The potential of house flies to act as a vector of avian influenza subtype H5N1 under experimental conditions

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Abstract. The objective of the present study was to determine the potential for house flies (*Musca domestica* L.) (Diptera: Muscidae) to harbour the avian influenza (AI) H5N1 virus. Laboratory-reared flies were experimentally fed with a mixture containing the AI virus. Exposed flies were washed with brain–heart infusion broth and followed by 70% alcohol before preparation of whole fly homogenate. The homogenate was inoculated into six 10-day-old embryonated chicken eggs (ECEs). Allantoic fluids were collected to determine the virus using the haemagglutination (HA) test, reverse transcription-polymerase chain reaction (RT-PCR) or quantitative real-time RT-PCR (RRT-PCR). In the first experiment, ECEs that were inoculated with the 50 AI virus exposed fly homogenates died within 48 h and HA and RT-PCR were positive for AI virus. In the second experiment, ECEs that were inoculated with only one fly died with positive HA test and RT-PCR. In the last experiment, a group of exposed flies was collected at 0, 6, 12, 24, 36, 48, 72 and 96 h post-exposure. Fly homogenates of each time point were tested by virus titration in ECEs and RRT-PCR. Virus titres declined in relation to exposure time. Furthermore, RRT-PCR results were positive at any time point. The present study shows that the flies may harbour the AI virus and could act as a mechanical vector of the AI virus.

Key words. *Musca domestica*, avian influenza H5N1, house flies, mechanical vector.

Introduction

Avian influenza (AI) is a highly contagious disease caused by the type A influenza virus of the family *Orthomyxoviridae*. Highly pathogenic avian influenza (HPAI) viruses, especially subtype H5N1, cause a multi-systemic disease associated with high morbidity and mortality in poultry, as well as in mammals and humans (Mahy & Van Regenmortel, 2008). The emergence of the AI H5N1 virus was first reported in 1996 in mainland China (Chen *et al.*, 2004) and later spread to live poultry markets in Hong Kong (Sims *et al.*, 2005). In early 2004, AI H5N1 virus outbreaks were reported in several Asian countries including Thailand. Since then, the viruses have spread to many areas around the world, causing major economic loss in poultry industries (OIE, 2008). Moreover, the impact from AI H5N1

virus outbreaks is not limited to poultry species as the virus also infects other animals and humans. As of September 2009, there are more than 442 human cases with 262 fatal cases. This leads to a serious concern regarding the pandemic potential of the AI H5N1 virus (WHO, 2009).

The influenza A virus genome comprises of eight segmented single-stranded negative sense RNA (Engelhardt & Fodor, 2006). They can be further sub-typed based on the two major antigens: haemagglutinin (HA) and neuraminidase (NA). To date, 16 subtypes of HA (H1–16) and 9 of NA (N1–9) have been identified (Fouchier *et al.*, 2005). In general, viruses of all HA and NA subtypes are found in aquatic birds. Among the 16 HA subtypes, only H5 and H7 are virulent in poultry (Alexander, 2000). By contrast, only three HA subtypes (H1, H2 and H3) and two NA subtypes (N1 and N2) have

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commonly been found in humans. However, over the past few years, several subtypes of AI viruses including H5N1, H7N7, H7N3 and H9N2 have directly crossed the species barrier to infect humans (Mahy & Van Regenmortel, 2008).

House flies, a synanthropic species, are commonly found associated with humans and the animals they raise (Greenberg, 1973). Importantly, house flies can be mechanical and/or biological vectors for a number of major pathogens in the animal production system such as *Aeromonas caviae* (Nayduch *et al.*, 2002), *Shigella* spp. (Greenberg, 1973), *Salmonella* spp. (Holt *et al.*, 2007), *Escherichia coli* (De Jesus *et al.*, 2004), *Vibrio cholerae* (Yap *et al.*, 2008), Newcastle disease virus (NDV) (Watson *et al.*, 2007), turkey coronavirus (Calibeo-Hayes *et al.*, 2003), porcine reproductive and respiratory syndrome virus (Otake *et al.*, 2003), rotavirus (Tan *et al.*, 1997), *Microsporum canis* (Cafarchia *et al.*, 2009) and metazoan parasites (Förster *et al.*, 2007). House flies transmit pathogens via mouthparts, vomit droplets, faeces and their body surface (Greenberg, 1973). Their ability to disperse contaminated pathogens correlates with the flying distance which was shown to be approximately 1–3 km per day (Herms *et al.*, 1969). Accordingly, house flies are considered as an important vector for spreading disease in the poultry production system. The objective of the present study was to determine the potential of the house fly as a vector of the AI H5N1 virus.

