UNIVERSITI PUTRA MALAYSIA

DETECTION OF AFRICAN SWINE FEVER VIRUS ON FLIES IN SELECTED PIG FARMS IN PENINSULAR MALAYSIA

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DETECTION OF AFRICAN SWINE FEVER VIRUS ON FLIES IN SELECTED PIG FARMS IN PENINSULAR MALAYSIA

LAU SHU XIN

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CERTIFICATION

It is hereby certified that we have read this project paper entitled "Detection of African swine fever virus on flies in selected pig farms in Peninsular Malaysia" by Lau Shu Xin and in our opinion, it is satisfactory in terms of scope, quality, and presentation as partial fulfilment of the requirement for the course VPD 4999 – Final Year Project.

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DEDICATION

This thesis is especially dedicated to:

My loving parents

Lau Yew Chin and Wong Siew Hium

My supportive supervisors

Associate Professor Dr. Ooi Peck Toung

Associate Professor Dr. Nor Yasmin Abd Rahman

Dr. Michelle Fong Wai Cheng

Dr. Nur Mahiza Md Isa

And

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PENGESANAN VIRUS DEMAM BABI AFRIKA PADA LALAT DI LADANG BABI TERPILIH DI SEMENANJUNG MALAYSIA

Oleh

LAU SHU XIN

2023

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Demam babi Afrika (ASF) adalah penyakit virus maut yang menyebabkan kerugian ekonomi yang ketara dalam industri babi di seluruh dunia disebabkan morbiditi dan kematian yang tinggi. Penyakit ini disebabkan oleh virus demam babi Afrika (ASFV) yang menjejaskan kedua-dua babi hutan dan babi domestik. Laluan penularan ASFV telah disiasat secara meluas untuk melaksanakan langkah-langkah pencegahan dan kawalan yang relevan dalam mengurangkan risiko penularan ASF di ladang babi. Selain daripada fomit yang tercemar atau babi yang dijangkiti sebagai sumber jangkitan ASF di Malaysia, vektor mekanikal lain seperti lalat, tikus atau burung masih

belum disahkan. Kajian ini menilai pengesanan ASFV pada lalat dan kepadatan lalat di lokasi yang berbeza dalam ladang. Empat ladang babi di Semenanjung Malaysia telah dipilih untuk kajian ini. Lalat dari kolam kumbahan, stor makanan, kandang babi dewasa dan kandang babi betina daripada empat-empat ladang telah dikumpulkan menggunakan perangkap lalat melekit selama 24 jam. Lima lalat genus yang sama dari setiap lokasi dikumpulkan bersama-sama dengan kepala dan badan di kolam sampel berasingan. *Real-time* PCR dijalankan untuk mengesan kehadiran DNA ASFV dalam lalat. 2254 lalat yang terdiri daripada *Musca* sp. (98%), *Chrysomya sp*. (1%) dan *Stomoxys* sp. (1%) telah disampel. Daripada 50 sampel, empat sampel *Musca* sp. yang terdiri daripada 50% kepala dan 50% badan adalah positif ASFV. Selain itu, statistik menunjukkan tiada perbezaan ketara dalam kepadatan lalat di empat-empat lokasi ladang babi. Kesimpulannya, pengesanan ASFV dalam lalat menunjukkan bahawa lalat adalah vektor mekanikal ASFV. Bilangan lalat yang tinggi yang dikumpulkan dari ladang juga menekankan kepentingan kawalan lalat sebagai salah satu langkah pencegahan dalam penularan ASF di ladang babi.

Kata kunci: *Virus demam babi Afrika (ASFV), lalat, vektor mekanikal, babi, Malaysia*

ABSTRACT

An abstract of the project paper presented to the Faculty of Veterinary Medicine in partial fulfilment of the course VPD 4999 – Final Year Project.

