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The potential of house fly, *Musca domestica* (L.) in the mechanical transmission of influenza A subtype H1N1 virus under laboratory conditions

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Abstract

A study on house flies was carried out to establish whether house flies can transmit the H1N1 virus mechanically due to their abundance, ability to transport pathogens and their behavioral traits of regurgitation and defecation. The objectives of this study were to examine the efficiency of house fly legs in picking up the influenza H1N1 virus particles, persistency of the virus particles on the legs at different time interval, viability of the virus dislodged from the legs and the presence of the virus in vomitus and fecal discharge of the house flies. The findings indicated that the persistency of H1N1 virus on fly legs could be detected up to 24 h in chilled and actively flying flies. Furthermore, the viability of virus was evidenced from immobilized flies exposed for 30 s. However, H1N1 virus was not detected in the vomitus and feces. Further, epidemiological studies are needed before the significance of house flies as transmitter of influenza virus can be determined.

Keywords: H1N1 virus, epidemiological studies, house flies, influenza, transmit.

INTRODUCTION

The house fly *Musca domestica* (L.) is a cosmopolitan species and its synanthrophic behavior of breeding in animal manure; human excreta, garbage and animal bedding have evolved the house fly to live in association with man (Thaddeus et al., 2001). House flies are a nuisance and mechanically transport a host of diseases to human such as trachoma, cholera, anthrax, diphtheria, tuberculosis and leprosy by food contamination due to their behavioral patterns of ovipositing and feeding on decaying matter, human excrement and animal manure (Lane and Crosskey, 1993). Transmission of pathogens by adult flies occurs through dislodgement from exo-skeleton, fecal deposition and regurgitation (Greenberg, 1973). Several studies have indicated that house flies play an important role in transmission of viruses. New-castle virus has been isolated from the surface of the fly body (Milushev et al., 1977). Medvecky et al. (1988) reported their experimental study on the role of house flies in transmission of pseudo rabies in pig, rabbit and lamb, while Emerson et al. (2000) and Forsey (2001) stated that house flies played an important role in transmission of eye disease among children. Satoshi et al. (2003)

conducted a laboratory study on a colony of 210 house flies fed on the feces of a pig infected with Porcine Reproductive Respiratory Syndrome Virus (PRRSV). All the samples from the pigs and house flies were tested positive for PRRSV nucleic acid by reverse transcriptase polymerase chain reaction, demonstrating that infectious PRRSV could remain viable in the intestinal visceral of a house fly up to 12 h following feeding on the faeces of a viraemic pig. These findings together with detection of infection from exterior surface indicated the ability of a house fly to transmit PRRSV mechanically.

Highly pathogenic avian influenza, or "fowl plaque" was identified as an infectious disease of birds and chicken in Italy in 1878. Avian influenza A (H5N1) virus strains that emerged in Asia in 2003 continued to evolve (Lupiani et al., 2009). There were 341 human fatalities due to Avian Influenza A/(H5N1) reported worldwide (Globalhealthfacts.org, 2012). Normally, influenza is not transmitted by insects, but since the virus is relatively environmentally stable, mechanical transmission by insects such as house fly could be possible. As the virus can survive long period in chicken feces and feed, flies may pick it up when landing on feces or infected dead bird and then carry it to other animals. Fomite can also be important in transmission of viruses and flies may act as a mechanical vector (Beard, 1998; USDA APHIS, 2002; WHO, 2004). Bean et al. (1985) isolated influenza virus of low virulence from chicken and the virus subsequently became virulent which was later identified as influenza H5N1 serotype. Subsequently, Sawabe et al. (2006) detected and isolated a highly pathogenic H5N1 virus from blow flies from an infected poultry farm in Kyoto, Japan. However, to date, no studies have been reported on the possibility of house flies in the transmission of Influenza A subtype H1N1 virus.

The objectives of our study were to examine the efficiency of house fly legs as a model in picking up the influenza H1N1 virus particles, persistency of the virus particles on the legs, viability of the virus dislodged from the legs and the presence of the virus in vomitus and fecal discharge. Our experimental work focused on the house flies because of their abundance, ability to transport pathogens and behavioral traits of regurgitation and defecation (Greenberg, 1973).

