to World Organisation for Animal Health (18) procedure. Briefly, twofold serial dilutions of serum sample were made in sterile phosphate-buffered saline (PBS) and a standard stock virus containing 4 HA units of H5N1 virus was added into each well and incubated for 40 min. Then, 0.5% of chicken red blood cells (RBCs) was added to all wells with gentle mixing, and the RBCs allowed to settle for 40 min. The HI end point was read as the last dilution with a complete inhibition of HA activity.

RNA extraction. Test samples were extracted using QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Briefly, swab solutions were mixed with Buffer AVL from the kit containing carrier RNA, vortexed, and incubated. Subsequently, absolute alcohol was added. The solution was transferred to QIAmp mini column and centrifuged. Columns were washed twice with washing buffer. RNA was eluted with nuclease-free water. Four microliters of the RNA template was used for analysis by RT-PCR or RRT-PCR assays, respectively.

RT-PCR assay. The presence of the HPAI-H5N1 virus in allantoic fluid was determined using RT-PCR assay as previously described (22) with primers specific for M, MF 5′-TGATCTTCTTGAAAATTTG-CAG-3′ and MR 5′-TGTTGACAAAATG ACCATCG-3′. RT-PCR assay was performed using AccessQuick™ RT-PCR System (Promega, Madison, WI) according to the manufacturer's protocol. Cycling conditions included a reverse transcription step at 48 C for 15 min, a denaturation step at 95 C for 2 min, amplification for 40 cycles,

Table 1. MDT and mortality of chickens inoculated with the homogenate of HPAI-H5N1 virus contaminated houseflies ($n = 10$).

	Number of chicken deaths each day							
Group[A]	1 DPI	2 DPI	3 DPI	4 DPI	5 DPI	6 DPI	7 DPI	MDT
1	0	0	0	0	0	0	0	0
2	0	1	2	3	2	1	1	4.3
3	0	0	1	0	4	2	3	5.6

[A]Group 1: negative control chickens; group 2: chickens inoculated with the homogenate of HPAI-H5N1 virus contaminated houseflies at immediately after contamination; group 3: chickens inoculated with the homogenate of HPAI-H5N1 virus contaminated houseflies at 24 hr after initial contamination.

including denaturation (94 C for 30 sec), annealing (55 C for 30 sec) and extension (72 C for 30 sec), and final extension at 72 C for 10 min. The PCR product was analyzed by 2% agarose gel electrophoresis (TopVision™ LE GQ Agarose, Fermentas, Hanover, MD) at 125 V for 45 min, stained with the 0.5 μg/mL ethidium bromide and analyzed by a gel documentation system (Vilber Lourmat, Marne-la-Vallée, France). The expected PCR product size of the M gene was 276 base pairs.

RRT-PCR assay. The presence of the H5N1 virus in swab solutions was determined using the RRT-PCR assay to detect M gene as previously described (30). Briefly, the SuperScript III Platinum One-Step RT-PCR System (Invitrogen, Grand Island, NY) was used in this study. The forward primer MF25 (5′-AGATGAGTCTTCTAACC-GAGGTCG-3′), reverse primer MR124 (5′-TGCAAAAACATCTT-CAAGTCTCTG-3′) and probe M64 (FAM-TCAGGCCCCCT-CAAAGCCGA-TAMRA) were used (30). The final concentrations of primers and probe were 10 and 2.5 μM, respectively. The reaction consisted of 4 μl of the RNA template sample, 7.5 μl of 2× reaction mix, 0.3 μl of SuperScript III RT Platinum® Taq Mix, 50 mM of $MgSO_4$, and RNase-free water in a final volume of 17 μl. One-step RRT-PCR was performed using Rotor-Gene 3000 (Corbett Research, Sydney, New South Wales, Australia). Cycling conditions included a reverse transcription step at 50 C for 30 min, an initial denaturation step at 94 C for 15 min followed by 40 cycles of amplification (95 C for 10 s, 54 C for 30 s, and 72 C for 10 s) (30). The fluorescence data was collected at the end of each annealing step, and data analyses of the RRT-PCR assay were performed using the Rotor-Gene analysis software, Version 6.0 (Corbett Research supporting program).

