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THE ROLE OF HOUSE FLIES (*Musca domestica*) IN TRANSMISSION OF AVIAN INFLUENZA VIRUS SUBTYPE H5N1

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สุวรักษ์ วรรณรัตน์ : บทบาทของแมลงวันบ้านต่อการนำเชื้อไวรัสไข้หวัดนก สายพันธุ์ เอช 5 เอ็น 1. (THE ROLE OF HOUSE FLIES (*MUSCA DOMESTICA*) IN TRANSMISSION OF AVIAN INFLUENZA VIRUS SUBTYPE H5N1) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : รศ.น.สพ.ดร.สมศักดิ์ ภัคภิญโญ , อ. ที่ปรึกษาวิทยานิพนธ์ร่วม : ศ.น.สพ.ดร.จิโรจ ศศิปรียจันทร์, ศ.ดร.สกล พันธุ์ยิ้ม, 78 หน้า.

วัตถุประสงค์ของการศึกษาในครั้งนี้เพื่อทดสอบความสามารถของแมลงวันบ้านต่อการนำเชื้อไวรัสไข้หวัดนก สายพันธุ์เอชห้าเอ็นหนึ่ง โดยในการศึกษาได้แบ่งการทดลองออกเป็นสาม ตอน คือ ตอนที่หนึ่งเพื่อศึกษาความสามารถของแมลงวันบ้านต่อการนำเชื้อไวรัสไข้หวัดนก โดยนำแมลงวันบ้านมาสัมผัสกับเชื้อไวรัสไข้หวัดนก เมื่อครบระยะเวลาที่กำหนดทำความสะอาดบริเวณพื้นผิวภายนอกลำตัวของแมลงวัน จากนั้นทำการฆ่าแมลงวัน และนำมาบดร่วมกับ brain heart infusion broth (BHI) เพื่อให้อยู่ในรูปของเหลวและฉีดของเหลวนี้ลงในไข่ไก่ฟัก อายุ 10 วันจำนวน 6 ฟอง สังเกตไข่ไก่ฟักทุกวัน เป็นระยะเวลา5 วัน ทำการเก็บน้ำไข่ฟักเพื่อไปตรวจหาเชื้อไวรัสไข้หวัดนกโดยวิธี HA test, RT-PCR assay ต่อไป จากนั้นทำการทดสอบเพื่อดูปริมาณของแมลงวันที่น้อยที่สุดที่สามารถนำเชื้อไวรัสไข้หวัดนกได้ โดยใช้แมลงวันจำนวนหนึ่งตัวมาทดสอบดูการนำเชื้อไวรัส และการทดลองส่วนสุดท้ายของตอนที่หนึ่งคือศึกษาระยะเวลาของเชื้อไวรัสไข้หวัดนกที่สามารถมีชีวิตอยู่ได้ในแมลงวัน โดยภายหลังจากที่แมลงวันบ้านสัมผัสกับเชื้อไวรัสแล้ว เก็บแมลงวันบ้านตามช่วงเวลาที่กำหนดนำมาฉีดลงในไข่ไก่ฟัก โดยผลการทดลองทั้งหมดของตอนที่หนึ่งพบว่าแมลงวันบ้านสามารถนำเชื้อไวรัสไข้หวัดนกได้และแมลงวันจำนวนน้อยที่สุดที่สามารถนำเชื้อไวรัสได้คือแมลงวันจำนวนหนึ่งตัว และเชื้อไวรัสสามารถมีชีวิตอยู่ภายในแมลงวันได้นานถึง 96 ชั่วโมง แต่ปริมาณของเชื้อไวรัสที่มีชีวิตลดลงแปรผันตามระยะเวลาที่เพิ่มมากขึ้น การทดลองตอนที่สองเพื่อดูความสามารถในการนำเชื้อไวรัสไข้หวัดนกในไก่ทดลองในการทดลองนี้ได้ใช้ไก่ไข่อายุ 32 วัน จำนวน 30 ตัวแบ่งไก่ทดลองออกเป็น 3 กลุ่ม กลุ่มละ 10 ตัว โดยนำของเหลวจากการ บดแมลงวันบ้านที่สัมผัสเชื้อไวรัสไข้หวัดนกป้อนปากไก่ทดลอง จากนั้นเก็บเชื้อไวรัสจากไก่ทดลองโดยการป้ายเชื้อจากท่อลมและทวารร่วมทุกวันเป็นระยะเวลา 14 วัน สังเกตอาการทางคลินิกร่วมกับอัตราการตายทุกวัน เมื่อครบระยะเวลาที่กำหนด เก็บชิ้นเนื้อเพื่อนำมาตรวจทางจุลพยาธิวิทยา ผลการทดลองในตอนนี้พบว่าของเหลวจากการบดตัวแมลงวันบ้านที่สัมผัสกับเชื้อไวรัสไข้หวัดนก สามารถทำให้ไก่ทดลองติดเชื้อไวรัสแสดงอาการป่วยและตายได้ การทดลองตอนที่สามเพื่อตรวจหาเชื้อไวรัสไข้หวัดนกในแมลงวันบริเวณพื้นที่ระบาดในประเทศไทยระหว่างปี พ.ศ. 2551 ผลการศึกษาคือไม่สามารถตรวจพบเชื้อไวรัสไข้หวัดนกในแมลงวันที่จับมาจากบริเวณพื้นที่ที่มีการระบาด อย่างไรก็ตามผลจากการศึกษาในครั้งนี้พบว่าแมลงวันบ้านเป็นพาหะเชิงกลของเชื้อไวรัสไข้หวัดนก และภายใต้การทดลองของเหลวจากการบดแมลงวันบ้านที่ติดเชื้อไวรัสไข้หวัดนกสามารถก่อให้เกิดโรคในไก่ทดลองได้

ภาควิชา....อายุรศาสตร์..... ลายมือชื่อนิสิต.....

สาขาวิชา...อายุรศาสตร์สัตวแพทย์.... ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

ปีการศึกษา..2554..... ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

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KEYWORDS : AVIAN INFLUENZA VIRUS / HOUSE FLIES / MECHANICAL VECTOR /

SUWARAK WANNARATANA : THE ROLE OF HOUSE FLIES (*MUSCA DOMESTICA*) IN TRANSMISSION OF AVIAN INFLUENZA VIRUS SUBTYPE H5N1. ADVISOR : ASSOC. PROF. SOMSAK PAKPINYO, Ph.D., CO-ADVISOR : PROF. JIROJ SASIPREEYAJAN, Ph.D., PROF. SAKOL PANYIM, Ph.D., 78 pp.

The objective of this study was to determine the potential role of the house flies in harboring and transmission avian influenza virus H5N1 (AIVs). To achieve this goal, this study was divided into three parts. The purpose of the first part was to determine the potential of the house flies as a vector of AIVs. In this part, the laboratory reared flies were experimentally fed with mixture containing the AIVs and then the external body surface of exposed flies was washed and sterile prior to preparation of homogenate whole fly between whole fly and brain heart infusion broth (BHI). The homogenate was inoculated into 10-day-old embryonated chicken eggs. Allantoic fluids were collected for determining virus concentration by HA test, RT-PCR assay. Moreover, the minimum of the house flies carrying AIVs was examined by inoculating one house fly homogenate into 10-day-old embryonated chicken eggs. In addition, the duration time of AIVs survival in house flies was determined by collecting exposed flies at different times of post exposure. The results in this part revealed that the house flies could carry the AIVs, one house fly was sufficient to carry and able to kill embryonated chicken eggs and the AIVs could survive within house flies for up to 96 hr. However, the virus titers were decreased over time. The second part that was the competence of the house flies as a transmission vector of AIVs in chickens under laboratory condition was determined. Three groups of 32-day-old chickens were individually inoculated AIVs infected house fly homogenate via oral route except the chickens in group 1 were inoculated with BHI, serving as a negative control group. Oropharyngeal and cloacal swabs were collected. Clinical signs and mortality were observed for 14 days post-inoculation (DPI). The experiment was terminated on 14 DPI and the tissues samples were collected for histopathological and immunohistochemistry evaluations. The results of this part indicated that AIVs infected house fly homogenate could induce the clinical signs and mortalities in inoculated chickens. Finally, the objective of last part was to examine the presence of the AIVs naturally infected house flies in epidemic area in Thailand during 2008. The result of this part revealed that none of AIVs naturally infected house flies was found. Nevertheless, all of the results in this study were conceivable illustrated to suggest that the house flies served as a mechanical vector of the AIVs and could transfer AIVs to chickens under experimental condition.

Department : Veterinary Medicine Student's Signature .....

Field of Study : Veterinary Medicine Advisor's Signature .....

Academic Year : 2011 Co-advisor's Signature .....

Co-advisor's Signature .....

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CHAPTER I
INTRODUCTION

Avian influenza or bird flu caused by influenza viruses type A is a highly contagious respiratory disease in birds. This virus has been isolated from wild bird species, especially from waterfowl, throughout the world (Forrest and Webster, 2010). Influenza virus belongs to member of Orthomyxoviridae family which is an enveloped RNA virus with a negative stranded, segmented genome. At present, there are five influenza virus genera within this family; *Influenzavirus A*, *Influenzavirus B*, *Influenzavirus C*, *Thogotovirus* and *Isavirus*. Virus particles demonstrated pleomorphic morphology to a spherical form with 80 to 120 nm in diameter (Bourmakina and García-Sastre, 2003). Influenza A virus consists of 8 gene segments which encode at least 11 proteins consisting of hemagglutinin (HA), neuraminidase (NA), matrix proteins (M1 and M2), nonstructural proteins (NS1 and NS2), neucleocapsid protein (NP) and polymerase proteins (PB1, PB1-F2, PB2 and PA). Influenza A viruses are categorized into subtypes based on the antigenic surface proteins. At present, 16 HA subtypes (H1 – H16) and 9 NA subtypes (N1 – N9) have been identified (Fouchier et al., 2005; Olsen et al., 2006) and all influenza A subtypes have been isolated from avian species (Alexander, 2007). Moreover, influenza A virus is able to infect several mammalian species such as swine, ferrets, whales, horses, dogs, and humans (Kalthoff et al., 2010). Influenza A infected animals showed various disease signs ranging from mild respiratory signs to high mortality rate depended on many factors including species susceptibility, infectious dose, and type of influenza pathogens (Gibbs, 2010, Forrest et al. 2010, Kalthoff et al., 2010).

In late 2002 to January 2003, the avian influenza virus subtype H5N1 was outbreaks in Hong Kong (Ellis et al., 2004) and first case report that this virus caused in human infections with fatality (Peiris et al., 2004). From late 2003 until 2004, the avian influenza subtype H5N1 outbreak caused devastating across Asia including in Vietnam, Thailand, Indonesia, Cambodia, Laos, Malaysia, China, Korea and Japan (Li et al., 2004; Peiris et al., 2007) and then throughout to the Siberia, Mongolia, Kazakhstan, Romania,

Croatia and Turkey, Eastern Europe, the Middle-Eastern and several Africa (Alexander, 2007).

House flies, *Musca domestica*, belonging to the order *Diptera*, are commonly associated with humans and animal husbandry (Harwood et al., 1979) by causing irritation and annoying to farmers and animals. Moreover, the house flies are considered to play an important role in the mechanical and/or biological vectors for many pathogens such as Shigella spp. (Greenberg, 1973), Salmonella spp. (Holt et al., 2007), *Escherichia coli* (De Jesús et al., 2004), *Vibrio cholerae* (Yap et al., 2008), Newcastle disease virus (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) and metazoan parasites (Förster et al., 2007) occurring through by fecal deposition, via mouthpart, vomit droplets and exoskeleton body surface (Harwood et al., 1979). Furthermore, their ability to disperse contaminated pathogen correlated to the flying distance which was shown to be approximately 1 to 3 km per day (Herms, 1969) and the relative proportion of the flight range is restricted by the landscape and the food supply (Chakrabari et al., 2007). Accordingly, house flies are considered as a very important vector for spreading disease in the poultry production system (Gerberich, 1952, Hald et al., 2004 and Hald et al., 2008). In general, the poultry manure from poultry operation is an excellent environmental medium for maintaining and developing of house flies by which the small pathogen particles attach readily to the external body surface or ingest into visceral organ; particular crop and then this pathogen was disseminate to the neighborhood farms (Graczyk et al., 1999). Therefore, the improvement of environmental sanitation, hygiene and biosecurity regulation by reducing or eliminating fly breeding sites is the easiest method for controlling the house fly population and reduces the risk of infection by contamination from the house flies.

Objectives

1. To determine the potential role of the house flies as a vector of avian influenza virus subtype H5N1.

2. To evaluate the minimum number of the house flies as an necessary to transmission of avian influenza virus subtype H5N1

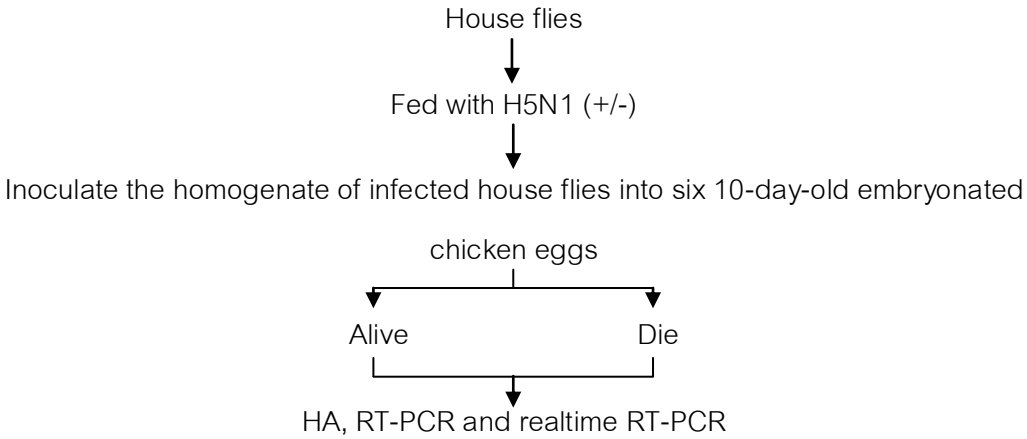
3. To determine the holding time of the avian influenza virus subtype H5N1 in house flies under experimental conditions.

4. To determine the competence of the house flies as a transmission vector of avian influenza virus subtype H5N1 in chickens under experimental condition.