DETECTION OF AFRICAN SWINE FEVER VIRUS ON FLIES IN SELECTED PIG FARMS IN PENINSULAR MALAYSIA

by

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2023

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African swine fever (ASF) is a fatal viral disease that causes significant economic losses in the swine industry worldwide due to its high morbidity and mortality. It is caused by African swine fever virus (ASFV) which affects both wild boars and domestic pigs. Transmission route of ASFV have been widely investigated to implement relevant preventive and control measures in reducing the risk of ASF in pig farms. Aside from contaminated fomites and/or infected pigs as infections sources, other possible mechanical vectors such as flies, rats or birds are not well known. This study assessed the detection of ASFV on flies and the different densities of flies in different locations within the farm. Flies from sewage pool, feed house, finisher pens

and sow pens of four pig farms in Peninsular Malaysia were collected using sticky fly traps over 24 hours. Five flies of the same genus from each location were pooled together with the head and body separated. Real-time PCR was conducted to detect the presence of ASF DNA virus in the flies. In this study, a total of 2254 flies consisting of *Musca* sp. (98%), *Chrysomya* sp. (1%) and *Stomoxys* sp. (1%) were sampled. Four pooled samples of *Musca* sp. consisting of 50% head and 50% body were positive for ASFV among 50 total pooled samples. There was no significant difference in densities of flies in the four locations inside the farms. In conclusion, the detection of ASFV in flies indicates that flies are mechanical vectors of ASFV. The high number of flies collected from the farms also highlights the importance of fly control as one of the preventive measures in the transmission of ASF in pig farms.

Keywords: *African swine fever virus (ASFV), flies, mechanical vector, swine, Malaysia*

1.0 INTRODUCTION

1.0 African Swine Fever

African swine fever (ASF) is a highly infectious and fatal viral disease in domestic pigs and wild boars. It causes huge economic losses to the swine industry worldwide due to its high morbidity and mortality. The aetiological agent is African swine fever virus (ASFV) which is an icosahedral, enveloped, double-stranded DNA virus from the *Asfaviridae* family and *Asfivirus* genus. In the sylvatic cycle in Africa and southern countries of European Union, soft argasid ticks of the *Ornithodoros* genus contributes as one of the sources of infection of ASF as it is both a biological vector and reservoir host for the virus (Nielsen *et al*., 2021). ASFV has been proven to replicate and survive in *Ornithodoros* spp. up to five years (Fila & Woźniakowski, 2020).

The sources of ASFV infection are clearly indicated in Figure 1 where infected domestic pigs, wild pigs, soft ticks, contaminated feed, water, semen, pork, personnel, and vehicles are the main sources of ASFV. Additionally, stable flies, leeches and other blood-sucking insects may possibly transmit ASFV too (Liu *et al.*, 2021).