Mean death time (MDT). The MDT was evaluated by the sum of the days from inoculated chicken deaths caused by HPAI-H5N1 infection divided by the total number of inoculated chicken deaths according to Sarmento *et al.* (26). All deaths were necropsied, and visceral organs were collected for histopathology and IHC to investigate HPAI-H5N1 virus.

Histopathology. The collected visceral organs of each chicken were fixed in 10% neutral buffered formalin for 48 hr and routinely processed. After fixation, the tissues were embedded in paraffin and cut at 5-μm thickness for histopathologic and immunohistochemical examination. For histopathologic examination, 5-μm tissue sections were stained with hematoxylin and eosin (H&E).

Immunohistochemistry. IHC for the determination of avian influenza-specific virus antigen was performed using a mouse-derived monoclonal antibody clone EVS 238 (HB65-like) (BV European Veterinary Laboratory, Woerden, The Netherlands) specific for type A influenza virus nucleoprotein according to Sreta *et al.* (31). In brief, after deparaffinization in xylene, hydration in ethanol, and washing in PBS, tissue sections were incubated for 30 min in 0.3% hydrogen peroxide in methanol to block endogenous peroxidase and then pretreated for 10 min in 0.05% proteinase K (Amresco, Solon, OH) to retrieve the antigen, then 1% bovine serum albumin (Invitrogen) was added and incubated for 45 min to reduce background staining. Subsequently, tissue sections were incubated with the mouse-derived monoclonal antibody at 4 C overnight and then incubated with EnVision® polymer reagent (DakoCytomation, Carpinteria, CA) for 45 min. The immunohistochemical signal was visualized using 3,3-diaminobenzidine tetrahydrochloride (Sigma-Aldrich, St. Louis, MO). Sections were counterstained in hematoxylin solution. Negative and positive controls were included in each test. The positive nucleoprotein (NP) viral antigen in tissue section was indicated by the substrate chromogen stained in the nucleus. All steps were done at room temperature unless otherwise stated.

RESULTS

Serology. All chickens were seronegative against HPAI-H5N1 virus prior to inoculation. All of the negative control chickens were also seronegative throughout the experimental period. There were no blood samples collected at 7 and 14 DPI from HPAI-H5N1 virus-inoculated chickens due to mortality.

Clinical observations, morbidity, and mortality. After 24 hr inoculation, one bird of group 2 showed clinical signs, was depressed, and died at 2 DPI, whereas the first bird of group 3 died without exhibiting clinical signs at 3 DPI. The clinical signs of the other chickens in group 3 were observed on day 4 postinoculation and later. The clinical signs of both inoculated groups included depression, listlessness, loss of appetite, and ruffled feathers. H5N1 virus caused 100% death of the chickens in groups 2 and 3 within 7 DPI, with the onset of death on days 2 and 3 postinoculation, and the MDT was 4.3 and 5.6, respectively (Table 1). All chickens in the negative control group lived and did not exhibit any clinical signs.

H5N1 detection in oropharyngeal and cloacal shedding. Recovery virus from OS and CS from inoculated chickens determined by RRT-PCR assay showed that all the inoculated chickens consistently shed virus during the observation period. H5N1 virus was frequently isolated from swabs at 24 hr before chicken death in accordance with the highest viral RNA copy numbers. The mean viral RNA load of the OS and CS of groups 2 and 3 ranged between 4.00–7.70 and 2.85–6.81 and 2.65–4.91 and 2.33–3.81 copy numbers/μl, respectively (Table 2). The mean infectious virus titers of the OS and CS of groups 2 and 3 ranged between 3.08–6.5 and 2.18–3.32 and 1.67–4.09 and 0–4.25 ELD_{50}/ml, respectively (Table 3). Moreover, the mean viral RNA shedding titers and the mean virus shedding titers from the OS of group 2 were higher than those of group 3 at any time of swab collection (Table 2). No virus shedding from the OS and CS of the negative control group was detected by RRT-PCR assay and virus isolation.

Necropsy findings. Gross lesions were observed in all inoculated chickens, and the predominant lesions were airsacculitis, pulmonary congestion, mild pancreatic congestion, and mild enteritis. There were no gross lesions in any negative control chicken.