Materials and methods

Virus

The highly pathogenic avian influenza (HPAI) A/Chicken/Nakorn-Pathom/Thailand/CU-K2/04 (H5N1) virus used in the present study was propagated in the allantoic cavity of 10-day-old embryonated chicken eggs using standard protocol (OIE, 2000). Allantoic fluid of the third passage of viral inoculated embryonated chicken eggs was used in this experiment. Virus titre was determined $10^{9.5}$ 50% of embryonated lethal dose/millilitre (ELD₅₀/mL) and the virus was stored at -80°C until used.

House flies

House flies were obtained as larvae from a layer chicken farm in Thailand ($5.4\text{--}20.6^{\circ}\text{N}$ and $97.1\text{--}106.0^{\circ}\text{E}$). The fly larvae were kept in a sand box containing larval medium which comprised of swine livers at room temperature until pupation. The pupae were harvested and stored in a plastic box at room temperature until the emergence of adults. The emergence of adult flies represent day 0. Before initiation of all experiments the adult stage flies were randomly selected and confirmed to be free of the AI H5N1 virus by the real-time reverse transcription-polymerase chain reaction (RRT-PCR) assay using specific primers and probes to the influenza A matrix (M) gene. The 2-day-old house flies were separated for use in the following experiments.

Experimental design

Three experiments were conducted to determine the potential of house flies to harbour the AI H5N1 virus. In all experiments, 2-day-old flies of mixed sex were starved for 12 h before use.

Experiment 1. To evaluate the potential of house flies to harbour the AI H5N1 virus, 100 house flies were separated into two groups ($n = 50$); control and treatment. The treatment group was fed with a virus mixture (1 mL of allantoic fluid containing the AI H5N1 virus with 1 mL of UHT (ultra high temperature) processed milk mixed with 10% sucrose solution). The final virus titre in the mixture was $1 \times 10^{9.2}$ ELD₅₀/mL. The mixture was placed on a cotton pad on top of the box of the treatment group. The control group was fed with the same amount of UHT milk mixed with 10% sucrose solution without the virus. House flies were allowed 15 min of feeding time and all flies were then euthanized with dry ice for 15 min. Each group was washed with 10 mL of brain–heart infusion (BHI) broth (Difco, BD Biosciences, Sparks, MD) by vortexing for 5 s. The BHI washing fluid (W1) was inoculated into six 10-day-old embryonated chicken eggs to evaluate the presence of the AI H5N1 virus on the external surface of the house flies. To assess the AI H5N1 virus within the internal viscera of house flies, the previously washed flies were disinfected by immersing into 10 mL of 70% ethanol, vortexed for 5 s and rinsed with 10 mL of BHI washing fluid (W2). The W2 washing fluid was inoculated into six 10-day-old embryonated chicken eggs to confirm the absence of the AI H5N1 virus on the external surface of house flies. The washed house flies were then homogenized with 900 μL of sterile BHI using sterile plastic pestles and sterile microcentrifuge tubes (Eppendorf South Asia, Malaysia) and centrifuged at 600 g for 15 min at 4°C . The supernatants were collected and gentamicin was added to a final concentration of 200 $\mu\text{g}/\text{mL}$ and incubated at 4°C for 40 min. Subsequently, virus isolation was performed by inoculating 100 μL of the supernatant into six 10-day-old embryonated chicken eggs. The embryos were observed twice a day for 5 days post-inoculation. The embryos that died within 24 h were discarded. Allantoic fluid was harvested from eggs and AI H5N1 virus identification was performed using the HA test and RT-PCR assay.

Experiment 2. This experiment was done to determine if one fly could harbor the virus. Experiments were performed in five replicates. Only one house fly was transferred into a plastic box and exposed to the mixture containing the AI H5N1 virus or without in a sterile cotton pad for 15 min as previously described in experiment 1. Subsequently, the exposed house fly was washed with 1 mL of BHI as W1 washing fluid. The W1 washing fluid was titrated by inoculation into a 10-day-old embryonated chicken and the W1 washing fluid titre was compared with the virus titre in fly homogenate. The steps for virus isolation and identification were performed as previously described in experiment 1.

Experiment 3. This experiment aimed to evaluate the correlation between the time period after exposure and AI H5N1 virus viability in AI H5N1 exposed house flies. The house flies were separated into two groups; each group was exposed to the mixture containing the AI H5N1 virus or without as previously described. After exposure to the virus, standard food (UHT milk mixed with 10% sucrose) was provided for all groups throughout experiment and five AI H5N1 exposed house flies from each group were examined for the presence of the AI H5N1 virus at 0, 6, 12, 24, 36, 48, 72 and 96 h post-exposure. The AI H5N1-exposed flies collected from each time period were washed three times and homogenized as previously described, to determine the presence of AI H5N1 viruses.