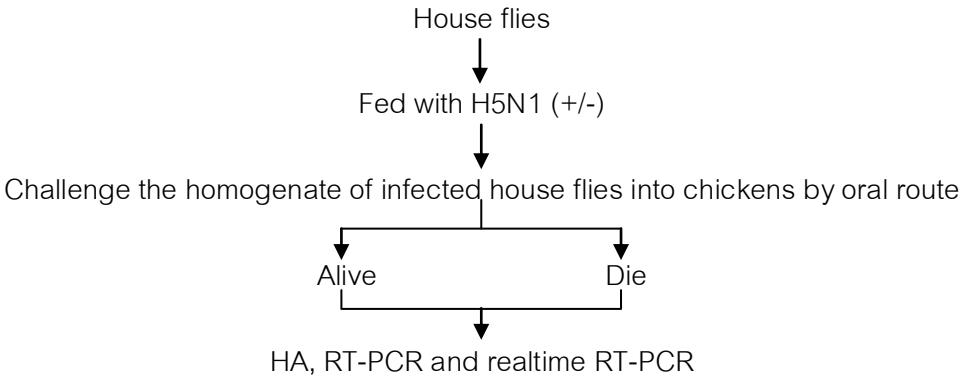
5. To examine for the presence of the avian influenza virus subtype H5N1 naturally infected house flies in epidemic area in Thailand during 2008-2009.

Conceptual framework

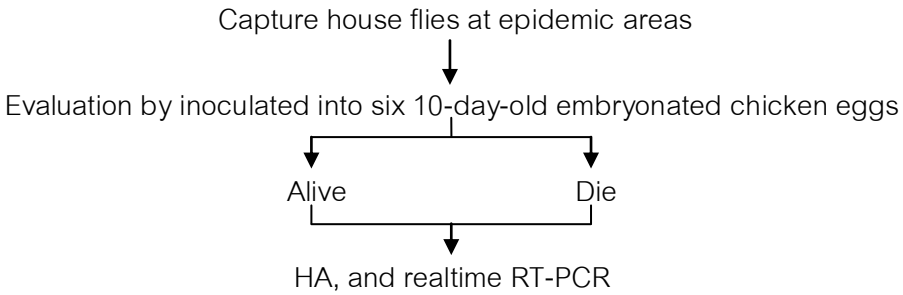
Part 1: To determine the potential of the house flies as a vector of avian influenza virus subtype H5N1 in experimental condition.



Part 2: To determine the competence of the house flies as a transmission vector of avian influenza virus subtype H5N1 in chickens under experimental condition.



Part 3: To examine for the presence of the avian influenza virus subtype H5N1 naturally infected house flies in epidemic area in Thailand during 2008-2009.



Expected values

1. To gain the knowledge of the role of house flies as a vector of avian influenza virus subtype H5N1.

2. To clearly understand the importance of the house flies serving as a mechanical vector of avian influenza virus subtype H5N1.

3. To gain the knowledge of the influence factor causing an outbreak and spread of avian influenza virus subtype H5N1.

4. To improve the efficiency of the biosecurity versus avian influenza virus subtype H5N1.

CHAPTER II

LITERATURE REVIEW

Avian influenza (AI) or bird flu is highly contagious disease, which causes serious respiratory problems and systemic disease in chickens and occasionally transmits to other host species such as pigs, horses, birds including humans (Suarez, 2000). In 1878, this disease was first reported by Perroncito in term as "fowl plaque" that affected chickens in Italy (Swayne and Harvolson, 2003). Until 1955, Schafer was demonstrated that the virus was belonging to member of influenza A virus (Schafer, 1955). Avian influenza virus has been isolated from at least 105 wild bird species especially in order *Anseriformes* (ducks, geese and swans) and *Charadriiformes* (gulls and shorebirds) (Olsen et al., 2006), which were known as the main natural reservoir of this virus. All influenza A virus subtypes have been isolated from wild birds, representing 16 HA (H1-H16) and 9 NA (N1-N9) (Fouchier et al., 2005). Hence, these wild birds play a major role in dissemination of this virus across the world, without developing symptoms (Ligon, 2005). Conversely, avian influenza virus causes the lethal disease in domestic chickens and turkeys that have impact to the poultry industries (Suarez, 2000). Over 100 years, avian influenza viruses have been limited spread and no influenza A cases were reported in human. Until 1997, avian influenza virus highly pathogenic subtype H5N1 was identified in Hong Kong from 18 infected people, resulting 6 people died (Claas et al., 1998). Moreover, in the early 21th century, the current outbreaks of a new subtype A (H5N1) were rapid spread in large-scale across the world; Asia, Europe, Africa and the Middle East (Loeffelholz, 2010). On 23rd January, 2004, the H5N1 was first reported in Thailand from layer chicken farm on Suphanburi province. During this time, the outbreak cases were reported more than 149 reported cases in 144 villages throughout the country, more than 62 million birds were destroyed (Capua and Alexander, 2004) and more than 400,000 workers were unemployed (Tiensin et al., 2005). Consequently, the government spent money about 5.3 billion Thai Baht for compensation to affecting farmers and the impact of this effect to the gross domestic product (GDP) was declines approximately 0.5% in this year (Tiensin et al., 2005).

Moreover, the H5N1 outbreaks have been continuing reported until year 2008. However, the H5N1 virus has been reported in several countries, in particular countries nearby Thailand such as Cambodia and Myanmar (OIE, 2011). In addition to causing problem in poultry industry, H5N1 virus poses to public health problems. Total number of the H5N1 infected people is 520 people and 320 people were death, which was reported by World Health Organization (WHO) (WHO, 2011).

Influenza A virus

Structure

Influenza virus belongs to member of Orthomyxoviridae family, which is divided into five genera; *Influenzavirus A*, *Influenzavirus B*, *Influenzavirus C*, classified based on the antigenic difference between their nucleoprotein (NP) and matrix (M) proteins, *Thogotovirus* and *Isavirus*. However, only influenza A virus can infect all avian species including chickens, ducks, wild birds and aquatic birds. Influenza A virus is enveloped, spherical to pleomorphic or elongation shape range in diameter 80-120 nm (Bourmakina). This virus possesses eight segments of negative sense single-stranded RNA viruses, which encodes 9 internal proteins; nucleoprotein (NP), two matrix proteins (M1 and M2), three polymerase protein (PB1, PB2 and PA) and three non-structural protein (NS1, NEP/NS2, PB1-F2), and two external glycoproteins; haemaglutinin (HA) and neuraminidase (NA) (Lamp, 2008). Likewise, the outside of this virus is enveloped, which consists of lipid bilayer comprising three transmembrane proteins (HA, NA and M2). HA and NA proteins are forming the surface spikes, which associate with virus entry and release of the virus particle, respectively. The M2 protein is proton-selective ion channel. The M1 protein lying beneath the membrane forms a shell surrounding the helical viral ribonucleoprotein (vRNP) and associates with nuclear export protein (NEP/NS2). The core of this virus particle comprises helical vRNP consisting of eight single-stranded negative viral RNA (vRNA) associated with NP and three polymerase proteins (PA, PB1 and PB2) (Fig 1) (Palese and Shaw, 2007). The function of each viral protein is indicated in Table 1.

Table 1. The function of viral proteins (modified from Lamb and Krung, 2001)

Segment	Nucleotide length (base pairs)	Encoded polypeptide	Nascent polypeptide length (amino acid)	Functions
1	2,341	PB2	759	Cap recognition of cell RNA, component of RNA polymerase
2	2,341	PB1	757	Endonuclease activity, component of RNA polymerase
		PB1-F2	87	Pro-apoptotic activity
3	2,233	PA	716	Component of RNA transcriptase and replication complex
4	1,778	HA	550	Surface glycoprotein, receptor binding, fusion activity, major antigen
5	1,565	NP	498	RNA binding, RNA synthesis, RNA nuclear import
6	1,413	NA	454	Surface glycoprotein, receptor destroying activity
7	1,027	M1	252	Matrix protein, interacts with vRNP, nuclear export, budding
		M2	97	Membrane protein, ion channel
8	890	NS1	230	Multifunction protein, viral IFN antagonist
		NEP/NS2	121	Nuclear export of vRNPs

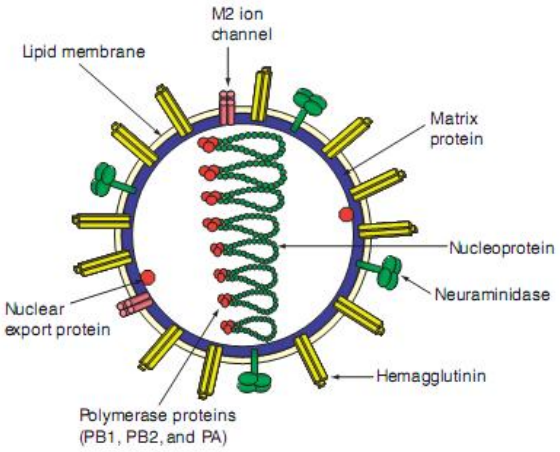


Figure 1. The structure of influenza A virus (copy from Shaw and Palese, 2010)

Virus antigenicity

HA protein

HA protein encoded by RNA segment 4, is a major surface antigen of influenza A virus. It is a trimeric rod shaped spike molecule comprising the hydrophobic carboxy terminus which embedded in the viral membrane. Another site is hydrophilic end which projected away from viral envelope. This site comprises the antigenic domain and the receptor binding site. Initially, the HA protein is synthesized in the endoplasmic retuiculum (ER) of the infected host cell as single polypeptide chain (HA0 or uncleaved HA). Subsequently, the HA0 is cleaved into two portions HA1 and HA2 by a serine protease enzyme and becoming an active form. In addition, the major function of HA is receptor-binding site (sialic acid or N-acetyl-neuraminic acid), which induces penetration of virus particle by acid pH-triggered fusion and presents major of antigenic epitopes molecule as inducing a neutralizing antibodies response (Shaw and Palese, 2010).

NA protein

NA protein, encoded by RNA segment 6, is the second major surface antigen. It consists of homotetramer head domain with a circular four-fold symmetry spike molecule containing the sialidase active enzyme and a stalk domain that is anchored in lipid envelope. The predominant function of sialidase enzyme is releasing of virus from the

infected cells and clearing around the sialic acid receptor by removing sialic acid residues from the virus envelope and cell surface leading to spread of virus particles (Lamb, 2008).

Influenza A viruses are categorized into subtypes based upon the antigenic differences of HA and NA antigens. At present, 16 HA subtypes (H1-H16) and 9 NA subtypes (N1-N9) have been identified (Fouchier et al., 2005; Olsen et al., 2006) and all of the virus subtypes were isolated from avian species. Moreover, these viruses can be classified into two pathotypes according to the pathogenesis in the domestic poultry and specific molecular genetics; low pathogenicity avian influenza (LPAI) and highly pathogenicity avian influenza (HPAI) (Forrest and Webster, 2010). The HPAI causes high mortality rate and possess multiple basic amino acids (arginine and lysine) at the HA cleavage site (Forrest and Webster, 2010). In contrast, the LPAI causes low mortality rate and the cleavage site have a single arginine and lysine. Up to now, most H5 or H7 subtypes have been shown to cause HPAI in susceptible species (Swayne and Halvorson, 2003).

Virus replication

Influenza A particle virus bind to receptor (sialic acids) on the host cell surface via the HA spike protein. This restriction binding is according to the type of linkage to galactose formed α2,3 or α2,6 configurations on the host cell surface. The α2,3 galactose linkage (SA α2,3 Gal) is generally located on the epithelial cell of avian species. Whereas, the α2,6 galactose linkage (SA α2,6 Gal) is located on the epithelial cell of the human trachea (Nicholls, et al., 2008). The virus enters through host cell by receptor-mediated endocytosis via pH-dependent fusion process. This fusion activity is cleaved the HA molecule undergo the endosomal acidification triggers a conformation change. Resulting in this fusion, endosomal and viral membranes are fused together then a pore of the membrane opens to release the viral RNP entering into the cytoplasm (Fig 2). The uncoating of influenza virus is depends on the presence of the M2 protein as an ion channel protein (Sidorenko and Reichi, 2004). The M2 ion channel mediates the influx of the proton from the lumen of endosome into the virus particle and brings the

dissociation of M1 from the vRNP into the cytoplasm (Fig 3) and then the vRNP are transported into the nucleus through nuclear pore complexes mediated by a nuclear localization signal (NLS) on the viral protein (Neumann et al., 2000 and Coloma, et al., 2009). In the nucleus, the negative- sense viral RNA (vRNA) serves as a template for synthesis a positive-sense messenger (mRNA) and a full-length complementary RNA (cRNA). Prior to transcription, this requires the 5' capped primer known as *cap snatching* process derived by cleaved from host cell polymerase II transcripts. In this process, the PB1 subunit is binding with 5' end of the vRNA, resulting to an allosteric change in the polymerase, leading to bind between PB2 and host pre-mRNAs. Moreover, the 3' end of vRNA can bind with PB1 subunit and activates the endonuclease enzyme of PB1. Then, the 5' end and the second portion of the host pre-mRNA will be cleaved into 10–13 nucleotides (nt). For the initial transcription, the guanine (G) residue is complementary with the cystine (C). Consequently, the RNA chain is elongated that is catalyzed by the polymerase enzyme of PB1 and proceeds until a stretch of uridine (U) residues is reached. The transcription will be stopped when polyadenylate (poly (A)) residues are added to the 3' end of the viral mRNA. The replication step involves the synthesis of positive sense cRNA from the vRNA and generates of newly negative-sense vRNA for packaging into progeny virions (Wang and Jiang, 2009, Palese and Shaw, 2007 and Lamb, 2008).

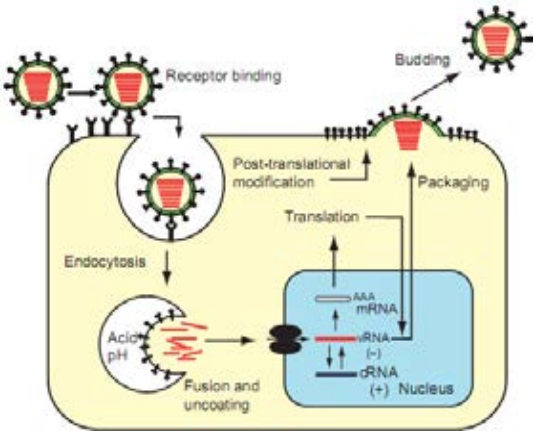


Figure 2. Replication of influenza virus (copy from Shaw and Palese, 2010).