MATERIALS AND METHODS

House fly (*M. domestica*)

The laboratory- bred WHO IJ2 strain of house fly (F202) used in this study was colonized and maintained in the ACL-2 (Arthropod Containment Level-2) insectarium of Medical Entomology Unit, Infec-tious Disease Research Centre, Institute for Medical Research, Kuala Lumpur. The house flies were reared in a wooden cage, on larval food consisting of damp ground mouse chow pellet with a photoperiod of 12:12, constant temperature of 26±2°C and relative humidity of 80±5%. The adults were fed on a diet of sugar and soaked cotton wool served as water source. Fermented mouse chow pellet served as breeding media for the adults. This colony was maintained in the insectarium without exposure to any pathogens and re-confirmed negative with influenza H1N1 virus infection by RT-PCR.

Virus

The influenza A virus, A/New Caledonia/20/99 strain, subtype H1N1 belonging to the family of Orthomyoxiviridae (Lamb and Kruger, 1996) used in this study was cultivated from a virus culture stock belonging to the Unit of Virology, Infectious Disease Research Centre, Institute for Medical Research, Kuala Lumpur.

Virus isolation in cell culture

An ampoule of Madin-Darby Canine Kidney (MDCK) cells stored in liquid nitrogen was transferred to a water bath until thawed completely. The content was pipetted slowly drop by drop into a 75 cm^2 tissue culture flask containing Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal calf serum (GIBCO, Invitrogen, USA) with 50 U/ml benzyl penicillin and 50 µg/ml streptomycin sulphate (Sigma, St Louis, USA) and incubated overnight at 36°C. The degree of cell confluence was checked under an inverted microscope. Once confluence, the growth medium from the cell culture flask was removed and the cells were washed twice with sterile phosphate buffered saline (PBS) without calcium and magnesium. 0.25% of trypsin-EDTA (Biowest) was added to the monolayer cells and the flask was placed in a 36°C incubator for about 5 min until the cells were completely detached from the surface. The detached cells were re-suspended in growth medium (DMEM) and the suspension was gently aspirated a few times through a fine Pasteur pipette to break up cell clumps. The number of cells were counted using a haemacytometer and normally 1×10^5 cells/ml were enough to obtain a confluent and monolayer cells within 2 to 3 days after incubation at 36°C. 1 ml of cells was transferred to each culture tubes and placed in the 36°C incubator until nearly confluent. The growth medium in the culture tubes was removed and the test specimens were added to the cells and allowed adsorption for 30 min at 37°C. Later, 1 ml of complete medium containing trypsin solution without fetal bovine serum (FBS) (Biowest) was added to the tubes and incubated in a stationary sloped (5°) position at 37°C. Cultures were observed daily for cytopathic effect (CPE) in which the cells become rounded, refractile and ultimately shrunk before detaching from the tubes.

Cultures were harvested when more than 90% of the cell monolayer showed cytopathic effect. The virus was reconfirmed as Influenza type A virus by reverse transcriptase-polymerase chain reaction (RT-PCR).

Reverse transcription-polymerase chain reaction (RT-PCR)

RNA extraction was performed using EZ-10 Spin Column Total RNA Minipreps Super Kit™ from Bio Basic Inc (Canada). In brief, 200 µ1 of infected virus solution was mixed with 400 µ1 RLT solu-tion and the manufacture's procedure was subsequently adhered to. The extracted RNA was stored at -70°C until further use. One step RT-PCR using cMaster RTplusPCR System™ and Cmaster RT Kit™ (Eppendorf™, Hamburg, Germany) was performed. RT-PCR was carried out in a 20 ul reaction mixtures containing 3.95 µ1 of RNase free water, 2.0 µ1 of RT-PCR buffer, 0.4 µ1 of dNTP mix, 0.2 µ1 RNAse inhibitor, 0.25 µ1 cMaster RT, 0.2 µ1 cMaster PCR and 10 µ1 of RNA template. 1.5 µ1 (20 pico mole) of forward primer NPF 5'-CAG-RTA-CTG-GGC-HAT-AAG-RAC-3' and reverse primer NPR 5'-GCA-TTG-TCT-CCG-AAG-AAA-TAA-G-3' (Lee et al., 2001) were used to amplify the 330 bp nucleoprotein gene of influenza virus. Reverse transcription was carried out at 42°C for 40 min follo-

wed by 45 PCR cycles, denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 0.4 min. The PCR products were examined by gel electrophoresis (Promega™, USA) stained with ethidium bromide (GelStar®).