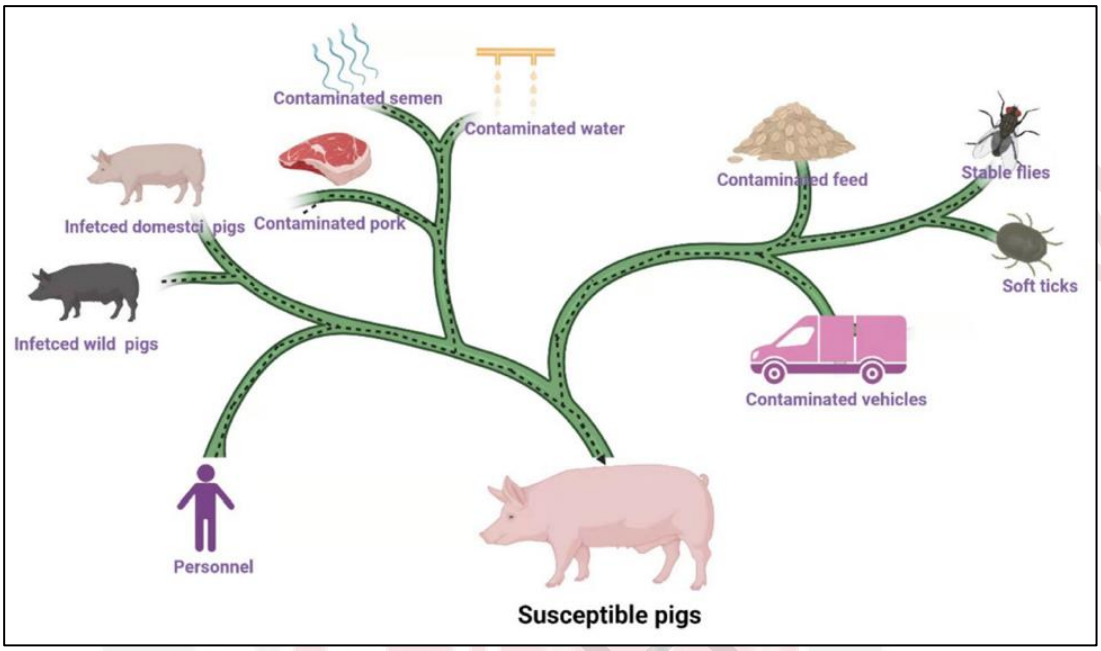


Figure 1: The source of ASFV infection. (Adapted from Prevention and Control Strategies of African Swine Fever and Progress on Pig Farm Repopulation in China by Liu *et al*., 2021, *Viruses*, 13(12), 2552)

In February 2021, ASFV genotype II was detected in wild boars and domestical pigs in the ASF outbreak in Sabah, Malaysia (Khoo *et al.*, 2021). There is limited research and information on the sources of infection for the outbreak in Malaysia. Theories circulating in the market stated that the outbreak is due to contaminated fomites, personnel as well as the fire sales of infected pigs. However, it is unlikely that the soft ticks are involved in the transmission. *Ornithodoros* spp. is one of the species excluded in the list of ticks found in Malaysia due to insufficient significant records (Kazim *et al*., 2022). As high biosecurity protocols are being implemented and practised in swine farms and slaughterhouses, it is of interest to investigate for the possibility of other vectors aside from *Ornithodoros* spp. involving in the transmission route of ASFV in Malaysia.

Flies (order Diptera) is known to be one of the common pests found in livestock farms despite high biosecurity levels (Fila & Woźniakowski, 2020). They are also vectors of pathogenic agents and causes nuisance, integument damages or even blood loss to livestock (Baldacchino *et al*., 2018). In this project paper, flies will be assessed as a potential mechanical vector for the mechanical transmission of ASFV.

1.2 Objective and Justification

The study was conducted with the following objectives:

1. To determine flies as a mechanical vector of ASFV.
2. To compare density of flies within different areas within the farm.

There are limited research studies in Malaysia that determines if flies can be a possible vector of ASFV. Identification of mechanical vectors that harbors and transmits ASFV aids in the surveillance of ASF via early detection in farms. Moreover, it facilitates the practice of relative prevention and control measures in eliminating these vectors as a source of infection. Hence, this study aims to determine if flies are mechanical vectors of ASFV to understand its epidemiology and contribute to the control and prevention of ASF in the swine industry of Malaysia.

1.3 Hypotheses

H_o 1: Flies are not mechanical vectors of ASFV.

H_a 1: Flies are mechanical vectors of ASFV.

H_o 2: There is no significant difference in density of flies in different areas of the farm.

H_a 2: There is a significant difference in density of flies in different areas of the farm.

2.0 LITERATURE REVIEW

2.1 Fly – A Livestock Pest

Flies are livestock pest as they cause significant problems in livestock production (Fila & Woźniakowski, 2020; Machtinger *et al.,* 2021). They are commonly found in farm buildings that has animals. Aside from flies, other livestock pests commonly found in the livestock farms are rats and birds. With the access to contaminated materials such as dead carcasses as well as the secretions and excretions of livestock, they play an important role in the spreading of many infectious diseases in the farm (Fila & Woźniakowski, 2020; Machtinger *et al.*, 2021).