Histopathology and IHC. Histopathologic lesions in all the chickens of groups 2 and 3 showed multiorgan necrosis with moderate to severe heterophilic and mononuclear inflammatory infiltration, deciliation and sloughing of the epithelial cells in the trachea, mild to moderate diffuse pulmonary congestion, multifocal to coalescing necrosis of the pancreas, perivascular infiltration of the mononuclear cells of the duodenum, and necrotizing splenitis. In contrast, no remarkable lesions were observed in all chickens of the negative control group.

By IHC, nucleoprotein of the HPAI-H5N1 virus was detected in all examined tissue sections, especially in the pancreas from both AIV-inoculated groups, whereas virus antigen was not detected in the examined sections from the negative control chickens (Fig. 1).

DISCUSSION

Poultry production in Thailand can be divided into four sectors, including industrial integrated system, semivertical integrated

Table 2. Viral RNA shedding titers determined by RRT-PCR from OS and CS of chickens inoculated with the homogenate of HPAI-H5N1 virus contaminated houseflies.

Group[A]	Viral RNA shedding titers[B]					
	1 DPI	2 DPI	3 DPI	4 DPI	5 DPI	6 DPI
2						
OS	4.53 ± 1.73	4.00 ± 2.27	4.86 ± 1.32	5.70 ± 0.83	6.41 ± 0.03	7.7
CS	3.31 ± 1.6	2.85 ± 1.27	4.59 ± 1.90	4.99 ± 2.25	3.48 ± 0.58	6.81
3						
OS	2.65 ± 1.07	3.30 ± 1.5	4.35 ± 1.44	4.91 ± 1.51	4.69 ± 2.55	3.98 ± 0.90
CS	2.33 ± 1.16	2.60 ± 1.42	3.50 ± 1.27	3.47 ± 1.39	3.81 ± 0.98	2.88 ± 0.30

[A]Group 2: chickens inoculated with the homogenate of HPAI-H5N1 virus-contaminated houseflies at immediately after contamination; group 3: chickens inoculated with the homogenate of HPAI-H5N1 virus contaminated houseflies at 24 hr after initial contamination.
[B]Mean viral RNA shedding titers of surviving chickens ± SD expressed as log RNA copy numbers per microliter.

system, extensive farming and backyard, or village poultry (32). In general, backyard chickens are freely raised in the backyard of houses that do not have a biosecurity system (32). In Thailand, the previous study showed that 57% of infected flocks from the first HPAI-H5N1 outbreak in 2004 were backyard chickens and 27%, 6%, 5%, 2%, and 3% of infected flocks were ducks, broilers, layers, quails, and other birds, respectively (32). Due to free raising and no biosecurity, the infected backyard chickens can defecate or be found dead anywhere, resulting in conditions in which houseflies can easily consume the contaminated feces or contaminated carcass. These contaminated houseflies possibly transmit to other backyard chickens. Furthermore, the environmental temperature (about 32–37 C) and humidity, especially during the summer season in Thailand, is the appropriate for development of houseflies leading to the increase of the number of houseflies in that area (6,16). Houseflies can be found throughout the year and are the predominant species in market areas, slaughter houses, and animal feces in the northern, northeastern, and central parts of Thailand reviewed by Echeverria *et al.* (6). Our previous study revealed that houseflies can serve as vectors in transmission of HPAI-H5N1 virus into 9–11 days of embryonated chicken embryos (33). Therefore, the potential role of houseflies to act as a vector of the HPAI-H5N1 virus is of great interest. Initially, this experiment was designed for chickens exposed with the freely contaminated houseflies in the BSL-3 isolators. Unfortunately, the regulation policy of NIAH, Thailand, does not allow this type of experiment. To solve this problem, we decided to inoculate the homogenate of contaminated houseflies into the chickens via the oral route instead of by free exposure.