Experimental design

Four experiments were conducted to determine the potential of house fly to transmit the virus. In all experiments, house flies of both sex starved overnight and aged 3 to 4 days old were used. All the experiments were conducted in a BSL-2 containment laboratory in accordance to established biosafety protocols.

Experiment 1: Influenza A H1N1 virus load acquisition by house fly legs

The experiment was conducted to detect the presence of Influenza A H1N1 virus mixed with bovine serum albumin to enhance adherence of virus particle on fly legs. A serial dilution of virus concentration in 10% bovine serum albumin was prepared. The dilution ratios used were 1:10, 1:4, 1:3, 1:2, 10:1 and 1:1 in the ratio of Influenza A infected tissue culture fluid: Bovine serum albumin. 50 µl suspension of each dilution was pipetted onto a sterile plastic surface placed in an ice box and exposed to flies. The titre of virus used in all the experiments was 3×10^6 $TCID_{50}$/ml. Flies were immobilized for 2 min at -20°C and were individually held over the virus suspension using forceps so that their legs came into contact with the suspension in order to simulate a walking motion on the virus suspension for 10 s. Ten flies were used per dilution ratio. The fly legs were then immediately washed with 200 µl of RNAnase free water to dislodge the virus particles from their legs. RNA was extracted from the dislodged virus solution and PCR was conducted to detect the virus.

Experiment 2: Persistency of Influenza A H1N1 virus on house fly legs

The presence of virus in the dilution shown as the brightest band on gel electrophoresis from experiment 1 was used to determine the persistency of virus on house fly leg. House flies were chilled at -20°C and individually held over the selected dilution of virus suspension so that their legs came into contact with influenza A virus for 10 s. Exposed flies were tested for the presence of the virus at interval of 30 s, 1 min, 5 min, 15 min, 30 min, 1 h, 2 h and 24 h post-exposure to the virus suspension. Exposed flies were divided into 2 groups: those chilled at 0°C and the actively moving flies. The survival of the virus on chilled flies and active flies were compared. After the respective time interval, the fly legs from both groups were immediately washed with 200 µl of RNAnase free water to dislodge the virus particles from the legs. RNA was extrac-ted from the dislodged virus solution and PCR was conducted. Two replicates were conducted with 10 flies per replicate.

Experiment 3: Viability of Influenza A H1N1 virus dislodged from house fly legs

The experiment was similar to experiment 2, except that the virus was isolated from flies sampled at 30 s and 5 min after a 10-s expo-sure. The fly legs were washed with 200 µl of RNAnase free water to dislodge the virus particles from both the immobilized and active flies. The solution was then filter sterilized through a 0.22 µm Spritzan Syringe Filter (TPP, Europe). The filtrate was inoculated into the MDCK cell line and observed daily for the presence of CPE before being harvested when more than 90% of the cell monolayer showed CPE. Two replicates were conducted with 10 flies per experiment. In the absence of CPE, the presence of virus was detected using immunofluorescence. Viral screening and identifica-tion IFA Kit (Millipore™, CA, USA) was used to stain the virus infec-ted cells. The slide was viewed under an immunofluorescence microscope at 400 to 500 nm range (blue light). Two passages of the virus were conducted to re-confirm the test results.

Experiment 4: Influenza A H1N1 virus in fly vomitus and fecal discharge

A total of 50 house flies (<7 days old) were collected from the laboratory colony and starved for 24 h prior to feeding on the virus. The flies were then immobilized for 2 min at -20°C and introduced into a plastic container (15 × 8 × 3 cm) consisting of a sterilized glass slide coated with 200 µl of tissue culture medium containing Influenza A virus. The flies were allowed to feed on the medium at room temperature for 1 h. After the exposure period, the flies were immobilized and removed into a sterile centrifuged container consisting of 3 ml RNAnase free water and vortexed vigorously for 1 min to dislodge the virus particles picked up by the fly legs, mouthparts, wings and the hairy body structure while feeding. RNA was extracted from the dislodged virus solution and PCR was conducted. The feces and vomitus were collected separately with 500 µl of RNAnase free water from the glass slide and the spots of vomitus and feaces were picked from the plastic container which was observed to contain large amount of vomitus and fecal discharges.