Virus isolation and titration

The AI H5N1 virus was isolated by inoculating six 10-day-old embryonated chicken eggs as previously described (OIE, 2000). Briefly, 100 µL of 10-fold, serially diluted supernatant of house fly homogenates was inoculated into the allantoic cavity of six 10-day-old embryonated chicken eggs. Embryos were examined twice a day for 5 days and allantoic fluid was harvested at 5 days post-inoculation or upon embryo death. The HA test and the RT-PCR assay were performed to determine the presence of the virus. Virus titre was determined according to the method of Reed & Muench (1938).

Haemagglutination (HA) test

Allantoic fluid was harvested from each embryonated chicken egg and the HA test was performed using a standard protocol (OIE, 2000). Briefly, 25 µL of phosphate-buffered saline (PBS) was added into a 96-well, V-shaped bottom microtitre plate followed by the addition of 25 µL of the samples. The solution was two-fold serially diluted with PBS. Twenty-five microlitres of PBS was added into all wells followed by the addition of 25 µL of 1% chicken red blood cells (RBCs) in PBS. The microtitre plate was gently tapped and RBCs were allowed to settle for 40 min at room temperature. The AI H5N1 virus was also included as positive controls. Samples with the HA titre ≥ 4 were considered positive for the AI H5N1 virus.

RNA extraction

RNA was extracted from the supernatant of house fly homogenates or allantoic fluid using the QIAamp Viral RNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Briefly, 140 µL of sample was mixed with 560 µL of Buffer AVL containing carrier RNA, vortexed for 15 s, and incubated at room temperature for 10 min. Subsequently, 560 µL of absolute alcohol was added to the sample. The solution was transferred to QIAamp Mini column and centrifuged at 11 000 g for 1 min. Columns were washed twice with sterile buffer. RNA was eluted with 50 µL

of nuclease-free water. The amount of 3 and 4 µL of the RNA template was used for analysis by RT-PCR and RRT-PCR assays, respectively.

Reverse transcription-polymerase chain reaction assay (RT-PCR)

The presence of the HPAI H5N1 virus in the allantoic fluids was determined using the RT-PCR assay, as previously described (Payungporn *et al.*, 2006), using the M-specific primer set, MF 5'-TGATCTTCTTGAAAATTTGCAG-3' and MR 5'-TGTTGACAAAATGACCATCG-3'. RT-PCR reactions were performed using the AccessQuick™ RT-PCR system (Promega, Madison, WI, USA) according to the manufacturer's protocol. RT-PCR condition consisted of a reverse transcription step for 15 min at 48 °C, an initial denaturation step for 2 min at 95 °C, an amplification step for 40 cycles (30 s at 94 °C, 30 s at 55 °C and 30 s at 72 °C) and a final extension step for 10 min at 72 °C. The PCR product was analysed by agarose gel electrophoresis. The expected PCR product size of the M gene was 276 bp. The allantoic fluid from non-exposed AI H5N1 house flies inoculated embryonated chicken eggs and the allantoic fluid of AI H5N1 stock virus were used as the negative and positive controls of this assay, respectively.

Real-time RT-PCR (RRT-PCR) assay

The RRT-PCR assay for M gene detection, modified from Spackman *et al.* (2002), was performed using the SuperScript III Platinum One-step RT-PCR system (Invitrogen, Carlsbad, CA, USA). The forward primer MF25 (5'-AGATGA GTCTTCTAACCGAGGTCG-3'), reverse primer MR124 (5'-TGCAAAAACATCTTCAAGTCTCTG-3') and probe M64 (FAM-TCAGGCCCTCAAAGCCGA-TAMRA) were used in the present study (Spackman *et al.*, 2002). Final concentrations of primers and probe were 10 and 2.5 µM, respectively. The reaction consisted of 4 µL of the RNA sample, 7.5 µL of 2× Reaction Mix, 0.3 µL of SuperScript III RT Platinum® Taq Mix, 50 mM of MgSO₄ and RNase-free water in a final volume of 17 µL. One-step RRT-PCR was performed using Rotor-Gene 3000 (Corbett Research, New South Wales, Australia). Cycling conditions included a reverse transcription step at 50 °C 30 min, initial denaturation step at 94 °C 15 min followed by 40 cycles of amplification (95 °C for 10 s, 54 °C for 30 s and 72 °C for 10 s) (Spackman *et al.*, 2002). The fluorescence data were collected at the end of each annealing step and the data analyses of the RRT-PCR assay was performed using the Rotor-Gene analysis software, Version 6.0 (Corbett research supporting program, Corbett Research, Sydney, Australia). The negative and positive controls of this assay were similar to the RT-PCR assay.