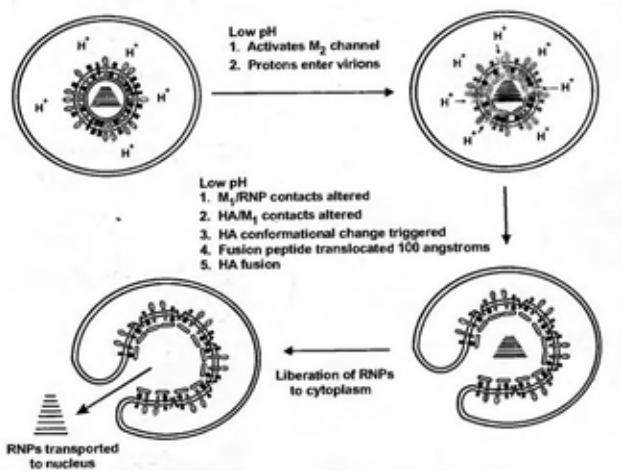


Figure 3. The schematic diagram of the proposed role of the M2 ion channel activity in virus entry (copy from Lamb and Krung, 2001)

Antigenic variation

The antigenic variation is the process of the virus that uses to be evading the host immune system. For the influenza A virus, there are two mechanisms of antigenic variation: antigenic drift and antigenic shift. Antigenic drift is a point mutation that arises in each gene segment, especially in HA or NA genes, during the replication cycle leading to spread in partially population. Antigenic shift is associated with major antigenic change during the infection stage caused by genetic reassortment between different virus strains. This mechanism introduces the newly virus strains to humans and/or animals. When the novel viruses are presented into the immunologically naive population, the high infection rate usually occurs and subsequently leads to pandemic outbreak (Palese and Shaw, 2007 and Forrest and Webster, 2010).

Host range and tissue tropism

Wild aquatic birds, particular in waterfowl (order *Anseriformes*) and shorebirds (order *Charadriiformes*), are the natural reservoir for all influenza A virus subtypes, but these viruses can infect other species, including chickens, turkeys, ducks, geese, quails, mammalian species and humans (Yassine, et al. 2010). The difference of naturally infected species depends on the host range restriction involving with sialic acid receptor (SA) and linkage type of galactose that will be formed on the host cell surface. Avian influenza virus subtype H5N1 preferentially binds to SA receptor consisting of

α2,3 galactose linkage (SA α2,3 Gal), located on the epithelial cells of the chickens trachea or duck intestine. Otherwise, human influenza viruses preferentially bind to SA receptor consisting of α2,6 galactose linkage (SA α2,6 Gal), usually found on human trachea. The different distribution of these two receptors might form a barrier to prevent interspecies transmission (Neumann and Kawaoka, 2006; Claas et al., 1998).

Clinical signs

In the susceptible species such as domestic chickens, the clinical sings of AIV infected chickens have ranged from asymptom to severe signs or death. The variation of the clinical signs is depending on acquired immunity, route of exposure, dose, and strains of virus (Pantin-Jackwood and Swayne, 2009). Generally, the LPAI results in mild respiratory signs slightly decline in egg production, ruffled feather, depression, occasionally diarrhea, loss appetite and low mortality rate, usually less than 5% (Swayne and Harvolson, 2003). For controversy, the HPAI, including HPAI H5N1 viruses causes high mortality and may cause death without prior clinical signs. However, the individual H5N1 virus infected birds show the nervous disorder, cyanosis of the comb, lethargy, recumbency and decrease in feed and water consumption (Pantin-Jackwood and Swayne, 2009).

Gross lesions

The gross lesions have extremely varied among host species and pathogenicity of viruses. In poultry, the lesions of HPAI H5N1 viruses are mainly found in the respiratory tract and multiple visceral organs characterized as necrosis, edema and hemorrhage. In chickens, hemorrhages may be found on serosal or mucosal surface especially at the epicardium, pectoral muscles and mucosa of the proventiculus and venticulus. However, no any gross lesions may be observed in sudden dead birds (Horimoto and Kawaoka, 2001 and Pantin-Jackwood and Swayne, 2009).

Since the outbreak in 1997, the HPAI H5N1 virus spread worldwide among bird species and it might transmit from infected birds to other species such as cats, dogs, tigers, and humans by close contact (Keawcharoen et al., 2004, Peiris et al., 2004 and

Songserm et al., 2006). Moreover, the routes of transmission of HPAI H5N1 virus can occur via directly contract including fecal-oral route, respiratory route and/or indirectly contact including vectors (Swayne and Halvorson, 2003). There are several possible transmission vectors of HPAI H5N1 virus including arthropods, wild birds and fomites (Tuttle, et al., 2010). Interestingly, the house flies can transmit several diseases such as shigellosis, salmonellosis, and cholera (Gerberich, 1952 and Graczyk et al., 2001). Moreover, the house flies can spread several pathogens to animals and/or humans by regurgitation or direct contact by adhering pathogens to their body hairs or other structures (Robinson, 2005).

The role of house flies as the mechanical and/or biological vectors for transmitting infectious diseases

House flies, *Musca domestica*, belonging to the order *Diptera*, are commonly associated with humans and animal husbandry (Harwood et al., 1979), are causing irritation and annoying to farmers and animals. Moreover, the house flies are considered to play an important role in the mechanical and/or biological vectors for many pathogens such as *Shigella* spp. (Greenberg, 1973), *Salmonella* spp. (Holt et al., 2007), *Escherichia coli* (De Jesús et al., 2004), *Vibrio cholerae* (Yap et al., 2008), Newcastle disease virus (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) and metazoan parasites (Förster et al., 2007).

House flies

The common house fly, *Musca domestica* Linnaeus (*M.domestica*), is a dominant synanthropic fly species and closes association with human and animal husbandry. Likewise, these flies are world-wide distributed insects and can be found in all areas around the world. Moreover, their habitat causes a serious problem in public health concerns since they can pick up and transport the several of pathogens (Greenberg, 1973).

Classification

The house fly is belonging to the order of Diptera, suborder Cyclorrhapha, subfamily Muscidae, genus *Musca* (Capinera, 2008). Of the many insect species, Diptera is a major class insect order. Moreover, subfamily Muscidae occurs worldwide in distribution, which comprises 5,000 species commonly known as blow flies and house flies (Carvalho and Mello-Patiu, 2008). The identification of many fly species is very important and the key to identify the species of fly used in this study was modified from Bruce and Maurice (1957) and Couri et al. (2006) as shown in Table 2.

Table 2. The specific structure to identify the species of fly used in this study.

Classification	Description
Order Diptera	Small to large insects with two fore membranous wings, the hind wings reduced to small and placed by knobbed called halteres and the mouthparts become formed to a proboscis
Suborder Cyclorrhapha	The antennae reduced into three segments with plumose bristle (arista) bearing at third enlarged segment
Family Muscidae	Wing vein, namely subcostal reach with costal vein usually nearly at straight angle
Genus *Musca*	Media vein with an angular forward bend towards radius vein
Species *Musca domestica* Linnaeus	Males has holoptic eyes and the body is not metallic blue or green

Morphology

Adult house flies

The morphology of adult fly is approximately 4-7.5 mm in length, which the female is usually larger than male. Normally, the body of insects is segments that are composed of a series of segments; six segments for the head, three segments for the thorax and eleven segments for the abdomen (Alford, 1999). Each segment is covered by three-layer skin; epidermis, procuticle and cuticle. The outer layer of cuticle becomes hardness formed from chitin or soft was caused by the addition of melanin (Gillott, 2005). The house fly has generally soft body and has various colors from brownish gray to black with four dark longitudinal stripes known as vittae at the thoracic dorsum (Fig 4). The color of abdomen is gray or yellowish with darks spot or blotches at the midline (Hurlstone, 1915 and Robinson, 2005).

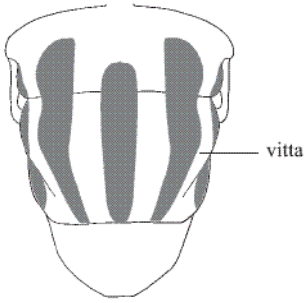


Figure 4. The thoracic dorsum of adult flies (copy from Carvalho and Mello-Patiu, 2008)

Face and antenna

The head of an insect is a capsule shape, which bears the mouthparts antennae and compound eyes. For the house fly, that head has a red pair of compound eyes, which male's eye are holoptic and nearly close to the dorsal midline, while female's eye are dichoptic and away from the dorsal midline (Hall and Gerhardt, 2002). Moreover, the house fly head has three ocelli, light-sensitive organ, which arranged in a triangle and located on anterior vertex or dorsal surface of the head (Fig 5) (Gullan, and Cranston, 2005). A pair of antenna is located beneath the lower edge of the fronts and each antenna comprises three segments, including the basal scape, which is the largest

segment and attach to the front; the second segment called pedicel and the last segment consists of arista called flagellomere. Arista is a single sharp hair with plumose hair (Fig 6) (Harwood et al., 1979 and Robinson, 2005).

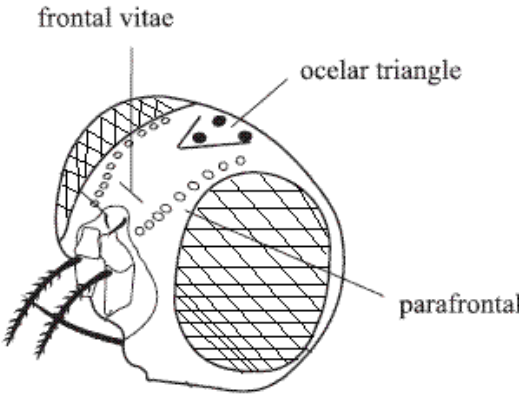


Figure 5. Morphology of head of house flies head (copy from Carvalho and Mello-Patiu, 2008)

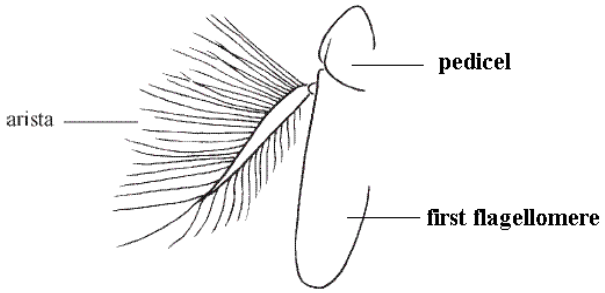


Figure 6. House fly antenna (copy from Gullan and Cranston, 2005)

Mouthpart

Normally, the mouthpart of an insect consists of five basic sections, including labrum or upper lip with a ventral surface called epipharynx, a tongue-like structure called hypopharynx, a mandible or jaw, a paired maxillae, and a lower lip (labium) (Fig 7) (Gillott, 2005). Since the house fly is non-biting fly, their mouthpart is adapted to suck or sponge up the liquid or semi-fluid food. Then, the mandible has disappeared and the elongated feeding tube is formed, which is called as proboscis. The proboscis consists of 3 parts, including a proximal part called rostrum or buccal, rostrum is cone shape and

bearing at the maxillary palps. The middle part, a cylinder in shape, is composed of 3 structures; labrum, hypoparynx and labrium, which food channel (called haustellum) is located between labrum and labrium, a distal part of proboscis called labellum which is very important for characterizing between biting and non-biting flies. The labellum comprises two lobes called labellae and its structure is enlarged oval shape with many fine channels namely pseudotrachea, which functions for absorption (William, 2005).

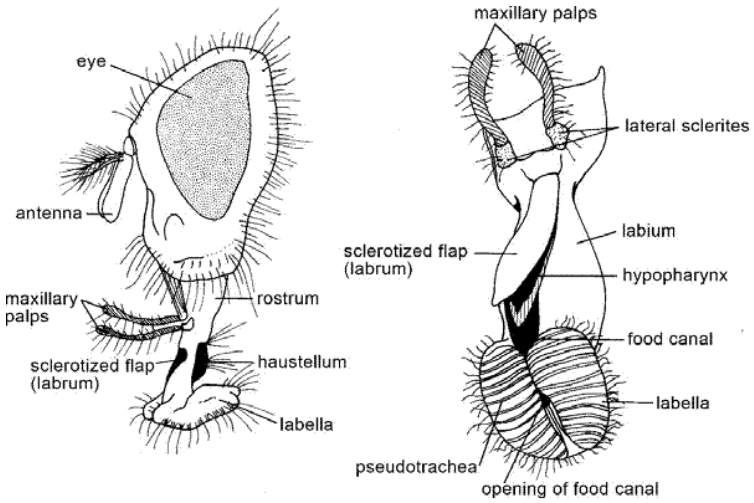


Figure 7. The structure of head and mouthpart of house fly (copy from Gullan and Cranston, 2005).

Thorax

The thorax of insect is composed of three thoracic segments; prothorax, mesothorax and metathorax. In many insects, the prothorax are usually large segment such as cockroaches and beetles, whereas the largest thoracic segment of house fly is mesothorax, which consists of two pair of wing; the fore wings are transparent membranous wings lies the dorsal of mesothorax and the hind wings called halteres are reduced, and lie on metathorax and then function are as balancing organs. The dominant mesothorax has oviform, which is fused with prothorax and metathorax (Romoser, 1998 and William, 2005).

Wings

The basic arrangement of wing venation is composed of eight veins, named from anterior to posterior; precosta (PC), costa (C), subcosta (Sc), radius (R), and media (M) (Fig 8). Most of these veins are branched. In most insects, the precosta and jugal veins are fused before reaching the wing margin. The delimited areas of the wing membrane caused by veins are called cell; these cells may be open (extending to the wing margin) or closed (entirely surrounded by veins) (Gullan and Cranston, 2005). The insect species can be characterized by wing venation. For the house fly, the wings have media veins (M_{1+2}) with a sharp angular forward bend towards to radius veins (R_{4+5}) in apical part (Fig 9) (Hurlstone, 1915 and Harwood et al., 1979).

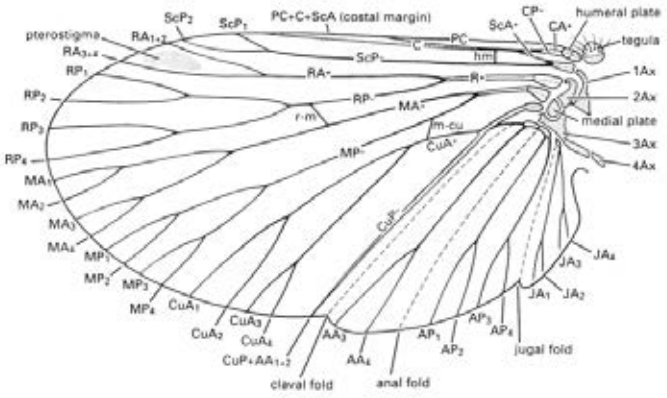


Figure 8. The main veins of the insect wing (copy from Gullan and Cranston, 2005)

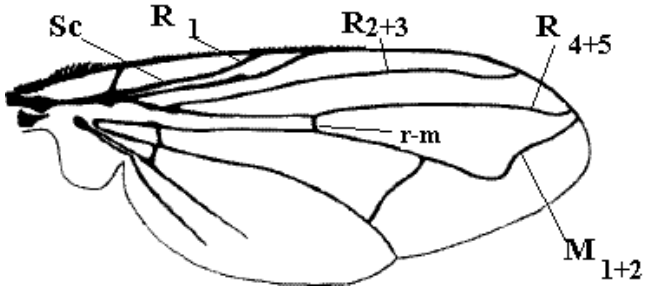


Figure 9. Wing venation of *musca domestica* L. (copy from Carvalho and Mello-Patiu, 2008)

Abdomen

Most of insects have 11 abdominal segments; several of these segments are reduced or incorporated into the thorax. In general, the abdomen of the house fly is reduced to five obvious segments. The posterior segments were modified to genital organs called aedeague or intromittent organ for male and ovipositor for female (Hurlstone, 1915 and Robinson, 2005).