RESULTS

Experiment 1: Influenza A virus load acquisition by house fly legs

The initial dilution of the virus in tissue culture fluid with the diluent bovine serum albumin indicated positive RT-PCR results in all the dilutions tested. A weak band was observed at 1:10 dilution (virus in tissue culture fluid: bovine serum albumin). The dilution of 1:2, gave a clear and sharp band at 330 bp, indicating the strong presence of Influenza A virus dislodged from the fly legs (Figure 1). Hence, the dilution of 1:2 was used in subsequent experiments.

Experiment 2: Persistency of Influenza A virus on house fly legs at different time intervals

Influenza A virus was detected by RT-PCR from the house fly leg that has been simulated to walking on infected virus medium. The virus was detected in all post-exposure intervals of 1 min, 5 min, 15 min, 30 min, 1 h, 2 h and 24 h in chilled and actively flying flies. However, as the bands detected were faint possibly due to low concentration of virus obtained from the single leg, only the results of the 24-h post-infection was shown (Figure 2). The results indicated that Influenza A nucleic acid can be detected up to 24 h on house flies legs.

Experiment 3: Viability of Influenza A virus dislodged from house fly legs

In the first passage, there was no cytopathic effect observed in all samples, but the virus was detected by immunoflou-

dilution of 1:2, gave a clear and sharp band at 330 bp, indicating the strong presence of Influenza A virus dislodged from the fly legs (Fig 1). Hence the dilution of 1:2 was used in subsequent experiments.

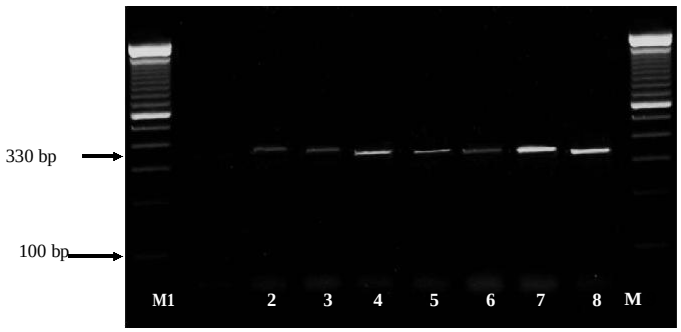

Figure 1. Agarose gel electrophoresis of RT-PCR amplified products for the detection of Influenza A virus at 330 bp. Lane M, size markers (100 bp ladder, Bioron, Germany) Lane 1 – 8 dilution of concentration of virus obtained from the single culture fluid; Lane 1- 1:10; lane 2- 1:4; lane 3- 1:3; lane 4- 1:2; lane 5- 10:1; lane 6- 1:1; lane 7- Influenza A infected tissue culture fluid; lane 8- positive control (Influenza A virus); lane M– size markers (100 bp ladder, Invitrogen Life Technology).

etected up to 24 h on house flies legs.

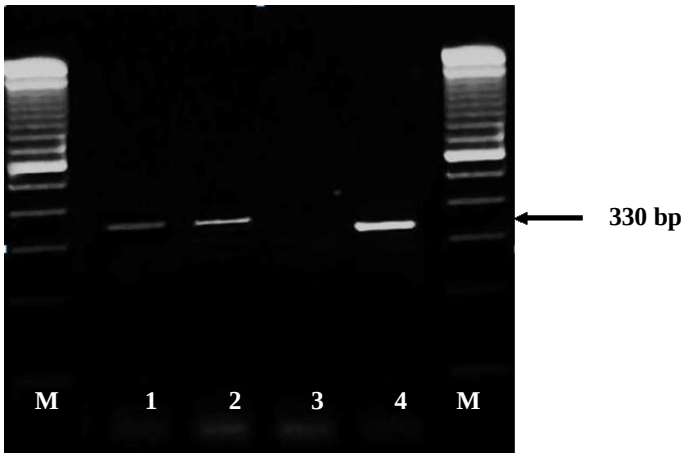

Figure 2. Agarose gel electrophoresis of RT-PCR amplified products for the detection of Influenza A virus at 330 bp after 24 h exposure. Lane M, size markers (100 bp ladder, Bioron, Germany). Lane 1- active flies after 24 h exposure to Influenza A virus; lane 2- chilled flies 24 h after exposure to Influenza A virus; lane 3- negative control (master = mix only); lane 4- positive control (Influenza A virus); lane M- size markers (100 bp ladder, Invitrogen Life Technology).