Filth flies are flies that have close association to the animal waste, feed waste or carrion. They breed and grow into larvae in animal feces too. Filth flies include the house fly (*Musca domestica*), stable fly (*Stomoxys calcitrans*), and horn fly (*Haemotobia irritans*). Flies can cause direct damage and indirect damage to the livestock production (Figure 2). Direct damages such as blood loss, integument damages and hypersensitivity to fly bites reduces meat production and overall farm profitability. Indirect damages such as animal disturbances and pathogen transmission are challenging to quantify from economic aspects. Painful bites from filth flies can cause irritation where the animals will express fly-repelling behaviours such as scratching the body against the wall, head shaking, leg stamping, thus leading to self-inflicted wounds at times (Machtinger *et al.,* 2021).

Filth Fly	Contact with Livestock[a]	Life Stage Primarily Impacting Livestock	Impacted Animals	Food Source	Production Impact[b]			
					Damage	Disturbance	Disease	Nuisance
New World screwworm fly (*Cochliomyia hominivorax*)	Intermittent	Immature	All livestock	Body tissues	X			
Horn fly (*Haematobia irritans*)	Temporary	Adult	Cattle Equines	Blood	X	X	X	
Stable fly (*Stomoxys calcitrans*)	Temporary	Adult	All livestock	Blood	X	X	X	X
Face fly (*Musca autumnalis*)	Temporary	Adult	Cattle, Equines	Exudates	X		X	X
House fly (*Musca domestica*)	Environmental Pest	Adult	All livestock, Poultry	Exudates and Non-animal			X	X
Fannia spp. (Little House fly, *Fannia canicularis*; Coastal fly *Fannia femoralis*; Latrine fly (*Fannia scalaris*)	Environmental Pest	Adult	Poultry, Swine (*F. scalaris only*)	Non-animal			X (*F. canicularis only confirmed*)	X

Figure 2: Pest fly associations with livestock, poultry and equine systems and their impact. [Adapted from Filth fly impacts to animal production in the United States and associated research and extension needs (Machtinger *et al.*, 2021)].

2.2 Pathogenic Agents Transmissible by Flies

According to World Health Organization (WHO), vectors are living organisms that can transmit infectious pathogens between humans, or from animals to humans. Vectors can be further divided into biological vectors and mechanical vectors. Biological vectors carry pathogens that can replicate and multiply within their bodies to an infective stage before being transmitted to a new host. Examples of biological vectors for diseases are mosquitoes and ticks. Mechanical vectors such as flies pick up infectious agents on the outside of their bodies (mouthparts, feet and bodies) and transmit them through physical contact (European Food Safety Authority, 2023).

Flies has been known to be vectors of many pathogenic agents present in animals especially livestock (Fila & Woźniakowski, 2020). The vector-borne diseases (VBD) not only cause high mortality among animals, some of the VBD has zoonotic significance (Table 1) and (Table 2).

Table 1: Pathogenic agents that are transmissible by flies to animals.

Flies	Virus	Bacteria	Parasite/Protozoa
Stomoxys calcitrans	- Equine infectious anemia virus (EIAV) - West Nile fever virus (WNFV) - Rift Valley fever virus (RVFV) - Lumpy skin disease virus (LSDV) - Bovine herpes virus (BHV) - Bovine leukosis virus (BLV) (Fila & Woźniakowski, 2020; Onmaz *et al.,* 2012)	- *Bacillus anthracis* - *Pasteurella multocida* - *Erysipelothrix rhusiopathiae* - *Francisella tularensis* - *Enterobacter sakazakii* (Fila & Woźniakowski, 2020)	- *Anaplasma sp.* (Garfias *et al.,* 2021)
Musca domestica	- Porcine respiratory and reproductive syndrome virus (PRRS) - Rotavirus (Baldacchino *et al.,* 2018; Fila & Woźniakowski, 2020)	- *Salmonella sp.* - *E.coli* - *Shigella sp.* - *Campylobacter sp.* (Issa, 2019)	- *Ascaris* - *Entamoeba* - *Ancylostoma* - *Trichuris* - *Strongyloides* - *Cryptosporidium* - *Giardia* - *Taenia* (Garfias *et al.,* 2021)
Haemotobia irritans	- Bovine leukemia virus (BLV) (Panei *et al.,* 2019)	-	- *Anaplasma sp.* (Garfias *et al.,* 2021)