Larvae

Larvae or maggot of house fly has 3 to 9 mm. in length, narrow and cylinder shape, creamy whitish in color, soft body, and legless segment. The head part is small and is located at the sharp end point, whereas the opposite blunt end point is an anus with posterior spiracular plate (Fig 10). The development of larva of house fly has three stages, which is characterized by spiracular plate. Generally, each spiracular plate comprises of three specific parts; peritreme, slit or opening cavity for gas exchange, and a scar or the remnant from a previous molt. The first stage of larva has one slit, whereas the second and the third stages of larvae have two and three slits, respectively. Moreover, the different shape of the peritreme, the position of the scar and the number of the slits are used to identify the larva species of the house flies (Fig 11) (Romoser, 1998).

Figure 10. The larva of the house fly (copy from Roger, 2002).

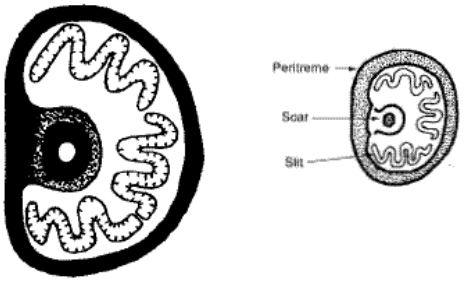


Figure 11. Reparatory spiracles of the larva of *Musca domestica* (copy from Roger, 2002)

Pupa

The pupa, the next stage from the larva, is approximately 6-8 mm. in length, barrel in shape and forms the exoskeleton. Normally, color of the pupa depends on stage of development. At the early stage after molting, the pupa has yellow color, and it is increasingly darken to red-dish brown until it is complete development. The mature pupa has become dark brown or black in color. (Robinson, 2005) (Fig 12).

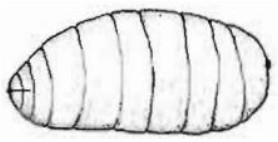


Figure 12. The pupa stage of the house fly (copy from Roger, 2002).

Egg

The house fly egg is white in color, elongates in shape and approximately 0.8-2.0 mm. in length. The house fly has highly reproductive capacity. The maximum eggs that female house fly can lay is about 75 to 150 per one batch (Roger, 2002).

Life cycle

The house fly has a complete metamorphosis, including eggs, larva, pupa, and adults or full wing insect. Under optimal condition, the life cycle of the house fly will be completed at least 7 days. After mating, the female house fly will oviposit in moist substrate. Each female house fly normally deposits 120 to 150 eggs per batch, with at least 6 batches of egg during her life time. Presumably, a couple of the house fly can produce the descendant of 191,010,000,000,000,000,000 house flies only in 5 months. The house fly egg is white in color, cylinder in shape, and 0.8–2.0 mm in length. After laying eggs, the egg will hatch within 8 to 12 hours, and then the development of larva stage will be completed within 4–13 days at the optimal temperatures (17-32 °C) with 50–70% humidity. At the late of the third larval stage, the larvae moves to the dry place and develops to the pupa stage, which completes development by approximately 2–6 days at 32–37°C (Fig 13) (Romoser, 1998 and Robinson, 2005).

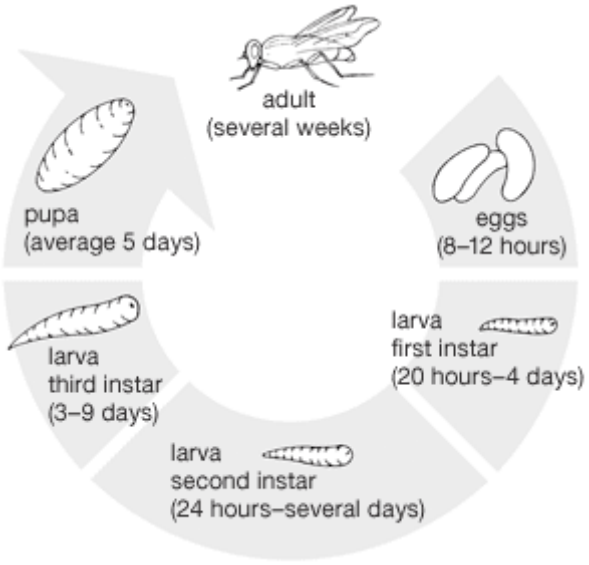


Figure 13. The life cycle of the house fly (Encyclopedia Britannica Facts matter).

Habitat

The breeding place and feeding behavior of house fly is an important role in transmission of many pathogens (Graczyk et al., 2001). Because the female house fly prepares the suitable necessary place for depositing eggs especially the excrement. Then the larvae hatch and feed on the manure containing the several organisms which may be contacted to the adult stage of house fly such as *Ascaris suum*, *Haemotopinus and Strongyloides ransomi* (Otranto et. al., 2005, Förster et al., 2007 and Förster et al., 2009). Furthermore, the feeding behavior of the house fly is a crucial role to disseminate several pathogens, particularly enteric diseases (Fotedar, 2001, Cirillo, 2006, Zweifel et al., 2008 and Fasanella et. al., 2010). The house fly has a sponging and lapping mouthpart to suck fluid or semi-liquid food. For the solid food, the house flies will regurgitate fluid from the crop to liquefied food and then ingest to the body (Harwood et al., 1979 and Robinson, 2005).

House fly and disease

The distribution of house fly is cosmopolitan and predominantly associated with human's habitat and livestock. The breeding place and feeding behavior of house fly is an important role in transmission of many pathogens (Graczyk et al., 2001). Because

the female house fly prepares the suitable necessary place for depositing eggs. Major breeding places include garbage, excrement and poultry litter. After hatching, the larva lives on the manure containing the several organisms, such as *Ascaris suum*, *Haemotopinus spp.* and *Strongyloides ransomi* (Otranto et. al., 2005, Förster et al., 2007 and Förster et al., 2009). Since adult house fly is attracted to a variety of decomposing organic materials and its mouthpart allows ingesting only the liquid or semi-liquid materials. Then, solid materials are liquefied by regurgitated digestion that contributes to spread of pathogens. Moreover, the body of the house fly has covered with a lot of fine hair that is suitable to pick up and carry pathogens (Robinson, 2005). In addition, the pathogens may be disseminated by the defecating habit during the feeding process. Thus, the feeding behavior of the house fly is a crucial role to disseminate the pathogens.

CHAPTER III

MATERIALS AND METHODS

Part 1: To determine the potential of the house flies as a vector of avian influenza virus subtype H5N1 in experimental condition.

In this part, three sections were investigated including:

1) To evaluate the potential of house flies can carry the avian influenza virus subtype H5N1.

2) To determine the minimum amount of house flies can transmit the avian influenza virus subtype H5N1

3) To evaluate the holding time of avian influenza virus subtype H5N1 can survive in the house flies.

Viruses

The highly pathogenic avian influenza (HPAI) strain A/Chicken/Nakorn-Pathom/Thailand/CU-K2/04 (H5N1) virus was used. The virus stock was propagated by third passage in the allantoic cavity of 10-day-old embryonated chicken eggs using standard protocol (OIE, 2000). The virus titer was performed using a previous protocol (OIE, 2000) as $10^{9.55}$ ELD_{50}/ml and the virus was stored at -80^{O}C until used.

House flies

The house flies were acquired as larvae from a layer chicken farm at Chachoengsao province in Thailand. The fly larvae were reared on a sand box containing larva medium which comprised swine livers at room temperature under natural sun light until pupation. The pupae were harvested and transferred into a plastic box within nested cage at room temperature for eclosion. Prior to initiation of all experiments, the adult house flies were randomly selected and confirmed to be free of H5N1 virus by real-time reverse transcriptase polymerase chain reaction (RRT-PCR) assay using specific primers and probes against influenza A matrix (M) gene. All flies were provided with water and food (a mixture of the ultra-high-temperature (UHT)

processing milk with 10% sucrose solution) *ad libitum*. The 3-day-old house flies were used and starved for 12 hours prior to use.

Experimental designs

The study was separated into 3 sections as previously mentioned. In all experiments, the house flies consumed with the virus mixture (1 ml of allantoic fluid containing avian influenza virus subtype H5N1 virus mixed with 1 ml of food solution) for 15 min except that house flies in negative control group consumed with mixture of sterile phosphate buffer saline (PBS) and food solution. The mixture was placed on a sterile cotton pad and the final titer of the avian influenza virus in the mixture was $5x10^{8.55}ELD_{50}$/ml. The house fly boxes of all experiments were a cylinder shape with 12 cm in diameter and were covered by plastic net on the top of the box.

Section 1: To evaluate the potential of house flies can carry the avian influenza virus subtype H5N1.

One hundred 3-day-old house flies were separated into two groups; control and treatment groups in plastic box. In the treatment group, flies were fed with 1 ml of the mixture containing the virus placed on a sterile cotton pad on top of the box. Conversely, the control group was fed with the same amount of the mixture without virus. Subsequently, the house flies were allowed 15 min of feeding time then all flies were euthanized with dry ice for 15 min. All flies of each group was washed with 10 ml of brain-heart infusion (BHI) broth (Difco, BD Biosciences, Sparks, MD) by vortexing for 5 sec. After washing, the BHI washing fluid (W1) was inoculated into six 10-day-old embryonated chicken eggs for evaluating the presence of avian influenza virus on the external surface of the house flies. To assess avian influenza 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 sec and rinsed with 10 ml of BHI washing fluid (W2). To confirm the absence of H5N1 virus on the external surface of house flies, the W2 washing fluid was inoculated into six 10-day-old embryonated chicken eggs. Fifthly washed house flies were placed in two sterile 1.5 ml microcentrifuge tubes (Eppendorf

South Asia, Malaysia) and homogenized with 900 µl of sterile BHI by using sterile plastic pestles and followed by centrifugation at 2000 rpm, 4 ^{O}C for 15 min. The supernatant was collected and gentamicin was added to a final concentration of 200 µg/ml and left at 4^{O}C for 40 min. Consequently, the virus isolation was performed by inoculating 100 µl of the supernatant into six 10-day-old embryonated chicken eggs. After inoculation, the embryos were observed twice a day for 5 days. The embryo was discarded if they died within 24 h. The allantoic fluid was harvested if the embryonated chicken egg died after 24 h at post inoculation. All allantoic fluids were stored at -80^{O}C until the hemagglutination (HA) test and RT-PCR assay were performed.

Section 2: To determine the minimum amount of house flies can transmit the avian influenza virus subtype H5N1

A 3-day-old house fly was transferred into a plastic box and exposed with the mixture containing with or without avian influenza virus in a sterile cotton pad for 15 min as previously described. Subsequently, an exposed house fly was washed with 1 ml of BHI as W1 washing fluid. The W1 washing fluid was titrated by inoculation into six 10-day-old embryonated chicken eggs. The virus titer of W1 washing fluid was compared with that of a house fly homogenate. After inoculation, the inoculated eggs were incubated at 37 ^{O}C for 5 day and observed twice a day. All allantoic fluids were harvested from all inoculated eggs exception that the embryos died within 24 h. The virus identification was performed as previously described. This section was performed in 5 replicates.

Section 3: To evaluate the holding time of avian influenza virus subtype H5N1 was survived in the house flies.

Forty-five house flies were equally separated into 9 boxes. Eight boxes were exposed with the mixture containing avian influenza virus as previously described, whereas the remaining box was fed with the mixture without the virus serving as a negative control group. The exposed house flies of each box were examined for the presence of the H5N1 virus at 0, 6, 12, 24, 36, 48, 72 and 96 h post exposure,

respectively. After exposure with virus, the standard food (UHT milk with 10% sucrose) was provided for all groups throughout experiment. The exposed flies collected from each time point were washed 3 times as previously described, homogenized and serially ten-folded diluted then inoculated into six 10-day-old embryonated chicken eggs of each dilution. The house flies of negative control group were only collected at 96 h post exposure. All allantoic fluids were harvested from all inoculated eggs and determined the presence of avian influenza virus.

Part 2: To determine the competence of the house flies as a transmission vector of avian influenza virus subtype H5N1 in chickens under experimental condition.

Viruses

The H5N1 virus stock was propagated and stored as previously described.

Chickens

Thirty 1-day-old female layer chickens were randomly and equally separated into 3 groups and placed in a separated unit. Group 1 was served as a sham negative control group. Group 2 and 3 were inoculated with house flies exposed with avian influenza subtype H5N1 that were euthanized at 0 and 24 h post exposure, respectively. At 14-day-old, serum sample of all chickens were collected to determine by HI test for confirming that no antibodies against avian influenza virus subtype H5 were present. At 30-day-old, the chickens in group 2 and 3 were transferred to isolator units under biosafety level 3 (BSL 3) facilities at National Institute of Animal Health (NIH), Bangkok, Thailand. The chickens of group 1 were raised in a separated unit. All chickens used in this study were handled and cared in accordance with the guidelines and approval of the Institution Animal Care and Use Committee of National Institute of Animal Health (Approval number EA-011/52R).

House flies

One hundred and fifty adult house flies were randomly and equally allocated into 3 groups. Fifty house flies of each group were equally divided into ten plastic boxes. All

house files used in this study were starved for 12 h prior to feeding with the mixture containing with or without H5N1 virus. The house flies of group 1 serving as a sham negative control group was fed with the mixture containing without H5N1 virus. In contrast, the house flies of group 2 and 3 were fed with the mixture containing the H5N1 virus approximately $5x10^{8.55}ELD_{50}$/ml for 15 min. After 15 min of exposure, the house flies of group 2 were immediately euthanized by placing them into a dry ice box for 2 min then collected, whereas the house flies of group 3 were held at room temperature for 24 h provided with H5N1 virus-free food as previously described. The preparation of house fly homogenate was performed as previously described.