rescence for the 30 s exposure in both immobilized and active flies (Figure 3). In the second passage, the influenza virus was detected in both the 30 s immobilized and active flying flies using the immunofluorescence technique; however, cytopathic effect was only observed for flies exposed for 30 s in immobilized state (Table 1).

Experiment 4: Presence of Influenza A virus in fly vomitus and fecal discharge

The vomitus specks were pinkish in colour while the fecal specks were yellowish. Results of RT-PCR indicated that no PCR product was detected at 330 bp, indicating that the Influenza A virus was not found in the vomitus, feces and the external body of the flies.

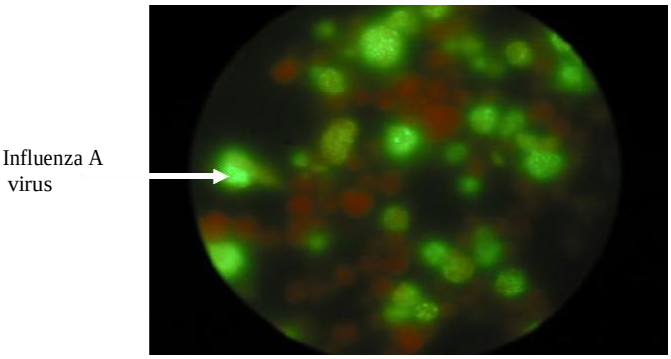

Fig 3. Detection of influenza A virus using technique

Figure 3. Detection of Influenza A virus using immunofluorescence technique.

DISCUSSION

Influenza A virus adherence to the fly legs persisted up to a period of 24 h, suggesting that the virus particles were trapped in the hairs and bristles on the legs of the house flies while the fly was in motion and therefore justifying the ability of house fly to transmit other viral infection like swine virus mechanically (Satoshi et al., 2003). In the experiment to detect the viability of virus at the different exposure periods in immobilized condition as well as in active state, virus in flies exposed for 5 min in both the immobilized and active flying state were not detected by immunofluorescence or tissue culture. The reason that CPE was not detected at 30 s in the actively flying insects could be due to the low viral load. as "However, the results showed that immunofluorescence was detected in both the actively flying and immobilized flies exposed for 30's in passages 1 and 2". "Furthermore, the results indicated that influenza A virus was still viable post exposure in immobilized flies suggesting that virus transmission through flies is possibl e". In the feeding experiment, while feeding, flies had the habit of regurgitating resulting in vomitus specks on the surface they explored. In addition, such surface also had fecal specks. It is noteworthy that the density of vomitus specks was higher than the fecal specks, indicating that the fly could probably spread infected organisms more often by vomitus rather than by feces. However, the current study demonstrated that Influenza A virus was not present in the vomitus and feces of the fly, probably due to the low viral load (<10 to 30 pico gram of RNA) detectable by RT-PCR.

Swabe et al. (2011) reported that crops and intestines dissected from fly at various times after virus exposure were used for virus isolation and titration. Virus was isolated from fly crops and intestines up to 24 h post-exposure and from feces and vomitus matter of 1 out of 3 blow flies at 48 h after exposure. At 14 days after exposure, no virus was isolated from any blow fly at 20 or 10°C. The H5N1 viral gene could be detected in blow flies up to 14

Table 1. Detection of Influenza A H1N1 virus using tissue culture and immunofluorescence techniques.