The results of this study demonstrated that houseflies can act as transmission vectors of the HPAI-H5N1 virus in chickens under experimental conditions. Our previous study showed that the HPAI-H5N1 virus titer continually declined over time (33). Moreover, Chakrabarti *et al.* (4) showed that the exotic Newcastle disease virus was detected from collected flies at the epidemic area before flock depopulation. This correlated with Sawabe *et al.* (27) who detected the H5N1 virus from collected flies at epidemic areas within 1 day of the H5N1 outbreak. Moreover, many researchers have found that the houseflies have the ability to carry and store several pathogens for at least 24 hr (14,34). Therefore, it seems reasonable to investigate the effect of different time points after houseflies have been exposed with the HPAI-H5N1 virus. Moreover, all of the infected chickens from the treatment groups died within 2 to 7 DPI and the MDT was 4.3–5.6 DPI, respectively. These results correspond with Jeong *et al.* (15) who demonstrated a 100% mortality rate of chickens after inoculation with avian influenza virus subtype H5N1 by the intranasal route. Furthermore, these infected chickens died within 3 to 6 days after inoculation, and MDT was 3 DPI. Previous studies have reported that avian influenza virus subtype H5N1 isolated in Thailand causes high mortality in chickens, quails, and ducks after inoculation by the intranasal route and MDT were approximately 1.4–2.3, 1.1–3.4, and 4.8–6.3, respectively (25).

However, the inoculated chickens of group 2 developed more severe clinical signs compared with those of group 3. This observation was related to the results of the virus shedding titer, which showed that virus infectivity titers from the swab samples of group 2 was higher than those of group 3 at every occasion postinoculation, and similar results were also supported by RRT-PCR assay. Surprisingly, the virus shedding titers determined by virus isolation were only detected in swab samples at 24 hr before chicken death, which showed the highest virus titer as being similar to the results determined by Brown *et al.* (1). Based on these results, it appears that the chickens in group 2 received higher amounts of

Table 3. Virus shedding titers determined by virus isolation from OS and CS of chickens inoculated with the homogenate of HPAI-H5N1 virus contaminated houseflies.

Group[A]	Virus shedding titer[B] (number of birds shedding virus/total collected birds)					
	1 DPI	2 DPI	3 DPI	4 DPI	5 DPI	6 DPI
2						
OS	3.08 ± 1.49 (8/10)	3.84 ± 1.28 (8/9)	3.85 ± 1.62 (5/7)	3.58 ± 1.23 (4/4)	4.73 ± 0.04 (2/2)	6.5 (1/1)
CS	2.76 (1/10)	3.32 ± 1.08 (6/9)	3.21 ± 0.85 (4/7)	3.05 ± 1.2 (3/4)	2.18 ± 0.18 (2/2)	2.75 (1/1)
3						
OS	1.68 ± 0.11 (2/10)	2.53 ± 1.42 (3/10)	1.67 ± 0.14 (4/9)	2.74 ± 0.81 (8/9)	3.48 ± 1.97 (5/5)	4.09 ± 0.94 (3/3)
CS	0 (0/10)	4.25 (1/10)	1.68 ± 0.11 (2/9)	3.11 ± 0.51 (4/9)	2.8 ± 0.76 (5/5)	2.88 ± 0.88 (2/3)

[A]Group 2: chickens inoculated with the homogenate of HPAI-H5N1 virus-contaminated houseflies at immediately after contamination; group 3: chickens inoculated with the homogenate HPAI-H5N1 virus contaminated houseflies at 24 hr after initial contamination.
[B]Mean virus shedding titers of surviving chickens ± SD expressed as log ELD_{50} per milliliter.