Experimental designs

Prior to inoculation, oropharyngeal and cloacal swabs were collected from each chicken to confirm that none of the chickens was infected with any subtype of avian influenza virus by using RRT-PCR assay. At 32 days old, each chicken of groups 1, 2 and 3 were individually inoculated via oral route with 1 ml of infected house fly homogenate derived from each plastic box of groups 1, 2 and 3, respectively. Oropharyngeal and cloacal swab were collected daily and placed in VTM consisting of Brain heart influsion broth (BHI) and antibiotics (penicillin G 200,000 IU/ml) then stored at -80 ^{O}C until virus isolation and titration were performed. The clinical signs and mortalities were observed daily for 14 days and the blood samples were collected at 7 and 14 days post inoculation (DPI). On 14 DPI, all remaining chickens were euthanized and visceral organs including trachea, lung, small intestine, pancreas and brain were collected. All visceral organs were fixed in 10% neutral buffered formalin to determine by histopathology and immunohistochemistry.

Part 3: To examine for the presence of the avian influenza virus subtype H5N1 naturally infected house flies in epidemic area in Thailand during 2008-2009.

Study sites

In 2008, Office International des Epizooties (OIE) reported four outbreaks of avian influenza subtype H5N1 in Thailand. The first outbreak was reported on January

8th, 2008 at Saklek district, Phichit province (16°50' N and 100°47'E); the second outbreak was reported on January 23rd, 2008 at ChumSaeng district, NakornSawan province (15°90' N and 100°29'E); the third outbreak was reported on October 28th, 2008 at TongSaleam district, Sukothai province (17°19' N and 99°33'E) and the last outbreak was reported on November 10th, 2008 at NongChang district, UthaiThani province (15°23' N and 99°50'E). The restriction area was 5 km in radius around the outbreak farm.

House flies collection

The house flies were captured from the restriction area in average 2 days after outbreak report. All of the house flies were collected in plastic bag 6x9 inch in size. Live flies were stored in an ice box and transferred to the Veterinary Diagnostic Laboratory, Faculty of Veterinary Science, Chulalongkorn University. All samples were stored at -80 °C until the morphology of house fly was identified and virus isolation was performed (Bruce and Maurice, 1957 and Couri et al., 2006).

Virus isolation

The captive house flies were homogenized as previously described. Prior to inoculation, the supernatant was filtrated with 0.45 µm in a diameter membrane. The virus isolation was performed according to OIE (2000) procedure by inoculating 200 µl of the supernatant into three 10-day-old embryonated chicken eggs. The allantoic fluid from each inoculated egg was harvested and tested for identification of the virus by using HA test and RRT-PCR assay. The allantoic fluid determined as the negative result was repeatedly inoculated into three 10-day-old embryonated chicken eggs. At 7 days of post inoculation, there was still negative to HA test and/or RRT-PCR assay; this sample was considered as the negative to avian influenza virus.

Virus titration

The virus titration was performed according to OIE (2000) procedure as previously described. Briefly, a ten-fold dilution of house fly homogenates or swab

solution was inoculated into six 10-day-old embryonated chicken eggs, starting from 10^{-1} to 10^{-10}dilutions. Inoculated eggs were incubated in a humidified incubator at 37^{O}C and were candled twice a day for 5 days. The virus titers were determined as ELD_{50}/ml according to the Reed and Muench (1938) method.

Hemagglutination (HA) test

The HA test was performed according to OIE (2000) procedure as previously described. Briefly, the 25 µl of sterile PBS pH 7.5 was added into all wells of a 96-well with V shaped bottom microtiter plate. Then, the 25 µl of infected allantoic fluid was added into the first column of plate. Following, two-fold serial dilution of infected allantoic fluid was done, exception with the last two columns serving as the controls. Then 25 µl of sterile PBS was added into all the wells followed by the adding 50 µl of 0.5% of chicken red blood cells (RBCs) to each well with gently tapped and allowed the RBCs to settle for 40 min at room temperature. For interpretation, the HA endpoint was read as the HA titer and HA titer equal or greater than 1:4 was considered as infectivity.

Hemagglutination inhibition (HI) test

The HI was performed according to OIE (2000) procedure as previously described. Briefly, two-fold serial dilutions of the serum were made in sterile PBS and 25 µl of standard stock virus containing 4 HA units of H5N1 virus, homologous with the tested serum (H5), was added into each wells. After adding, the test serum was incubated at room temperature for 30 min. Then, 50 µl of 0.5% of chicken RBCs was added into all wells with gentle mixing and allowed the RBCs to settle for 40 min at room temperature. The HI end point was read as the last dilution with a complete inhibition of HA activity.

RNA extraction

The RNA was extracted from the supernatant of house fly homogenates or allantoic fluid by using QIAamp Viral RNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Briefly, 140 µl of sample was mixed with 560 µl of Buffer

AVL containing carrier RNA, vortexed for 15 sec, and incubated at room temperature for 10 min. Subsequently, 560 µl of absolute alcohol was added to the sample. The solution was transferred to QIAmp Mini column and centrifuged at 8,000 rpm for 1 min. Columns were washed twice with washing buffer. RNA was eluted with 50 µl of nuclease-free water. Three or four µl of the RNA template was used for analysis by RT-PCR or RRT-PCR assays, respectively.

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

The presence of the avian influenza virus in the allantoic fluids was determined by using RT-PCR assay, with specific primers for M gene as previously described (Payungporn et al., 2004). The M-specific primer set comprised MF 5'-TGATCTTCTTGAAAATTTGCAG-3' and MR 5'-TGTTGACAAAATGACCATCG-3'. The reverse transcription (RT) and multiplex polymerase chain reaction (PCR) were performed simultaneously in a single-step reaction using AccessQuick ™ RT-PCR system (Promega, USA) according to the manufacturer's protocol. A combination of 5.0 µl of the RNA sample with a reaction mixture containing 12.5 µl of AccessQuick Master Mix, 5 µl of AMV Reverse transcriptase and RNase-free water was used in a final volume of 25 µl. RT-PCR condition consisted of reverse transcription step for 15 min at 48^{O}C, initial denaturation step for 2 min at 95^{O}C, amplification step for 40 cycles (30 sec at 94^{O}C, 30 sec at 55^{O}C and 30 sec at 72^{O}C) and final extension step for 10 min at 72^{O}C. A total of 10 µl of PCR product was added into the loading gel and run on a 2% agarose gel (TopVisionTM LE GQ Agarose, Fermentus, USA) at 125 Volts for 45 min and stained with ethidium bromide. 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 stock virus were used as the negative and positive controls of this assay, respectively.

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

The presence of the avian influenza virus in the house fly homogenates or swab solution were determined by using the RRT-PCR assay to detect M gene, modified from

Spackman et al., (2002). This study was performed by using the SuperScript III Platinum One-step RT-PCR system (Invitrogen, California, USA). The Forward primer MF25 (5'-AGATGA GTCTTCTAACCGAGGTCG-3'), reverse primer MR124 (5'-TGCAAAAACATC TTCAAGTCTCTG-3') and probe M64 (FAM-TCAGGCCCCC-TCAAAGCCGA-TAMRA) were used in this 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 2x 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, 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 sec, 54°C for 30 sec and 72°C for 10 sec) (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 were performed using the Rotor-Gene analysis software, Version 6.0 (Corbett research supporting program). The allantoic fluid from non-exposed AI H5N1 house flies inoculated embryonated chicken eggs and the stock virus were used as the negative and positive controls of this assay, respectively.

Histopathology

Tissue samples collected at necropsy from each chickens were fixed in 10% neutral buffered formalin solution for 48 h. and routinely processed as follows. After fixation, the tissues were embedded in paraffin and cut at 5 µm in thickness for histopathological and immunohistochemical examination. For histopathological examination, 5 µm tissue sections was stained with hematoxylin and eosin (H&E) to evaluate histopathological lesion score according to modified from McAuley et al. (2007). Briefly, each tissue section consisting of trachea, lung, small intestine, pancreas, spleen and brain was observed using a microscope magnification of 400X for 3 fields/section in blinded fashion chosen. The lesion score was evaluated based on epithelial deciliation, degenerative in crypt epithelial cells in the mucosa, lymphocyte

and macrophage infiltration, hyperemia and necrosis as follows: 0 = no lesions, 1 = mild lesion or necrosis, 2 = moderate lesion or necrosis, 3 = severe lesion or necrosis

Immunohistochemistry (IHC)

The IHC of avian influenza specific virus antigen detection was performed by using a mouse-derived monoclonal antibody clone EVS 238 (HB65 like) (B.V.EUROPEAN VETERINARY LABORATORY, The Netherlands) specific for type A influenza virus nucleoprotein according to Sreta et al. (2009) as previously described. In brief, after deparaffinization in xylene, hydrated in ethanol, and washed in PBS, 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, USA) for antigen retrieval and then incubated for 45 min in 1% bovine serum albumin to reduce background staining. Subsequently, the sections were incubated with the mouse-derived monoclonal antibody at 4°C overnight and then incubated with EnVision® polymer reagent (Dako Cytomation) for 45 min. The immunohistochemical signal was visualized using 3,3-diaminobenzidine tetrahydrochloride (Sigma-Aldrich, St. Louis, MO). Sections were counterstained in hematoxylin. The negative and positive controls were included in each test. The positive NP viral antigen in slide tissue was indicated by substrate chromagen stained in the nucleus.

Mean death time (MDT)

The MDT was evaluated by the sum of days from chicken inoculation to death divided by the total number of inoculated chicken deaths according to Sarmento et al. (2010).

Statistical analysis

In section 2 of part 1, Student's paired t-test was used to compare the virus titer between a house fly homogenate and W1 washing fluid. A *P*-value was considered as a significant difference ($P<0.05$). In section 3 of part 1, analysis of variance (ANOVA) and linear regression analysis were used. ANOVA with a Tukey-Kramer multiple comparison

tests was used to determine the correlation among the repeated measures. A *P*-value was considered as a significant difference ($P<0.05$). The linear regression analysis was used to evaluate the correlation between virus titer and the copy numbers at the time of post exposure of H5N1 virus. In addition, descriptive statistics was used to analyze the data in section of part 2.

CHAPTER IV
RESULTS

Part 1: To determine the potential of the house flies as a vector of avian influenza virus subtype H5N1 in experimental condition.

Section 1: To evaluate the potential of house flies can carry the avian influenza virus subtype H5N1.

Embryonated chicken eggs inoculated with W1 washing fluid from treatment groups and house fly homogenates from treatment group died within 2 days post inoculation. Allantoic fluid of inoculated eggs from the treatment group and W1 washing fluid from the treatment group were positive for the H5N1 virus by HA test and RT-PCR assay. In contrast, all embryonated chicken eggs inoculated with the W2 washing fluid and all embryonated chicken eggs of negative control group remained healthy with no evidence of the H5N1 virus infection.

Section 2: To determine the minimum amount of house flies can transmit of the avian influenza virus subtype H5N1.

The avian influenza virus was detected from an individual house fly of all replicates (Fig 14). Each inoculated eggs of all replicates from treatment groups died within 2 days post inoculation. Likewise, all embryonated eggs inoculated with W1 washing fluid from all of the treatment groups were died at the nearby time. The average virus titer of house fly homogenate was 5.43 ± 0.33 log ELD_{50}/fly, which was significantly ($P<0.05$) higher than W1 washing fluid (external surface, 4.28 ± 0.55 log ELD_{50}) (Table 3). The virus titers between a house fly homogenate and W1 washing fluid at different times were decreased after 48 h and 24 h, respectively (Table 4). The HA test and RT-PCR assay were positive in all groups and in all replicates. In the negative control group, all embryonated chicken eggs were alive and HA test and the RT-PCR assay were negative.

Table 3. The virus titers (log ELD_{50}/ml) from a house fly homogenate and W1 washing fluid determined by virus titration

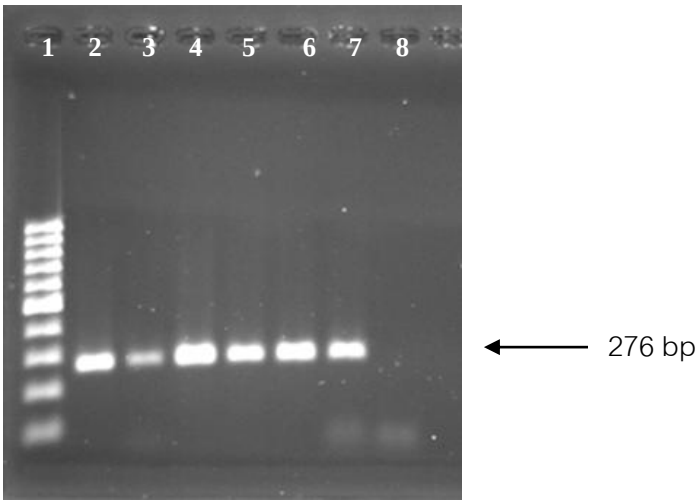
Groups	Virus titer (log ELD_{50}/ml)	
	Homogenate	W1*
1	5.37	3.62
2	5.62	3.75
3	5.75	4.74
4	5.49	4.60
5	4.90	4.70
Negative	0	0

W1*, 1^{st} washing fluid represented virus of external surface of a house fly

Table 4. H5N1 virus titer between house fly homogenates and W1 of avian influenza virus subtype H5N1 exposed house flies at different times of post exposure in embryonated chicken eggs.

Times of post exposure (h)	H5N1 virus titer (log ELD_{50}/fly)	
	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

Figure 14 The RT-PCR assay of individual house fly exposed with avian influenza virus subtype H5N1virus with M primer set (276 bp). Lane 1; marker 100 bp, 2-6; samples from individual fly replicate 1-5, respectively, 7; positive control (CUK-2) and 8; negative control.



Section 3: To evaluate the holding time of avian influenza virus subtype H5N1 was survived in the house flies.