Glossina spp.	-	-	- *Trypanosoma* spp. (Baldacchino *et al*., 2018; Garfias *et al*., 2021)
Tabanus spp.	- Bovine leukosis virus (BLV) (Baldacchino *et al.,* 2018)	- *E.coli* - *Klebsiella pneumoniae* (Yin *et al.,* 2022)	- *Trypanosoma evansi* - *Anaplasma marginale* (Garfias *et al.,* 2021)

Table 2: Pathogenic agents that are transmissible by flies to pigs.

Virus	Bacteria	Parasite / Protozoa
- Porcine respiratory and reproductive syndrome virus (PRRS) - Transmissible gastroenteritis virus (TGEV)	- *Campylobacter jejuni* - *Shigella* spp. - *E. coli* O157 - *Yersinia enterocolitica*	- *Toxoplasma gondii* - *Cryptosporidium parvum*

Adapted from Controlling risks of pathogen transmission by flies on organic pig farms (Meerburg *et al*., 2017).

2. 3 Fly Species Involved in ASFV Transmission

Flies of the order Diptera are true flies that has only a pair of wings. The hind pair of wings have been greatly reduced to a small, club-like organs called halteres as a stabilizing organ to assist in flight (Taylor *et al.,* 2015). Classification of Diptera is generally divided into three sub-orders, Cyclorrhapha, Brachycera and Nematocera. The adult of these sub-orders can be identified and differentiated morphologically by

wing venation, antennal structure as well as ecological habitats (Wall and Shearer, 2001).

There is limited research on other fly species such as *Musca* sp., *Chrysomya* sp. or *Tabanus* sp. that could be a mechanical vector of ASFV. Most of the studies done regarding the detection of ASFV on flies and mechanical transmission of the virus to the pigs were on *Stomoxys calcitrans* (Mellor *et al*., 1987; Olesen*, et al.,* 2018a; Olesen *et a..,* 2018b). The reason of having *Stomoxys calcitrans* as a study target was due to the feeding nature of the fly which feeds on blood. ASF infected pigs will experience viremia where high virus load can be found in the blood, providing an ideal ASFV food source to the flies.

Blood-feeding or hematophagous flies were suspected to be a vector of ASFV when an ASF outbreak spiked up in high biosecurity farms during the summer in certain EU member states (Olesen, *et al*., 2018b) as flies are more active at higher temperature. 87 adult stable flies (*Stomoxys calcitrans*) infected with ASFV had successfully transmitted the virus to two healthy Large White cross Landrace pigs within 24 hours of feeding (Mellor *et al.,* 1987). These flies were infected via direct feeding from an infected host or viremic blood-soaked cotton wool pads. This study suggested that *Stomoxys calcitrans* were mechanical vectors of ASFV.

In contrary, another study showed negative results for blowflies (Calliphoridae), specifically *Lucilia sericata* and *Calliphora vicina* as possible reservoir and mechanical vector of ASFV in a laboratory setting (Forth *et al*., 2017). The larvae of these necrophagous flies were continuously fed on ASFV positive-wild boar spleen tissue since hatching, yet no ASFV was detected in and on any larvae or washer fluid tested. Larvae's salivary secretion could inactivate the virus (Forth *et al.,*

2017). Hence, it was concluded that the larvae of blowflies are not mechanical vectors of ASFV.

2.4 ASFV Localization on Flies

The specific location of detectable ASFV DNA on the flies were further studied (Olesen *et al.,* 2018b). The body parts of the flies with recoverable ASFV could reflect the risk and routes of transmission of the virus from the infected flies to the naïve pigs. The mouth parts, heads, and bodies (thorax and abdomen) of *Stomoxys calcitrans* were analysed independently for ASFV with the body having the highest proportion of recoverable ASFV DNA (Olesen *et al.,* 2018b). This study showed that out of the 73 stable flies, the smallest proportion of detectable ASFV DNA was in the mouth parts (10%) and the largest proportion was in the bodies (79%) whereas the remaining was at the heads (43%).