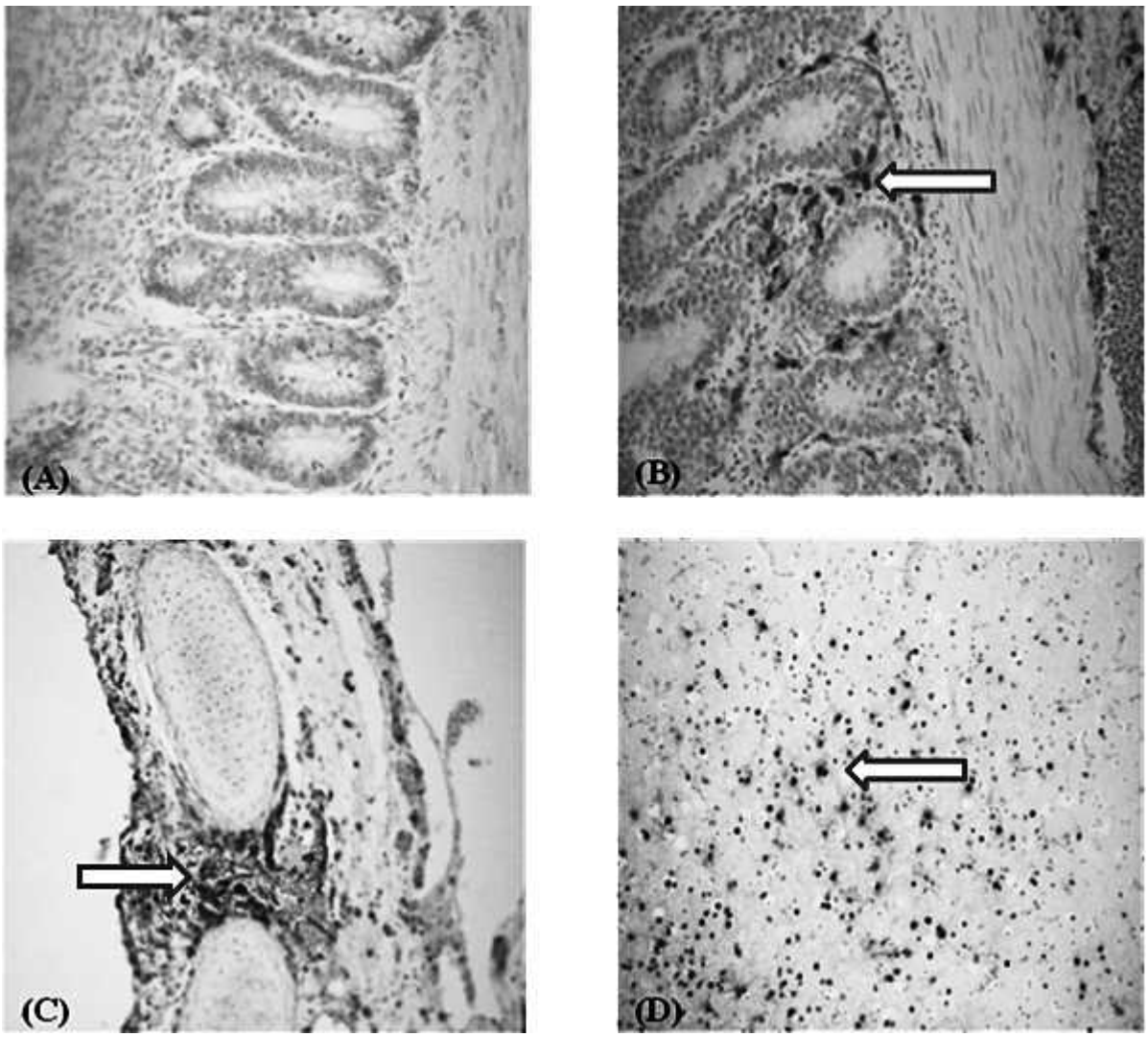

Fig. 1. Distribution of H5N1 viral antigen in tissues of chickens inoculated with the homogenate of HPAI-H5N1 virus contaminated houseflies. Photomicrographs showed the detection of type A influenza viral antigen (indicated by arrows) on a hematoxylin-stained background. (A) Section from duodenum of negative control chicken (original magnification 20×). (B) Section from duodenum of chicken in group 2 inoculated with the homogenate of HPAI-H5N1 contaminated houseflies immediately after contamination (original magnification 20×). (C) Section from trachea of chicken in group 2 inoculated with the homogenate of HPAI-H5N1 virus contaminated houseflies immediately after contamination (original magnification 40×). (D) Section from the brain of chicken in group 3 inoculated with the homogenate of HPAI-H5N1 contaminated houseflies at 24 hr after initial contamination (original magnification 10×).