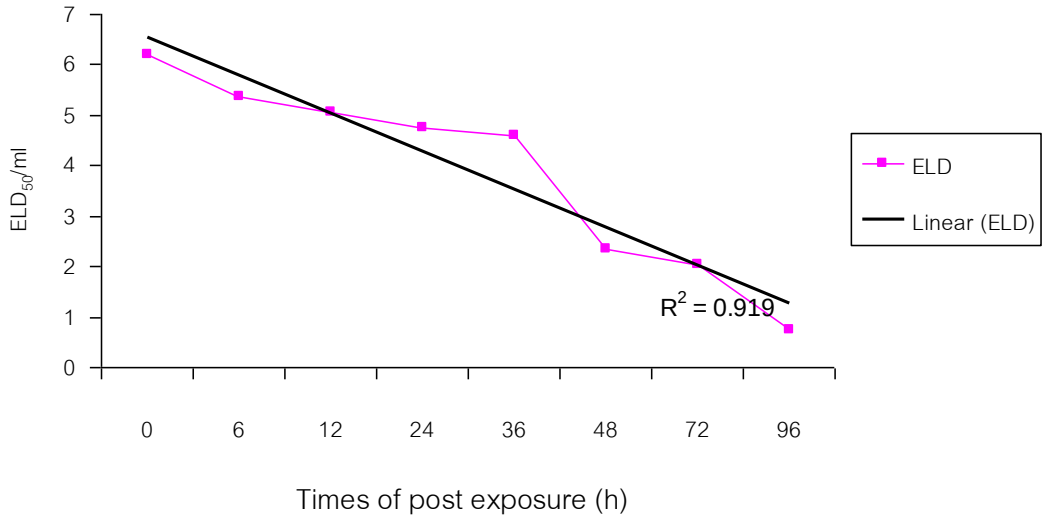
The results of the virus isolation revealed that the avian influenza virus subtype H5N1 could survive in house flies and remained infectivity up to 96 h time of post exposure (Fig 15). RRT-PCR assay were positive up to 96 h times of post exposure indicating that all house flies could carry the H5N1 virus but the virus could not replicate in the house flies. The virus titer among the five replicates at the same time of post exposure was not significantly different ($P>0.05$) but the significant difference was observed between at 0 and 48 h post exposure and later, and 6 h post exposure and later and 48 h post exposure and later. In contrary, the H5N1 virus was not detected from sham-inoculated negative control group by both virus titration and RRT-PCR assay (Table 5).

Table 5 The average H5N1 virus titers determined by virus titration and by RRT-PCR assays. The virus titers were detected from H5N1 virus exposed house fly homogenates at different times of post exposure (n = 5).

Times of post exposure (h)	Avian influenza virus titer (log ELD_{50}/ml)*	RRT-PCR assay (log copies/µl)*
0	6.21±0.09[a,b]	9.62±2.39
6	5.38±0.02[b]	9.26±1.34
12	5.06±0.51[b]	8.74±0.86
24	4.76±1.28[b]	8.76±0.84
36	4.60	8.19
48	2.35±0.17[c]	7.9±0.30
72	2.02±0.42[c]	7.4±0.95
96	0.75±1.06[c]	7.7±0.12
Negative	0	0

*Average virus titers ± SD calculated from H5N1 exposed house fly. The different superscript letters within the same column were significantly different ($P<0.05$).

Figure 15 Average avian influenza virus titers (log ELD_{50}/ml) detected from five house flies at the different times of post exposure (y=-0.7537x+7.2829; R^2=0.919)



Part 2: To determine the competence of the house flies as a transmission vector of avian influenza virus subtype H5N1 in chickens under experimental condition.

Clinical observations, morbidity and mortality

In order to determine the competence of the house flies as a transmission vector of avian influenza virus, chickens of groups 2 and 3 were inoculated with the infected house flies in different times of post exposure with virus as 0 and 24 h, respectively. After 24 h inoculations, one chicken of group 2 became sick, depressed and died at 2 days post inoculation. Beside the first chicken of group 3 died without exhibiting clinical sings after 72 h of post inoculation. The clinical signs of other chickens of group 3 were observed on day 4 post inoculation and later. However, the last chicken of group 2 showed severe clinical signs with incoordination and tremors at 24 h before death. The inoculated chickens of group 2 developed severe clinical signs compared with those of group 3. During the observation period, the clinical signs of both inoculated groups were observed, including depression, listlessness, loss of appetite, and ruffled feather (Fig 16). For the mortality, the virus caused 100% death to all chickens of groups 2 and 3 within 7 days post inoculation, while the onset of death was

48 h and 72 h after inoculation and the mean death time (MDT) was 4.3 and 5.6, respectively (Table 6). Otherwise, all chickens in sham-inoculated negative control group survived and did not exhibit any clinical signs of post inoculation.

Table 6. The mean dead time (MDT) and mortality of chickens inoculated with H5N1 exposed house flies and sham-inoculated negative control chickens (n = 10).

Group*	No. of chicken deaths per day							MDT
	1DPI†	2DPI	3DPI	4DPI	5DPI	6DPI	7DPI	
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

*Group1, sham-inoculated negative control chickens; group 2, chickens inoculated with H5N1 exposed house flies at 0 h; group 3, chickens inoculated with H5N1 exposed house flies at 24 h.

†Days post inoculation.

Figure 16. The clinical signs including ruffled feather of chickens inoculated with house flies exposed with H5N1 virus

Serological analysis

Sera were collected from each chicken at 14-day-old in order to investigate the presence of the specific antibody against the homologous virus. The results were negative by HI assay indicating that all chickens in this experiment had not been exposed with the avian influenza H5N1 virus prior to study. However, the blood collection at 7 and 14 days post inoculation of the inoculated groups did not achieve because the chickens died before time of collection.

Oropharyngeal and cloacal shedding

The recovery virus from oropharyngeal and cloacal swabs from the inoculated chickens determined by RRT-PCR assay showed that all inoculated chickens consistently shed virus during the validation period. Moreover, the H5N1 virus from the swabs was mostly isolated at 24 h before chicken death corresponding with the peak of the viral RNA copy numbers. The highest copy numbers from the oropharyngeal swabs of group 2 was 7.699 log copy numbers/ml detected by RRT-PCR and the virus titer was 6.5 log ELD_{50}/ml (Table 7), whereas those of group 3 was 7.565 log copy numbers/ml and 4.25 log ELD_{50}/ml, respectively (Table 8). In versus, the highest copy numbers from the cloacal swabs of group 2 was 6.807 log copy numbers/ml and 4.75 log ELD_{50}/ml (Table 9), whereas those of group 3 was 5.22 log copy numbers/ml and 4.25 log ELD_{50}/ml (Table 10), respectively. The average virus titers from cloacal and oropharyngeal swabs of groups 2 and 3 after inoculation presented in table 8 and figure 17 and figure 18, respectively. Moreover, the comparison of virus titers between the routes of virus shedding revealed that the virus titer of oropharyngeal route was higher than that of cloacal route suggesting that the inoculated chickens shed virus via oropharynx rather than the cloacal. For the negative control group, no virus shedding from oropharyngeal and cloacal swabs was determined by RRT-PCR assay and virus isolation.

Table 7. The virus shedding results determined by RRT-PCR (log copy numbers/µl) and by virus isolation (log ELD_{50}/ml) from oropharyngeal swabs of individual chickens of group 2

No	log	1 DPI	2 DPI	3 DPI	4DPI	5DPI	6 DPI
11	copy numbers/µl	6.38					
	ELD_{50}/ml	4.47					
12	copy numbers/µl	4.614	5.164	5.854	5.155	6.396	7.699
	ELD_{50}/ml	1.6	2.87	1.74	3.45	4.7	6.5
13	copy numbers/µl	3.146	4.567	5.53	6.634		
	ELD_{50}/ml	0	2.6	2.6	5.36		
14	copy numbers/µl	1.498	1.375	4.217			
	ELD_{50}/ml	2.15	2.87	4.7			
15	copy numbers/µl	5.041	3.883	3.844			
	ELD_{50}/ml	4.5	3.6	4.5			
16	copy numbers/µl	4.824	0.215	5.835	6.161	6.433	
	ELD_{50}/ml	0	3.64	0	2.75	4.75	
17	copy numbers/µl	5.186	5.248				
	ELD_{50}/ml	5.4	4.76				
18	copy numbers/µl	5.828	5.585				
	ELD_{50}/ml	2.65	6.5				
19	copy numbers/µl	6.648	7.461	6.146			
	ELD_{50}/ml	2.25	3.87	5.69			
20	copy numbers/µl	2.182	2.464	2.626	4.86		
	ELD_{50}/ml	1.6	0	0	2.75		

Table 8. The virus shedding results determined by RRT-PCR (log copy numbers/µl) and by virus isolation (log ELD_{50}/ml) from oropharyngeal swabs of individual chickens of group 3

No	log	1 DPI	2 DPI	3 DPI	4DPI	5DPI	6 DPI
21	copy numbers/µl	1.766	4.185	6.093	5.973	6.79	
	ELD_{50}/ml	0	0	0	2.75	4.63	
22	copy numbers/µl	2.777	1.326	5.647	6.21		
	ELD_{50}/ml	0	0	1.6	4.15		
23	copy numbers/µl	3.013	2.831	5.134	5.693	7.565	
	ELD_{50}/ml	0	0	1.87	1.83	6.4	
24	copy numbers/µl	2.645	2.326	4.635	2.942	1.535	3.124
	ELD_{50}/ml	1.6	1.83	0	2.5	2	5.15
25	copy numbers/µl	1.852	5.212				
	ELD_{50}/ml	0	4.16				
26	copy numbers/µl	5.14	5.303	5.25	6.618		
	ELD_{50}/ml	1.75	1.6	0	3.6		
27	copy numbers/µl	1.303	2.606	3.591	5.517		
	ELD_{50}/ml	0	0	0	2.62		
28	copy numbers/µl	2.061	1.682	1.56	2.407	2.873	3.894
	ELD_{50}/ml	0	0	0	0	2.37	3.36
29	copy numbers/µl	3.288	5.182	4.274	5.041	4.666	4.927
	ELD_{50}/ml	0	0	1.6	1.75	2	3.76
30	copy numbers/µl	2.617	2.369	2.962	3.785		
	ELD_{50}/ml	0	0	1.6	2.75		

Table 9. The virus shedding results determined by RRT-PCR (log copy numbers/µl) and by virus isolation (log ELD_{50}/ml) from cloacal swabs of individual chickens of group 2

No	log	1 DPI	2 DPI	3 DPI	4DPI	5DPI	6 DPI
11	copy numbers/µl	5.438					
	ELD_{50}/ml	2.76					
12	copy numbers/µl	3.896	2.314	4.233	5.623	3.068	6.807
	ELD_{50}/ml	0	0	0	2.25	2.36	2.75
13	copy numbers/µl	4.599	0.678	5.238	7.013		
	ELD_{50}/ml	0	2	2.75	4.2		
14	copy numbers/µl	2.775	3.378	6.318			
	ELD_{50}/ml	0	2.6	3.7			
15	copy numbers/µl	2.513	2.675	6.771			
	ELD_{50}/ml	0	2.75	4			
16	copy numbers/µl	1.919	3.407	1.954	1.759	3.883	
	ELD_{50}/ml	0	0	0	0	2	
17	copy numbers/µl	0.025	4.19				
	ELD_{50}/ml	0	4.4				
18	copy numbers/µl	4.92	4.888				
	ELD_{50}/ml	0	4.75				
19	copy numbers/µl	3.272	2.061	5.43			
	ELD_{50}/ml	0	3.4	2.15			
20	copy numbers/µl	3.703	2.037	2.199	5.551		
	ELD_{50}/ml	0	0	0	2		

Table 10. The virus shedding results determined by RRT-PCR (log copy numbers/µl) and by virus isolation (log ELD_{50}/ml) from cloacal swabs of individual chickens of group 3

No	log	1 DPI	2 DPI	3 DPI	4DPI	5DPI	6 DPI
21	copy numbers/µl	2.009	1.74	2.63	2.25	3.26	
	ELD_{50}/ml	0	0	0	0	2.5	
22	copy numbers/µl	1.049	0.489	5.176	3.241		
	ELD_{50}/ml	0	0	0	3.75		
23	copy numbers/µl	2.941	1.294	2.807	0.682	4.41	
	ELD_{50}/ml	0	0	0	0	3	
24	copy numbers/µl	2.604	1.585	4.911	4.799	5.22	2.965
	ELD_{50}/ml	0	0	1.6	0	2.5	0
25	copy numbers/µl	1.614	4.784				
	ELD_{50}/ml	0	4.25				
26	copy numbers/µl	3.956	2.228	4.265	4.647		
	ELD_{50}/ml	0	0	0	3.4		
27	copy numbers/µl	4.405	4.111	1.985	4.618		
	ELD_{50}/ml	0	0	1.75	3.75		
28	copy numbers/µl	1.695	4.326	4.751	4.674	2.827	3.121
	ELD_{50}/ml	0	0	0	0	2	3.5
29	copy numbers/µl	2.1	2.701	2.823	3.346	3.338	2.542
	ELD_{50}/ml	0	0	0	2	4	2.25
30	copy numbers/µl	0.893	2.758	2.114	3.013		
	ELD_{50}/ml	0	0	0	2.67		

Figure 17. The average virus titers (log ELD_{50}/ml) determined by virus isolation from oropharyngeal and cloacal swabs of group 2.

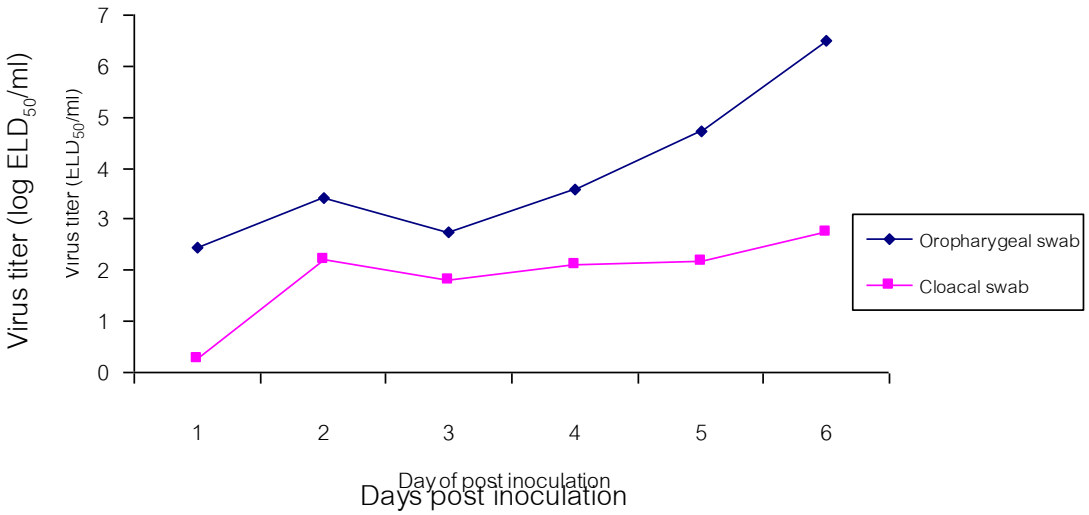
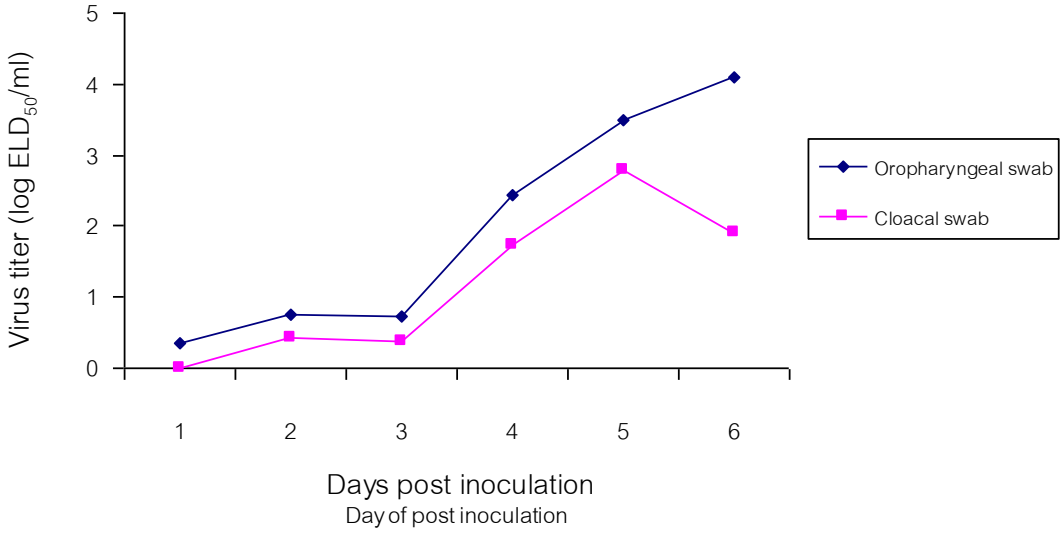