2.5 Duration of ASFV that Remains Detectable in Flies

The duration of ASFV that remains detectable in flies is crucial in determining the window of transmission of infection to occur from flies to naïve pigs. The longer the duration of ASFV that remains detectable in flies, the higher the chances of the flies transmitting the ASFV to the pigs.

In the mouth parts of *Stomoxys calcitrans,* ASFV DNA was only recoverable within 12 hours post-feeding of ASFV viremic blood but not after 24 hours. On the other hand, ASFV DNA remained detectable in the head and body of the *Stomoxys calcitrans* up to 72 hours post-feeding of the ASFV viremic blood, which was about two days longer than in the mouth parts (Olesen *et al.,* 2018b). Nevertheless, the load

of detectable ASFV DNA on the mouth, head and bodies reduces with time. For example, the recoverable ASFV DNA in the bodies declined from 80-100& at 12-36 hours post feeding to 14-25% at 48-72 hours post feeding (Olesen *et al.*, 2018b). The decreasing level of ASFV DNA in the stable flies over time implied that *S. calcitrans* are not biological vectors for the virus as there is a negative indication of ASFV replication.

In another study, the duration of detectable ASFV DNA on flies were only 48 hours. High titer of ASFV in the infected flies persisted for the first two days after feeding but failed to be recovered between three- and eight-days post-feeding. This suggested that the window of transmission could possibly be up to two days (Mellor *et al.*, 1987). Hence, the average duration of the detectable ASFV DNA in flies is about 12 to 72 hours.

2.6 Routes of ASFV Transmission by Flies

Two ASFV transmission route by flies were being identified, which were oral (ingestion) and biting (mechanical transmission). A naïve pig can be infected via ingestion of 20 ASFV blood-fed *Stomoxys calcitrans* under laboratory setting (Olesen *et al.,* 2018a). The estimated amount of infectious ASFV that may be present in stable flies were relatively high as a single fly could contain enough virus to cause infection to the healthy pigs after oral uptake (Olesen *et al.* 2018a). Nevertheless, the possibility of a pig to ingest 20 flies in one uptake in a natural setting is relatively low.

On the other hand, virus can also be transmitted mechanically via biting from the hematophagous flies (*Stomoxys calcitrans*) during feeding (Mellor *et al.,* 1987). Another study indicated that all six pigs that were inoculated with ASFV via

intramuscular (IM) route were successfully infected with the virus irrespective of the dose (Howey *et al.,* 2013). In spite of that, a single fly with ASFV positive detection of ASFV may not be satisfactory to cause an infection via biting due to the low levels of ASFV DNA identified in the mouth parts (Olesen *et al.* 2018a).

3.0 MATERIALS AND METHODS

3.1 Sample Collection

Four pig farms in Peninsular Malaysia were selected for this field study. The farms had a mixed ASF status of post-ASF outbreak or ASF-free farms. The management system of the four farms was a mixture of closed and opened house systems. Four locations within the farms were chosen for the placement of sticky fly traps which were "Sewage Pool", "Feed House", "Finisher Pen" and "Sow Pen" (Appendix I). Five sticky fly traps of size 26.8 cm x 18.6 cm were placed within each location of each farm for 24 hours. A total of 2254 flies were collected from the four farms. The flies were removed from the sticky fly traps with sterile forceps. The forceps were disinfected with 70% alcohol between each house and farm. Finally, the flies were placed inside separate ziplock bags and transported inside polystyrene boxes filled with ice to maintain a temperature of 2 – 8 °C back to the laboratory. The samples were processed within two days of collection.