HPAI-H5N1 virus than the chickens in group 3 did. The different amounts of virus possibly resulted from the survival of the virus in houseflies, which indicated that the survival rate of the HPAI-H5N1 virus in contaminated house files in group 2 was higher than that in group 3. Moreover, the virus shedding titers determined by virus isolation and RRT-PCR assay of the OS was higher than those of the CS suggesting that the H5N1 virus was preferentially shed from the respiratory tract more than from the digestive tract in accordance with the results previously published by Brown *et al.* (1) and Jeong *et al.* (15). One of the possible reasons for this difference is that the avian influenza virus frequently binds to α-2,3-linked sialic acid receptor, which is generally located at the respiratory tract of poultry, including chickens (8). Another possible reason is that the route of inoculation used in this study was oral drop by using pipette. This may cause high virus replication in the respiratory tract.

For the histologic examination, the collected tissue samples were based on the replication sites of avian influenza virus, which resulted in pathologic changes in those tissues as previously described (20). The histopathologic lesions in this study were observed in most of the tissue section samples from the treatment groups, whereas no histopathological lesions were observed in the negative control group (25). Moreover, the IHC result revealed that the NP viral antigen was detected in all tissue section samples from both inoculated groups, especially in the pancreas, whereas no NP viral antigen was detected in the tissue section samples from the negative control group. This result agreed with previous findings (15,20). Thus, these results confirm that all chickens of treatment groups died because of the HPAI-H5N1 virus from the homogenates of infected houseflies. Therefore, houseflies can carry a sufficient amount of H5N1 virus to cause the disease in chickens under experimental conditions. Regarding this point of view, the results of this study are related to Calibeo-Hayes *et al.* (2), which demonstrated that oral inoculation with turkey coronavirus- (TCV-) infected houseflies could infect turkeys, suggesting that houseflies can carry the TCV. Similarly, swine in a native swine farm became infected with porcine

reproductive and respiratory syndrome virus (PRRSV) when PRRSV-infected houseflies entered the farm (23).

Moreover, houseflies are usually found in nonhygienic human and animal areas, in particular poultry operations, and play an important role in the transmission of several pathogens (7). Interestingly, Graczyk *et al*. (10) described how the average flying distance of the individual housefly was approximately 3.2 km per day; therefore, the houseflies could disseminate pathogens into neighboring areas. Furthermore, Calibeo-Hayes *et al*. (2003), and reviewed by Chakrabarti *et al*. (4), revealed that poultry could consume the adult houseflies. Therefore, improvement of environmental sanitation, hygiene, and biosecurity to reduce or eliminate fly breeding sites is the appropriate method for controlling housefly populations and reducing the risk of infection by contamination from houseflies. This is the first article indicating that houseflies could serve as a transmission vector for the HPAI-H5N1 virus in chickens under experimental conditions.

REFERENCES

1. Brown, J. D., D. E. Stallknecht, and D. E. Swayne. Experimental infection of swans and geese with highly pathogenic avian influenza virus (H5N1) of Asian lineage. Emerg. Infect. Dis. 14:136–142. 2008.

2. Calibeo-Hayes, D., S. S. Denning, S. M. Stringham, J. S. Guy, L. G. Smith, and D. W. Watson. Mechanical transmission of turkey coronavirus by domestic houseflies (*Musca domestica* Linnaeus). Avian Dis. 47:149–153. 2003.

3. Capua, I., and D. J. Alexander. Human health implications of avian influenza viruses and paramyxoviruses. Eur. J. Clin. Microbiol. Infect. Dis. 23:1–6. 2004.

4. Chakrabarti, S., D. J. King, C. Afonso, D. Swayne, C. J. Cardona, D. R. Kuney, and A. C. Gerry. Detection and isolation of exotic Newcastle disease virus from field-collected flies. J. Med. Entomol. 44:840–844. 2007.

5. De Jesus, A. J., A. R. Olsen, J. R. Bryce, and R. C. Whiting. Quantitative contamination and transfer of Escherichia coli from foods by houseflies, Musca domestica L. (Diptera: Muscidae). Int. J. Food Microbiol. 93:259–262. 2004.

6. Echeverria, P., B. A. Harrison, C. Tirapat, and A. McFarland. Flies as a source of enteric pathogens in a rural village in Thailand. Appl. Environ. Microbiol. 46:32–36. 1983.