Statistical analysis

Student's paired *t*-test was used in experiment 2 to compare the virus titre between a house fly homogenate and W1 washing fluid. In experiment 3, ANOVA with a Tukey–Kramer

multiple comparison test was used to determine the correlation among the repeated measures. A *P*-value was considered as a significant difference (*P* < 0.05) for all tests. In addition, the linear regression analysis was used to evaluate the correlation between virus titre and the time of post-exposure.

Results

Experiment 1

Embryonated chicken eggs inoculated with W1 washing fluid from treatment groups and house fly homogenates from the treatment group died within 2 days post-inoculation. Allantoic fluid from the treatment and W1 washing fluid were positive for the AI H5N1 virus using the HA test and RT-PCR assay. All embryonated chicken eggs inoculated with the W2 washing fluid (after surface sterilization) and negative control group remained healthy with no evidence of AI H5N1 virus infection.

Experiment 2

The AI H5N1 virus was detected in a single house fly in all replicates. Thirty embryonated chicken eggs from all replicates of treatment groups died 2 days post-inoculation. The average virus titre of the house fly homogenate was 5.43 ± 0.33 log ELD₅₀/mL, which was significantly (*P* < 0.05) higher than that of 1 mL of W1 washing fluid (external surface, 4.28 ± 0.55 log ELD₅₀/mL). The virus titres between a house fly homogenate and W1 at different times decreased after 48 and 24 h, respectively (Table 1). The HA test and RT-PCR results were also positive in all groups and in all replicates. In the negative control group, all embryonated chicken eggs were alive and the RT-PCR results were negative.

Experiment 3

The results of the virus isolation revealed that the H5N1 virus could survive in house flies and remained infective up to 72 h post-exposure (Fig. 1). Whereas, the results of the RRT-PCR assay were positive up to 96 h post-exposure (Table 2), indicating that all house flies carried the AI H5N1 virus but

Table 1. Avian influenza (AI) H5N1 virus titre between house fly homogenates and W1 of the AI H5N1 virus exposed house flies at different times of post-exposure in embryonated chicken eggs.

Time post exposure (h)	AI H5N1 virus titre (log ELD ₅₀ /mL)	
	House fly homogenates	W1 washing fluid
0	6.31	4.70
24	6.15	1.90
48	1.87	0
96	1.70	0
Negative	0	0

Fig. 1. Mean of the H5N1 virus titre (log ELD₅₀/mL) detected from five house flies at different times of post-exposure.

Table 2. AI H5N1 virus titre and RRT-PCR assays detected from five AI H5N1 virus exposed house fly homogenates at different times of post-exposure.

Time post exposure (h)	AI H5N1 virus titre (log ELD ₅₀ /mL)	RRT-PCR assay (log copies/μL)
0	6.21 ^{a,b}	9.62
6	5.38 ^b	9.26
12	5.06 ^b	8.74
24	4.76 ^b	8.76
36	4.60	8.19
48	2.35 ^c	7.9
72	2.02 ^c	7.4
96	0.75 ^c	7.7
Negative	0	0

The different superscript letters in the same column means significant difference (*P* < 0.05).

the virus could not replicate in the house flies and the results from three replicates were not statistically significantly different (*P* > 0.05). The AI H5N1 virus was not detected from the sham-inoculated negative control group by both virus titration and RRT-PCR assay.

Discussion

The highly pathogenic avian influenza virus subtype H5N1 causes serious problems not only in poultry industries but also poses a threat to human health. Generally, the poultry production system in Thailand can be divided into four sectors namely, the industrial integrated system, the semi-vertical integrated system, extensive farming and backyard. In extensive farming (e.g. layer hens, broilers, ducks and free-grazing ducks) and backyards (e.g. chickens, ducks and fighting cocks) they live in close proximity with their owners and are freely in contact with the other animals (Tiensin *et al.*, 2007). Then, the manure of this poultry unit was a perfect breeding place for house flies. Moreover, Thailand's climate is appropriate for the development of house flies. Previous studies showed