Exposure time	1st passage		2nd passage	
	T/ culture	IF	T/culture	IF
30 s ACT	No CPE	+	No CPE	+
30 s IMB	No CPE	+	CPE	+
5 min ACT	No CPE	-ve	No CPE	-ve
5 min IMB	No CPE	-ve	No CPE	-ve

ACT, Flies flying actively; IMB, immobilized flies; T/culture, tissue culture; IF, immunofluorescence technique; CPE, cytopathic effect.

days after exposure; no viable virus was detected 48 h post- exposure. These findings are important since the ability of influenza virus to reside within the body of a house fly may protect it against certain environmental factors known to be detrimental to influenza survivability outside the host such as ultraviolet light and drying. Furthermore, house flies frequently inhabit the interior of transport vehicles and livestock trailers. This may enhance exposure of the insects to influenza infected animal, and allow the movement of the insects to travel over greater geographical distances up to 7 km (Nazni et al., 2005). Hence, in the process of flying, house fly may serve as a mechanical vector of influenza virus. Because of the paucity of study on the potential of house fly to transmit Influenza A H1N1 virus, the findings from this study could only be compared with studies on other related viruses such as the H5N1 subtype transported by flies. Highly pathogenic H5N1 virus was detected and isolated from blow flies in an infected poultry farm in Kyoto, Japan (Sawabe et al., 2006). The virus genes were detected from the intestinal organs, crop and gut of two blow fly species, *Callliphora nigribarbis* and *Aldrichina grahami*, by RT-PCR. The authors suggested that it is possible that blow flies could be mechanical transmitters of H5N1 virus.

Earlier investigation in United States indicated that a virulent H5N1 virus was detected in house fly in a poultry farm (Bean et al., 1985). In a recent study, Wanaratana et al. (2010) showed that the virulent AI H5N1 virus con-sumed by house flies as food contaminated with this virus could carry the virus within their bodies and remained infective up to 72 h post infection and their RT-PCR was positive up to 96 h post infection. However, they showed that viruses could be detected in external surfaces of house flies for only up to 24 h post-exposure. Tyasasmaya et al. (2012) had also isolated highly pathogenic avian influenza virus H5N1 (AIV H5N1) from field collected house flies in Java, Indonesia. In a recent review by Sawabe (2011), H5N1 virus in blow flies could be detected up to 14 days after exposure, but no viable virus was detected at 48 h post- exposure. They further mentioned that con-tamination of the body surface of blow fly was much less compared to house flies where the body surface could be easily contaminated by the viruses. The author also stated that the transmission mechanism could be complicated due to the habit of blow flies which prefer to lick carcasses and droppings of chicken and pigs. These behavioural traits are not only present in blow flies but also in most flies including the house flies. Elsewhere, Sievert et al. (2006) reported the existence of avian influenza virus in the house flies, while studies in Malaysia showed that rotavirus can be mechanically transported by fly contaminated surfaces (Tan et al., 1997).

The prevalence of Influenza H1N1 is a serious public health problem in human. It is important to note that flies often occur in millions in human habitations, indicating that flies will definitely play an important role in transmission of virus particles. Field and laboratory studies on mechanical transmission of virus by flies would contribute greatly to the control of influenza outbreaks. Therefore, further studies on field-collected house flies are needed before a final conclusion can be drawn regarding the significance of house flies as a transmitter of influenza virus and impact on the human population.

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REFERENCES

Bean WJ, Kawaoka, JM, Wood JE, Pearson RG, Webster (1985). Characterization of virulent and avirulent A/chicken/ Pennsylvania/83 influenza A viruses: potential role of defective interfering RNAs in nature. J. Virol. 54 (1):151-160

Beard CW (1998). Avian influenza. In foreign Animal Diseases, Richmond, V.A; United States Animal Health Association .pp. 71-80

Emerson PM, Bailey RL, Mahdi OS, Walraven O, Lindsay SW (2000). Transmission ecology of fly *Musca sorbens*, a punitive vector of trachoma. Transaction of Royal Society Trop. Med. Hyg. 94(1): 28-32 Forsey T, Drouugar S (2001) Transmission of chlamydia by the housefly. Brit. J. Ophtalmol. 65: 147-150

Globalhealthfacts.org. (2012). Avian Influenza A/(H5N1) cumulative number of confirmed human deaths. as of January 18, 2012, US Global Health www.globalhealthfacts.org/data/topic/map.aspx?ind=33

Greenberg B (1973) Flies and Diseases: Volume II; Biology and Disease transmission, Princeton University Press, Press, Princeton, New Jersey.