7. Fasanella, A., S. Scasciamacchia, G. Garofolo, A. Giangaspero, E. Tarsitano, and R. Adone. Evaluation of the house fly Musca domestica as a mechanical vector for an anthrax. PLoS One. 5:12219. 2010.

8. Forrest, H. L., and R. G. Webster. Perspectives on influenza evolution and the role of research. Anim. Health Res. Rev. 11:3–18. 2010.

9. Förster, M., S. Klimpel, H. Mehlhorn, K. Sievert, S. Messler, and K. Pfeffer. Pilot study on synanthropic flies (e.g. *Musca, Sarcophaga, Calliphora, Fannia, Lucilia, Stomoxys*) as vectors of pathogenic microorganisms. Parasitol. Res. 101:243–246. 2007.

10. Graczyk, T. K., M. R. Cranfield, R. Fayer, and H. Bixler. House flies (Musca domestica) as transport hosts of Cryptosporidium parvum. Am. J. Trop. Med. Hyg. 61:500–504. 1999.

11. Greenberg, B. Flies and disease. In: Biology and disease transmission, vol. 2. Princeton University Press, Princeton, NJ. pp. 90–275. 1973.

12. Harwood, R. F., M. T. James, and W. B. Herms. Muscoid flies and louse flies. In: Entomology in human and animal health, 7th ed. R. F. Harwood and M. T. James, eds. Macmillan, New York. pp. 255–275. 1979.

13. Herms, W. B. The house fly and its relatives. In: Herm's medical entomology, 7th ed. R. F. Harwood and M. T. James, eds. Macmillan, New York. pp. 249–265. 1969.

14. Holt, P. S., C. J. Geden, R. W. Moore, and R. K. Gast. Isolation of Salmonella enterica serovar Enteritidis from houseflies (Musca domestica) found in rooms containing Salmonella serovar Enteritidis-challenged hens. Appl. Environ. Microbiol. 73:6030–6035. 2007.

15. Jeong, O. M., M. C. Kim, M. J. Kim, H. M. Kang, H. R. Kim, Y. J. Kim, S. J. Joh, J. H. Kwon, and Y. J. Lee. Experimental infection of chickens, ducks and quails with the highly pathogenic H5N1 avian influenza virus. J. Vet. Sci. 10:53–60. 2009.

16. Ngoen-klan, R., K. Moophayak, T. Klong-klaew, K. N. Irvine, K. L. Sukontason, C. Prangkio, P. Somboon, and K. Sukontason. Do climatic and physical factors affect populations of the blow fly *Chrysomya megacephala* and house fly *Musca domestica*? Parasitol. Res. 109:1279–1292. 2011.

17. Nielsen, A. A., H. Skovgard, A. Stockmarr, K. J. Handberg, and P. H. Jorgensen. Persistence of low-pathogenic avian influenza H5N7 and H7N1 subtypes in house flies (Diptera: Muscidae). J. Med. Entomol. 48:608–614. 2011.

18. [OIE] Office International des Epizooties. Influenza. In: Manual of standards for diagnositic tests and vaccines. OIE, Paris. pp. 212–219. 2000.

19. [OIE] Office International des Epizooties. Update on highly pathogenic avian influenza in animals (Type H5 and H7) [Internet] 2011. [Accessed 2012]. Available from: http://www.oie.int/animal-health-in-the-world/update-on-avian-influenza/2011

20. Pantin-Jackwood, M. J., and D. E. Swayne. Pathogenesis and pathobiology of avian influenza virus infection in birds. Rev. Sci. Tech. 28:113–136. 2009.

21. Paul, M., S. Wongnarkpet, P. Gasqui, C. Poolkhet, S. Thongratsakul, C. Ducrot, and F. Roger. Risk factors for highly pathogenic avian influenza (HPAI) H5N1 infection in backyard chicken farms, Thailand. Acta. Trop. 118:209–216. 2011.

22. Payungporn, S., P. Phakdeewirot, S. Chutinimitkul, A. Theamboonlers, J. Keawcharoen, K. Oraveerakul, A. Amonsin, and Y. Poovorawan. Single-step multiplex reverse transcription-polymerase chain reaction (RT-PCR) for influenza A virus subtype H5N1 detection. Viral Immunol. 17:588–593. 2004.