Figure 18. The average virus titers (log ELD_{50}/ml) determined by virus isolation from oropharyngeal and cloacal swabs of group 3

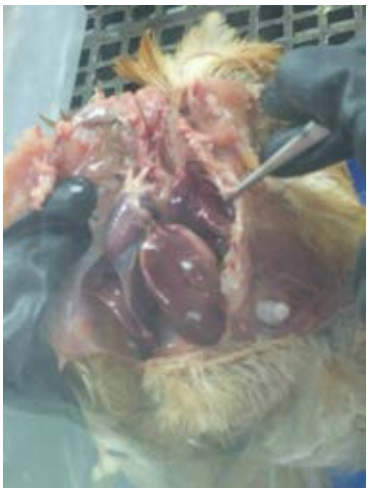


Necropsy findings

Gross lesions were observed from all chickens of inoculated groups and the predominant lesions of inoculated groups were airsacculitis, pulmonary congestion, mild pancreas congestion and mild enteritis. Some chicken carcasses were autolysis

because all chickens of inoculated group were sudden death at the night. For the negative control group, there were no any gross lesions were observed in all chickens (Fig 19).

Figure 19. Show necropsy of dead chickens inoculated house flies exposed with H5N1 virus.



Histopathology and immunohistochemistry

Histopathological lesions showed that all chickens of inoculated groups were multiorgan necrosis with moderate to severe heterophilic and mononuclear inflammatory infiltration, deciliation and sloughing of epithelial cells in trachea, mild to moderate diffuse pulmonary congestion, multifocal to coalescing necrosis of the pancreas, perivascular infiltration of mononuclear cell at duodenum. In contrary, no obviously histopathological lesions were observed in all chickens of group 1 (negative control group

For immunohistochemistry, the nucleoprotein of the H5N1 virus was detected in all examined tissue sections especially in pancreas from both inoculated groups, whereas the virus antigen was not detected in all examined sections from negative control group (Fig 20).

Figure 20. Distribution of H5N1 virus antigen in tissue of infected chickens determined by immunohistochemistry

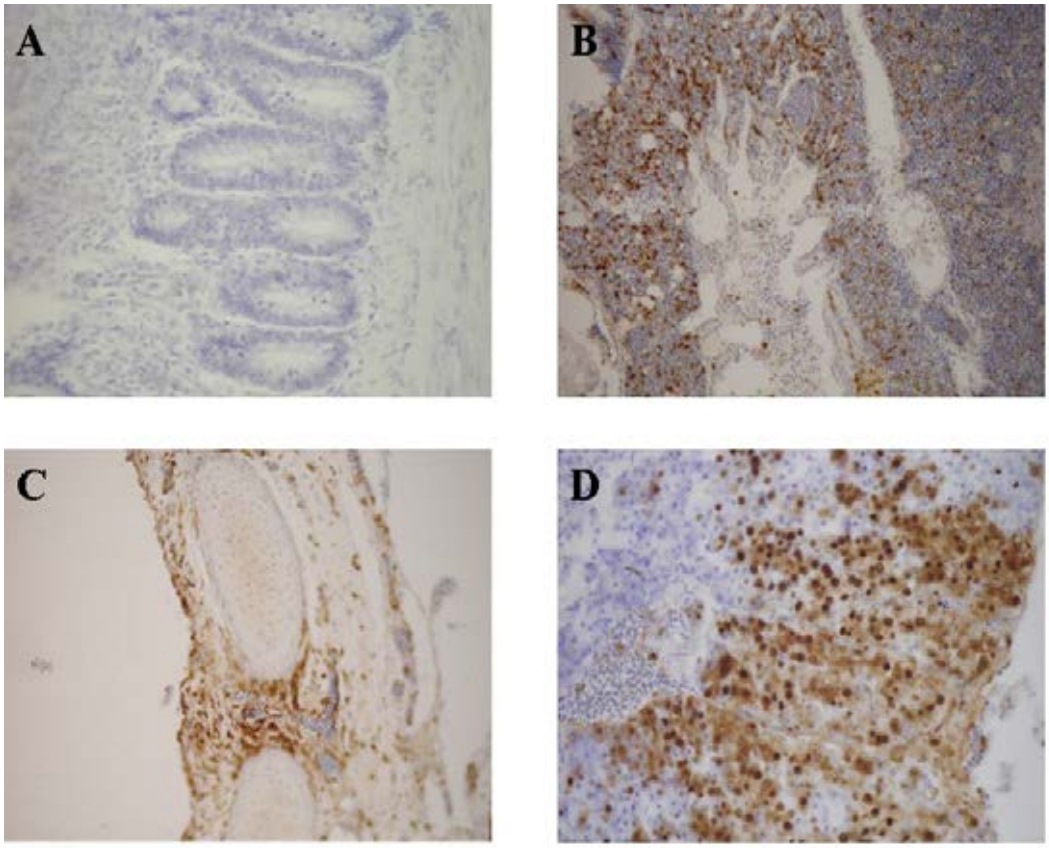


Fig 1. Distribution of H5N1 virus antigen in tissues of chickens inoculated with H5N1 exposed house flies. Photomicrographs showed the detection of AIV antigen (red-brown staining) on a hematoxylin-stained background. A, Section from duodenum of negative control chickens (original magnifications 20x); B, Section from lung of chickens inoculated with H5N1 exposed house flies in group 2 (original magnifications 20x); C, Section from trachea of chickens inoculated with H5N1 exposed house flies in group 2 (original magnifications 40x) and D, Section from pancreas of chickens inoculated with H5N1 exposed house flies in group 3 (original magnifications 10x).

Part 3: To examine for the presence of the avian influenza virus subtype H5N1 naturally infected house flies in epidemic area in Thailand during 2008-2009.

Flies sampling

Sixty two flies identified as three species were trapped within a radius of 10 km surrounding from H5N1 virus the epidemic area in 2008. Most of collected flies were identified as *Musca domestica*, *Chrysomyia megacephala* and *Sarcophaga* sp. (Table 11). The first collected site was the Saklek district, PhiChit province that was a medium size of village of 5,766 inhabitants that was located approximately 344 km, north of Bangkok. This epidemic area was surrounded by a group of small local villages with the clumping of grazing ducks. The second collected site was ChumSaeng district, Nakornsawan province, a small size of village of 3,368 inhabitants that was located on the bank of the NAN River and approximately 280 km, north of Bangkok. This epidemic area was surrounded by a group of rural villages and the rice field. The third collected site was TongSaleam district, Sukothai province that was located approximately 495 km, north of Bangkok. This a small size of a community village, and rarely found backyard chickens, whereas there are a number of fighting cocks outside from the epidemic area approximately 10 km. The last collected site was NongChang district, UthaiThani province that was a rural village, neighbors by National Park, located approximately 270 km north of Bangkok. Overall, the total collected site were fifteen areas including (3 houses and 1 flock of grazing duck from Saklek district, 4 houses from ChumSaeng district, 2 houses and 1 local fresh market from TongSaleam district and 4 houses from NongChang district. The collected files were pooled as approximately 5 flies/group based on the area and species of the flies. However, there was no H5N1 outbreak report in 2009. Therefore, the house flies were not collected in this year.

Table 11. The species and numbers of the collected flies surrounding from the epidemic area (Saklek district, ChumSaeng district, TongSaleam district and NongChang district)

Species	No. of collected fly	Proportion	Collected location
Musca domestica	57	91.93	15 flies from Saklek district, 12 flies from ChumSaeng district, 21 flies from TongSaleam district and 14 flies from NongChang district
Chrysomyia megacephala	4	6.45	grazing duck herd at Saklek district
Sarcophaga sp.	1	1.61	market at TongSaleam district

Virus isolation

After species identification, 5 pooled of *Musca domestica* and 1 pooled of *Chrysomyia megacephala* from Salek district, 3 pooled of *Musca domestica* from ChumSaeng district, 5 pooled of *Musca domestica* and one *Sarcophaga* fly from TongSaleam district and 3 pooled of *Musca domestica* from NongChang district were homogenized and then inoculated with 200 µl of homogenate pooled flies into three 10-day-old embryonated chicken eggs (Table 12). The second serial passage was performed when the result of the 1st passage showed the absence of virus. Subsequently, the allantoic fluids of the 2nd inoculated embryonated chicken eggs were tested for the presence of avian influenza virus by RRT-PCR assays using primers and probe specific to influenza matrix A gene. The result of this study demonstrated that all of the allantoic fluids from the 2nd passage showed no evidence of avian influenza virus (Table 12).

Table 12. The results of avian influenza virus isolation and real-time RT-PCR assay of collected flies.

Date of collection	Site of collection (province)	No. of pooled flies	Virus isolation (2^{nd} passage)	RRT-PCR
25/01/08	ChumSaeng	3 pool of *Musca domestica*	-*	-
04/02/08	Saklek	5 pool of *Musca domestica*	-	-
		1 pool of *Chrysomyia megacephala*	-	-
13/11/08	NongChang	3 pool of *Musca domestica*	-	-
18/11/08	TongSaleam	5 pool of *Musca domestica*	-	-
		One *Sarcophaga* sp. fly	-	-

-*, No evidence of the avian influenza virus.

CHAPTER V

DISCUSSION AND CONCLUSIONS

Highly pathogenic avian influenza virus subtype H5N1 causes serious problems not only in poultry industries but also poses a threat to human health (Olsen et al., 2006). Nowadays, the avian influenza A viruses is still a major disease problem of global concern because this virus is widely spread in broad host range and rapid evolution (WHO, 2011). Generally, the poultry production system in Thailand can be divided into four sectors namely, industrial integrated system, semivertical integrated system, extensive farming and backyard chickens. In extensive farming (e.g., layer hens, broiler, ducks, and free-grazing duck) and backyard (e.g., chickens, duck, and fighting cocks), poultry were kept in close association with the human and freely contact with the other animals (e.g. cats, dogs and wild birds) (Tiensin, et al., 2007). The manure of these poultry was an appropriated breeding place for house flies. Moreover, Thailand's temperature is optimum for development of house flies. The previous study showed that house flies can act as a vector for transmitting disease to poultry farm (Greenberg, 1973). Therefore, the conceivable explanation that the potential role of house flies to act influenza virus subtype H5N1 in experimental condition was determined. On the basis of this objective, the experiment was divided into three sections for clarify the questions. The purpose of the first sections was to evaluate that the ability of house flies to carry the avian influenza virus subtype H5N1. The result showed that the house flies consumed food containing the virus could carry this virus via the internal organ. This finding was related to Barin et al. (2010) that house flies could serve as a mechanical vector of the Newcastle disease virus in the laboratory experiment.

The purpose of the second section was to determine the minimum number of house flies for transmitting the avian influenza virus subtype H5N1. We allowed a fly to consume the food contaminated with avian influenza virus for 5 replicates. This result clearly revealed that an individual house fly could carry the H5N1 virus (Table 3). This

finding was in accordance with the study determined by Otake et al. (2004) which reported that the individual house fly could carry the porcine reproductive and respiratory syndrome virus (PRRSV) and caused infection in a naïve pig. Moreover, this study also demonstrated that the house fly could carry the H5N1 virus within their bodies for a long period of time. The H5N1 virus was detected in both external surfaces and internal organs of the house fly. The numbers of H5N1 virus in the internal organs of house fly were significantly higher than those of the external surface ($P<0.05$). Nevertheless, the H5N1 virus from whole fly homogenate could be detected up to 96 h post exposure (Table 4). Furthermore, our finding was consistent with the finding done by Otake et al. (2003), which found that the numbers of viable PRRSV in the internal organs of house flies were higher than those of the external surface. Interestingly, Chakrabarti et al. (2008) found higher numbers of Exotic Newcastle disease virus (ENDV) from the whole house fly homogenate than that from the external body surface. The purpose of the third section was to evaluate the retention time of avian influenza virus subtype H5N1 surviving in the house flies. The results of this section revealed that the H5N1 virus could be detected within the infected house flies for up to 96 h post exposure (Table 5). The present study was similar to Barin et al. (2010), which found that the Newcastle disease virus (NDV) remained viable in the crop and gut tissues of the infected house flies for up to 72 h post exposure under laboratory condition. In contrast, Nielsen et al. (2011) found that the low pathologenic avian influenza virus subtype H7N1 and H5N7 could be isolated from the alimentary tract of experimental infected house flies only for 24 h after exposure. Corresponding with the Otake et al. (2003), this previous study showed that the PRRSV could survive within the intestine of house flies for up to 12 h post exposure. Moreover, another previous study showed that NDV could survive within the digestive tract of experimental infected house flies for up to 96 h post exposure (Watson et al., 2007). This difference may be caused by the difference of virus strains (Förster et al., 2007). However, the real-time RT-PCR results from our study showed that the viral RNA could be found in all time points. However, the infectious virus titer continuously decreased according to the increasing times of post exposure (Fig 15). This result revealed that the H5N1 virus could not replicate within the house