Lamb RA, Krug RM (1996). Orthomyxoviridae: the viruses and their replication. In: BN Fields, DM, Knipe PM, Howley RM, Chanock JL, Melnick TP, Momath, B Roizman (Eds.), Fields Virology, 3rd ed., Lippincott-Ravee, Philadelphia. PA.

Lane RP, Crosskey RW (1993). Houseflies, Blowflies and their allies (Calypterate Diptera). In Lane RW and Crosskey RW (eds) Medical insects and arachnids. Champman and Hall, London, pp. 403-428

Lee MS, Chang PC, Shien JH, Cheng MC, Shieh HK (2001). Identification and subtyping of avian influenza viruses by reverse transcription-PCR. J. Virol. Methods 97(1-2):13-22.

Lupiani B, Reddy MS (2009). The history of avian influenza. Comparative Immunology. Microbiol. Infectious Dis. 32: 311–323

Medvecky I, Kovacs L, Kavacs F, Papp L (1988). The role of the house flies *Musca domestica* in the spread of Aujesky's disease (pseudorabies). Med. Vet. Entomol. 2: 81-86

Milushev I, Greganov G, Shishkov N (1977). The role of the house flies in the epizootiology pseudorabies in birds. Vet. Med. Nausaki 4: 97-100

Nazni WA, Luke H, Wan Rozita WN, Abdullah AG, Sa'diyah I, Azahari AH, Zamree I, Tan SB, Lee HL, Sofian MA (2005). Determination of the flight range and dispersal of the housefly *Musca domestica* (L) using mark release and recapture methods. Trop. Biomed. 22(1): 53-61

Satoshi O, Scott A, Dee RD, Moon K, Rossow D, Carlos T, Mac DF, Carlos P (2003). Survival of porcine reproductive and respiratory syndrome virus in the house flies. Canadian J. Vet. Res. 67(3): 198-203

Sawabe K, Hoshino K, Sasaki T, Isawa H, Hayash, T, Tsuda Y, Kuruhashi H, Tanabayashi K, Hotta A, Saito T, Yamada A, Kobayashi M (2006). Detection and isolation of highly pathogenic H5N1 avian influenza A viruses from blow flies collected in the vicinity of an infected poultry farm in Kyoto, Japan. Amer. J. Trop. Med. Hyg. 75(2): 327-332.

Sawabe K, Hoshino K, Isawa H, Sasaki T, Kim KS, Hayashi T, Tsuda Y, Kurahashi H, Kobayashi M (2011). Blow flies were one of the possible candidates for transmission of highly pathogenic H5N1 Avian Influenza Virus during the 2004 outbreaks in Japan. Influenza Res. Treatment, Article ID 652652, p. 8.

Sievert K, Alvarez R, Cortada R, Valks M (2006). House flies carrying avian influenza virus (AIV). International Pest Control 48 (3): 114–116.

Tan SW, Yap KL, Lee HL (1997). Mechanical transport of rotavirus by the legs and wings of *Musca domestica* (Diptera: Muscidae). J. Med. Entomol. 34(5): 527-531

Thaddeus KG, Ronald K, Robert HG, Michael RC (2001). The role of non biting flies in the epidemiology of human infectious diseases. Microb. infect. 3: 231-235

Tyasasmaya T, Wuryastuty H, Wasito R, Sievert K (2012). Experimental evaluation of the house flies (*Musca domestica spp.*) as a possible vector for avian influenza virus H5N1. In: Current Status of Veterinary Biologicals and Opportunities and Challenges for the Future www.ivvdc2012.org/assets/IVVDC/ivvdc-abstracts.pdf

US Department of Agriculture, Animal (USDA), And Plants Health Inspection Services (APHIS), veterinary Vaccines for terrestrial animal. Highly pathogenic avian influenza. US poultry vaccines for terrestrial animal.(2002). Highly Pathogenic Avian Influenza, a threat to http://www.aphis.usda.gov.oa/pubs/avianflu.html.

Wanaratana S, Panyim S, Pakpinyo S (2011). The potential of house flies to act as a vector of avian influenza subtype H5N1 under experimental conditions. Medical and Veterinary Entomology 25 (1): 58-63

World Health Organization (WHO) (2004). Avian influenza fact sheet. http//www.who.int/crs/don/2004-01-15-en/.