23. Pitkin, A., J. Deen, S. Otake, R. Moon, and S. Dee. Further assessment of houseflies (Musca domestica) as vectors for the mechanical transport and transmission of porcine reproductive and respiratory syndrome virus under field conditions. Can. J. Vet. Res. 73:91–96. 2009.

24. Reed, L. J., and H. Muench. A simple method of estimating fifty percent endpoints. Am. J. Hyg. 27:493–497. 1938.

25. Saito, T., C. Watanabe, N. Takemae, A. Chaisingh, Y. Uchida, C. Buranathai, H. Suzuki, M. Okamatsu, T. Imada, S. Parchariyanon, N. Traiwanatam, and S. Yamaguchi. Pathogenicity of highly pathogenic avian influenza viruses of H5N1 subtype isolated in Thailand for different poultry species. Vet. Microbiol. 133:65–74. 2009.

26. Sarmento, L., J. Wasilenko, and M. Pantin-Jackwood. The effects of NS gene exchange on the pathogenicity of H5N1 HPAI viruses in ducks. Avian Dis. 54:532–537. 2010.

27. Sawabe, K., K. Hoshino, H. Isawa, T. Sasaki, T. Hayashi, Y. Tsuda, H. Kurahashi, K. Tanabayashi, A. Hotta, T. Saito, A. Yamada, and M. Kobayashi. Detection and isolation of highly pathogenic H5N1 avian influenza A viruses from blow flies collected in the vicinity of an infected poultry farm in Kyoto, Japan, 2004. Am. J. Trop. Med. Hyg. 75:327–332. 2006.

28. Sawabe, K., K. Tanabayashi, A. Hotta, K. Hoshino, H. Isawa, T. Sasaki, A. Yamada, H. Kurahashi, C. Shudo, and M. Kobayashi. Survival of avian H5N1 influenza A viruses in *Calliphora nigribarbis* (Diptera: Calliphoridae). J. Med. Entomol. 46:852–855. 2009.

29. Shahid, M. A., M. Abubakar, S. Hameed, and S. Hassan. Avian influenza virus (H5N1); effects of physico-chemical factors on its survival. Virol. J. 6:38. 2009.

30. Spackman, E., D. A. Senne, T. J. Myers, L. L. Bulaga, L. P. Garber, M. L. Perdue, K. Lohman, L. T. Daum, and D. L. Suarez. Development of a real-time reverse transcriptase PCR assay for type A influenza virus and the avian H5 and H7 hemagglutinin subtypes. J. Clin. Microbiol. 40:3256–3260. 2002.

31. Sreta, D., R. Kedkovid, S. Tuamsang, P. Kitikoon, and R. Thanawongnuwech. Pathogenesis of swine influenza virus (Thai isolates) in weanling pigs: an experimental trial. Virol. J. 6:34. 2009.

32. Tiensin, T., M. Nielen, T. Songserm, W. Kalpravidh, P. Chaiteweesub, A. Amonsin, S. Chotiprasatintara, A. Chaisingh, S. Damrongwatanapokin, S. Wongkasemjit, C. Antarasena, V. Songkitti, K. Chanachai, W. Thanapongtham, and J. A. Stegeman. Geographic and temporal distribution of highly pathogenic avian influenza A virus (H5N1) in Thailand, 2004–2005: an overview. Avian Dis. 51(Suppl. 1):182–188. 2007.

33. Wanaratana, S., S. Panyim, and S. Pakpinyo. The potential of house flies to act as a vector of avian influenza subtype H5N1 under experimental conditions. Med. Vet. Entomol. 25:58–63. 2011.

34. Watson, D. W., E. L. Nino, K. Rochon, S. Denning, L. Smith, and J. S. Guy. Experimental evaluation of *Musca domestica* (Diptera: Muscidae) as a vector of Newcastle disease virus. J. Med. Entomol. 44:666–671. 2007.

35. Yap, K. L., M. Kalpana, and H. L. Lee. Wings of the common house fly (Musca domestica L.): importance in mechanical transmission of Vibrio cholerae. Vibrio cholerae. Trop. Biomed. 25:1–8. 2008.

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