flies, suggesting that the house flies act as a mechanical vector rather than a biological vector. Likewise, Calibeo- Hayes et al. (2003) found that the house flies served as a mechanical vector of the turkey Corona virus (TCV) and Barin et al. (2010) demonstrated that the house flies implicated as a mechanical vector of the Newcastle disease virus (NDV). However, the avian influenza virus can survive in an environment for a period of time (Swayne & Halvorson, 2003). The optimum humidity, temperature and organic materials including nasal discharge and feces can prolong the survival of many viruses (Pantin-Jackwood & Swayne, 2009). The avian influenza virus subtype H5N2 virus can survive in feces at 4 ^{O}C for 30-35 days and at 20 ^{O}C for 7 days (Swayne & Halvorson, 2003). In contrast, avian influenza virus subtype H5N1 virus can survive at 25 ^{O}C for 24 h, and at 40 ^{O}C for 15 min (Chumpolbanchorn et al., 2006). Jeong et al. (2009) found that chickens experimentally inoculated with $10^{6.5}$ EID_{50} of A/Chicken /Korea/IS/06 (H5N1) could shed virus from oropharynx and cloaca for up to 3 days after inoculation at titers of $10^{4.6}$ and $10^{2.4}$ $TCID_{50}$/ml, respectively. Other poultry species, including ducks and quails, which were experimentally infected with avian influenza virus subtype H5N1, could shed virus from oropharyngeal swabs for up to 4 and 6 days, respectively, at titers of $10^{3.2}$ and $10^{6.0}$ $TCID_{50}$/ ml, respectively. Moreover, Tsukamoto et al. (2007) demonstrated that the chickens experimentally inoculated with $10^{2.5}$ to $10^{4.5}$ EID_{50} of A/chicken/Yamaguchi/7/04 (Ck/Yama/7/04) (H5N1) via the intranasal route died within 4 days post inoculation. Interestingly, the results of this study showed that an individual house fly can uptake the virus in approximately $10^{5.43}$ ELD_{50}/ml out of $5x10^{8.55}$ ELD_{50}/ml of infectious mixture or approximately 0.02% of the total virus. Thus, this data suggests that house flies can carry the amount of H5N1 virus closely to the amount of virus shedding from experimentally infected avian species such as chickens, quails and ducks. Interestingly, an individual house fly can fly up to 11.8 km. within 24 h (Greenberg, 1973). Therefore, house flies can serve as potential mechanical vectors, which spread avian influenza virus subtype H5N1 to poultry farms in the nearby area. Recently, Sawabe et al. (2009) demonstrated that blow flies (*Calliphora nigribarbis*) served as the mechanical vectors for H5N1 virus in Japan. Moreover, all results of this part have already been published in Wannaratana et al. (2010).

In the second part of this study, we determined the competence of the house flies as a transmission vector of avian influenza virus subtype H5N1 in chickens under laboratory condition. In the present study, chickens were divided into 3 groups. The group 1 was a sham negative control group and group 2 and 3 were treatment groups, which differed in the holding time of avian influenza virus in the house flies. Our previous study demonstrated the correlation between times of post exposure and the survival rate of the H5N1 virus in the house flies, which found that the virus titer continually declined when the time passing. Therefore, the virus titer at 0 and 24 h of exposure was significantly different (Wannaratana et al., 2010). Similar to Chakrarti et al. (2008), this previous study showed that the Newcastle disease virus titer was gradually declined after time passing. For this reason, we chose the different times of post exposure between 0 and 24 h for evaluating the effect of the times of exposure in chicken under experimental condition. Therefore, the chickens in groups 2 and 3 were orally inoculated with house fly homogenate, which were exposed with H5N1 virus at the different times of post exposure (0 and 24 h post exposure). The results showed that the chickens from both groups were highly susceptible with H5N1 exposed house flies. The clinical signs including depression, listlessness, loss of appetite, and ruffled feather, were observed starting at 24 h and 96 h post inoculation in chickens from groups 2 and 3, respectively. This clinical signs were similar to the previous reports of H5N1 infected chickens (Isoda et al., 2006, Keawcharoen et al., 2008, Forrest et al., 2010 and Fujimoto et al., 2010). Furthermore, all of chickens from both inoculated groups died within 2 to 7 days post inoculation and the MDT of groups 2 and 3 were 4.3 and 5.6 days post inoculation, respectively. These results corresponded with Jeong et al. (2009) that demonstrated the 100% mortality of chickens after inoculated with avian influenza virus subtype H5N1 by intranasal routes. Furthermore, these chickens died within 3 to 6 days after inoculation and MDT was 3 days post inoculation. The other studies reported that the avian influenza virus subtype H5N1 isolated in Thailand caused the high mortality in chickens, quails and ducks after inoculation by intranasal route and MDT were about 1.4-2.3, 1.1-3.4 and 4.8-6.3, respectively (Saito et al.,2009).

Our results showed that the virus was consistently detected from all swab samples in both inoculated groups when were tested with RRT-PCR assay. Moreover, this study also showed that the H5N1 virus was preferentially shed from the oropharynx more than from the cloaca. This suggested that the H5N1 virus was shed at higher virus titer from the respiratory tract when compared with the digestive tract. This result was similar to the previous findings (Jeong et al., 2009 and Brown et al., 2008). The difference in preference routes of virus shedding between cloaca and oropharynx might be caused by the characteristic of viruses. Moreover, the previous studies revealed that the avian influenza virus frequently bound to α-2,3-linked sialic acid receptor which generally located at the respiratory tract of many poultry, including chickens (Horimoto and Kawaoka, 2001 and Forrest et al., 2010). Our results also found that the virus titer was rarely detected from the swab samples at any times of post exposure except at 24 h before chicken death, which showed a peak of the virus titer as similar to Brown et al. (2008). In addition, the average virus shedding titer increased following by the prolonged times of post inoculation which was similar to Brown et al. (2009). Noteworthy, the selection of the appropriate method for collecting the avian influenza virus in routine surveillance program is of importance. Comparing the virus shedding titer between both inoculated groups was clearly indicated that the virus shedding titer of group 2 was significantly higher than that of group 3 at all times of post inoculation. Based on this result, it is reasonable to assume that the chickens from group 2 received higher amount of H5N1 virus than chickens of group 3. The difference in amount of virus, which chicken in both group were received, may cause from the survival of virus in the infected house flies. Thus, rate of H5N1 virus loss correlated with times of post exposure.

For histological examination, the collected tissue samples were selected on the basis of the site of viral replication and pathological change in tissue of H5N1 infected chickens as previously reported (Perkin and Swayne, 2001 and Patin-Jackwood and Swayne, 2009). The results showed that the histopathological lesions were observed in most of tissue section samples whereas no histopathological lesions were observed in

the negative control group corresponded with the previous reports (Perkin and Swayne, 2001, Liu et al., 2005, Isoda et al., 2006 and Saito et al., 2009). However, no histopathological lesions were observed in the brain of infected chickens, while the previous study showed that the H5N1 virus preferentially replicated in the brain of the infected chickens (Patin-Jackwood and Swayne, 2009, Jeong et al., 2009 and Kwon and Swayne, 2010). This difference possibly caused by the time at collecting tissue sample. Since all of the infected chickens in this study died at the during night period, researchers could not access to this isolator to bring the dead chickens to necropsy and collect the tissue samples resulting in the autolysis of the brain. Secondly, the infected chickens of both inoculated groups died in the early period after infection. Theses dead birds were found the respiratory failure caused by cytokine storm before the inflammatory cell migrated into the brain tissue (Tisoncik et al., 2012). Therefore, no histopathological lesions were observed in the brain of infected chickens.

Nevertheless, the immunohistochemistry revealed that the NP viral antigen was detected in all tissue section samples especially in pancreas of the inoculated groups, whereas no viral antigen was detected in all tissue section samples in negative control group. This result was related with the previous publications (Perkin and Swayne, 2001, Keawcharoen et al., 2008, Löndt et al., 2008 and Jeong et al., 2009). Moreover, based on the results of part I, we possibly assumed that the infected house flies in groups 2 and 3 could consume approximately 10^6 and 10^4 ELD_{50}/ml of H5N1 virus during 15 min feeding period, respectively. Whereas, the minimum infectious dose of the H5N1 virus that can cause disease in chickens was 10^2 EID_{50}/ml (Forrest et al., 2010). Regarding to this point of view, the results of this section demonstrated that the house flies could carry the H5N1 virus, which was sufficient to cause the disease in chickens under experimental condition. This is in comparison with the results of Calibeo-Hayes et al. (2003), which demonstrated that the oral inoculation with Turkey corona virus (TCV) infected house flies could cause infection in infected turkeys, suggesting that house flies could transmit TCV. Similarly, naïve swine became infection with PRRSV when house flies containing with PRRSV entered into naïve swine farm (Pitikin et al., 2009). Many

previous studies indicated that the house flies, which are closely associated with humans and animals in particular poultry operation, are considered to play an important role in the carrier of many pathogens (Kobayashi et al., 1999 and Fasanella et al., 2010) and are able to disseminate pathogens into surrounding areas.

Moreover, Tsuda et al. (2009) reported that *Calliphora nigribarbis* was capable of flying ranging from 750 to 1150 meters within 4.5 h and Graczyk et al. (1999) showed that the average of flying distance of the individual house fly was about 3.2 kilometers per day. In conclusion, this study suggested that the house flies (*Musca domestica*) could serve as a mechanical vector of avian influenza virus subtype H5N1.

The objective of the third part was to examine the presence of the avian influenza virus subtype H5N1 naturally infected house flies in epidemic area in Thailand during 2008-2009. In regard to the objective, four avian influenza virus subtype H5N1 outbreaks were officially reported in Thailand during the 2008 to 2009; three outbreaks were found in backyard poultry and one outbreak was found in the poultry farm at Nakornsawan province. All of epidemic areas were located in the central region of Thailand surrounding with rice field and several animal species, including dogs, cats, poultry and capture wild birds such as Red-Whishered Bulbul or *Pycnonotus jocosus*.

Overall, 62 flies composing of three species were collected from fifteen different locations with 10 km in a radius from the epidemic area except for the grazing duck flock, which was located 15 km in a radius from the epidemic area. House fly or *Musca domestica* was the major species among all of the collected files (91.93%) while 6.45% and 1.61% of collected flies were *Chrysomyia megacephala* and Sarcophaga sp. respectively. This finding corresponded with Echeverria et al. (1983). Echeverria et al. (1983), which reported that approximately 90% of flies collected from Ban Pong village, Soongnern district, Nakornrajsima province, Thailand was *Musca domestica*. Förster et al. (2007) also demonstrated that the *Musca domestica* was the dominant captured flies in rural area with the domestic animal related place (dog pound, poultry house, cattle barn, horse stable and pig-pen) in Dormagen town, Germany. In contrast to Sawabe et al. (2006) which showed that 80% of collected flies at Tamba town, Japan were

Calliphora nigribarbis without the presence of *Musca domestica*. The possible explanation for this difference might occur from the difference of temperature at collected site; the temperature at Tambo town was -2.0 to 19.2 ^{O}C while the other places were 25 to 35 ^{O}C (at Nakornrajsima province) and 20 to 30 ^{O}C (at Dormagen town). *Calliphora nigribarbis* proliferates under cold environment whereas *Musca domestica* can generate under tropical environment (Graczyk et al., 1999, Hogsette, 1999 and Graczyk et al., 2001). Therefore, the relationship between the collected area and the species of collected flies was of importance (Echeverria et al., 1983 and Hald et. al., 2008). *Musca domestica* are commonly associated with humans and animals and the breeding place of the domestic house flies is usually found at the human excrement, garbage, landfill sites and animal manure (Graczyk et al., 1999) whereas the breeding place of *Chrysomya megacephala* and Sarcophaga sp. was found at decomposed tissue and carrion or fresh carcass, respectively (Greenberg, 1973). However, these data supported our finding that the house flies were collected as major species compared with other species.

The synathropic flies, particularly domestic house flies, are able to carry several etiological pathogens of humans and animals (Fotedar 2001, Szalanski et al., 2004, Zweifel et al., 2008 and Yap et al., 2008) via the regurgitation of vomit and via the body surface (Fasanella et al., 2010). Therefore, the results of virus isolation from 16 pooled of *Musca domestica*, 1 pooled of *Chrysomyia megacephala* and one *Sarcophaga* sp. collected flies showed no evidence of the avian influenza virus determined by RRT-PCR assays. Whereas Sawabe et al. (2006) revealed that the avian influenza subtype H5N1 was detected from *Calliphora nigribarbis* at Tambo town, Japan. In addition, Chakrabarti et al. (2007) detected the exotic Newcastle disease virus from collected flies (*Musca domestica, Phaenicia cupria* and *Fannia canicularis*) at the epidemic area. The possible explanations of this contrast were (i) the amounts of infective virus in collected house flies was very low until the diagnostic test could not detect. Since the researcher could reach into epidemic areas approximately 2 days later after outbreaks were reported. At that time, the infected chickens and suspected chickens in epidemic areas

had already been destroyed. Moreover, the Swayne and Beck (2005) showed that the shedding titer of H5N1 virus from infected chickens was approximate $10^{4.27-7.72}$ EID_{50}/ml for respiratory secretion and $10^{2.5-4.5}$ EID_{50}/g feces from cloaca. According to this information, the highest H5N1 virus titer that the house flies in epidemic areas could carry in day 2 after outbreaks was below 10^{1} EID_{50}/ml. Therefore, the virus isolation and the RRT-PCR assay results in this study showed no presence of the H5N1 virus in collected flies (ii) the amounts of collected flies in this study was less than those of other previous reports, (iii) the sampling location was at remote area and take a long time to submit the samples to laboratory, and (iv) due to the regulation policy of restriction area of the government, the strangers is not allowed to the epidemic area. Normally, the flight ranges of the house flies traveling in the village was approximately 2 km within 24 h (Nazni et al., 2005) and the maximum record of the flight range of the house flies was 11.8 km within 24 h (Greenberg, 1973). The difference in flight distance between two previous reports may be caused by the food source of the house flies. However, in this study, the flight range of infected flies in the epidemic areas might be 4 km within 2 days while the collected fly site was 10 km in a radius. Then, the results from this study showed no evidence of the H5N1 virus in the flies collected from epidemic areas when determined by virus isolation and RRT-PCR assays. However, this information in this study has implicated for controlling flies population and improves the priority of biosecurity regulation in poultry productions.

Conclusion

The data presented in this study, indicating that (i) the house flies (*Musca domestica*) served as potential mechanical vectors, which could disseminate avian influenza virus subtype H5N1 under experimental condition (ii) the house flies played an important role for transferring the avian influenza virus subtype H5N1 to chickens and caused death under experimental condition (iii) the viral shedding titer from oropharyngeal swabs of chickens inoculated with H5N1 exposed house flies was higher than those from cloacal swabs, thus the oropharyngeal swabs should be considered for avian influenza virus surveillances (iv) the house fly control and elimination should be

performed to improve the biosecurity strategies. However, further study will be done for determining the actual role of the house fly as a vector of avian influenza virus in natural condition and the strategy of collected flies in epidemic areas should be improved.

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