that house flies could act as a vector for transmitting disease to poultry farms and flies readily move between poultry buildings (Lysyk & Axtell, 1986). Therefore, the potential of house flies to act as a vector for the AI H5N1 virus was determined in the present study. Here we demonstrated that house flies that consumed food contaminated with AI H5N1 could carry the virus within their bodies for a long period of time at least 72 h post-exposure. The virus was detected both in the homogenates of whole flies and the external surfaces of flies at high levels. Moreover, virus titres of a whole fly homogenate compared with that of washing fluid revealed that the viruses could be detected in homogenates of whole flies for up to 96 h post-exposure, whereas these viruses could be detected in external surfaces of house flies for only up to 24 h post-exposure (Table 1). The capacity of a house fly to carry the AI H5N1 virus via whole fly homogenate was significantly higher than that of the external surface ($P < 0.05$). Our finding is consistent with Otake *et al.* (2003) that found viable porcine reproductive and respiratory syndrome virus (PRRSV) in the internal organs of house flies higher than the external surface. A separate study detected higher levels of Exotic Newcastle disease virus (ENDV) from the whole house fly homogenate than the level of virus from the body surface (Chakrabarti *et al.*, 2008).

In general, the avian influenza virus can survive in an environment for a period of time (Swayne & Halvorson, 2003). Optimum humidity, temperature and organic materials including nasal discharge and faeces can prolong the survival time of many viruses (Pantin-Jackwood & Swayne, 2009). The AI H5N2 virus can survive in faeces at 4 °C for 30–35 days and at 20 °C for 7 days (Swayne & Halvorson, 2003). In contrast, the AI H5N1 virus can survive at 25 °C for 24 h, and at 40 °C for 15 min (Chumpolbanchorn *et al.*, 2006). Jeong *et al.* (2009) found that experimental chickens inoculated with $10^{6.5}$ EID₅₀ of A/Chicken /Korea/IS/06 (H5N1) can shed a virus from oropharyngeal and cloaca for 3 days at virus titres of $10^{4.6}$ and $10^{2.4}$ TCID₅₀/mL, respectively. Other poultry species infected with H5N1 including ducks and quails can shed virus up to 4 and 6 days, respectively at titres of oropharyngeal swabs is $10^{3.2}$ and $10^{6.0}$ TCID₅₀/mL, respectively. Pantin-Jackwood & Swayne (2009) demonstrated that Pekin ducks intranasally inoculated with the AI H5N1 virus could shed virus from oral and cloaca at titres of $10^{6.5}$ and $10^{3.3}$ EID₅₀, respectively. Interestingly, the results of the present study showed that an individual house fly can uptake the virus in approximately $10^{5.43}$ ELD₅₀/mL out of $1 \times 10^{9.2}$ ELD₅₀/mL of infectious mixture or approximately 0.02% of the total virus when it was kept at room temperature. Thus, our data suggest that house flies can carry a similar amount of the AI H5N1 virus to the amount of virus shed from H5N1 experimentally infected avian species such as chickens, quails and ducks. However, transmission studies should be performed. In addition, an individual house fly can fly up to 11.8 km within 24 h (Greenberg, 1973). Therefore, house flies may serve as potential mechanical vectors which spread the AI H5N1 virus to poultry farms in the nearby area similar to blow flies (*Calliphora nigribarbis*) that was shown to be mechanical vectors for AIV in Japan (Sawabe *et al.*, 2009).

The results of experiment 3 revealed that the AI H5N1 virus could be detected within the fed house flies for up to 72 h post-exposure. Otake *et al.* (2003) showed that the PRRSV could survive within the intestinal viscera of house flies for up to 12 h post-exposure. Moreover, Watson *et al.* (2007) found that the NDV remained viable in the crop and gut tissues of the house flies for up to 96 h post-exposure. It should be noted that different results of viable virus remaining in the digestive tract depend on the dimension of anatomy, the type of virus and the route of transmission (Förster *et al.*, 2007). However, the real-time RT-PCR results showed that the viral RNA can be found in all time points of this experiment. In addition, the virus infection titre continuously decreased according to the increasing exposure time. This study found that the AI H5N1 virus could not replicate within the house flies, a mechanical vector rather than a biological vector.

In conclusion, the present study demonstrated that the house flies could carry the H5N1 virus, which remained infective for at least 72 h. The results from the present study indicated that house flies may act as a potential mechanical vector for spreading the AI H5N1 virus. As a consequence, improving biosecurity including fly management control should also be considered for the effective control of the AI H5N1 virus in epidemic areas. However, the actual ability of infected house flies in disseminating the AI H5N1 virus to the chicken has not been determined in this study. Therefore, we suggest that the experimental infection with AI H5N1 virus exposed house flies in chickens should be further performed.

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