3.2 Sample Identification

The flies were grouped based on their morphological characteristics. A stereomicroscope was used to observe the morphological structures of the flies under magnification for identification and classification purposes according to the guidelines "Guide to families of Diptera of veterinary importance" [Wall, R., & Shearer, D. (2001)] (Appendix II). The genus of the flies identified were verified with a parasitologist.

3.3 Sample Pooling

Five flies of the same genus from one location within each farm were randomly selected and pooled together. *Musca* sp. identified within four locations of four farms made up to 16 *Musca* sp. pooled samples. *Chrysomya* sp. identified within 5 locations contributed to 5 *Chrysomya* sp. pooled samples. *Stomoxys* sp. identified in 4 locations gave 4 *Stomoxys* sp. pooled samples. The head and body (thorax and abdomen) of the flies were separated using sterile scissors and pooled respectively into two 2 ml microcentrifuge tubes filled with 800 µL of PBS. A total of 50 pooled samples were used (Figure 3).

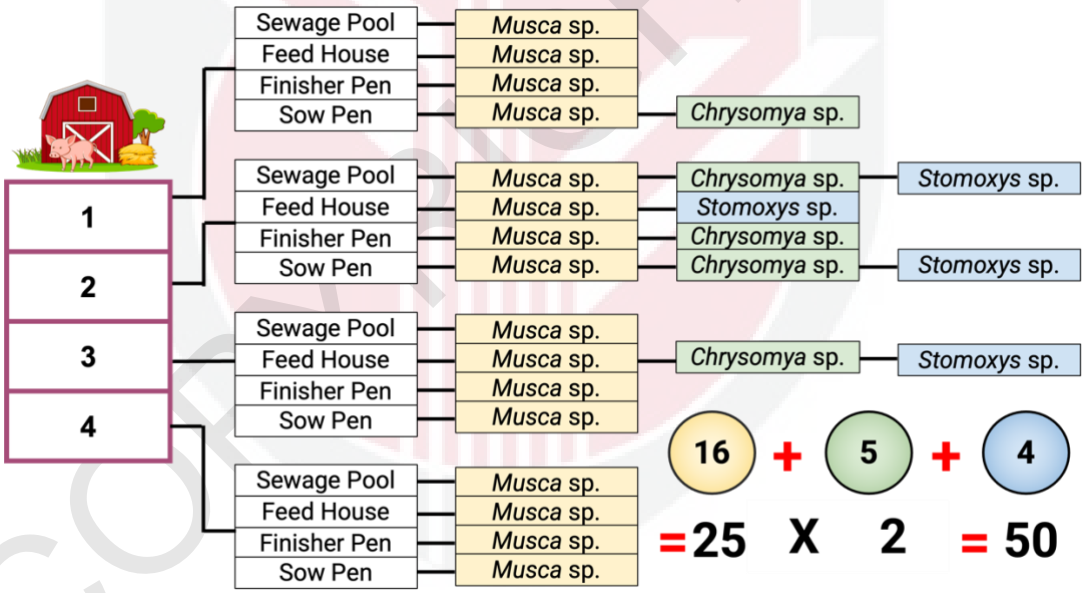


Figure 3: 50 pooled samples of flies with head and body separated in different pools.

3.4 Homogenisation of samples (head and body)

All fly samples were stored in -20 °C to obtain the optimal DNA yield and quality. Four 15 ml tubes [2 tubes of 70% alcohol, 1 tube of double distilled water

(DDH20), and 1 tube of Phosphate Buffer Solution (PBS)] were prepared. The homogenizer was primed with four solutions, which were alcohol 1 followed by alcohol 2, then DDH20, and finally PBS prior to homogenization (Appendix III). Then, the homogeniser was cleaned with the four solutions after each homogenisation of the pooled samples to prevent cross contamination. The homogenised samples were then centrifuged at 9000 rpm for 1 minute. 200 µL of supernatant from each pooled sample was pipetted into a new 1.5 ml microcentrifuge tube to be used for DNA extraction.

3.5 ASFV DNA Extraction

The DNA was extracted using RealPCR DNA/RNA Magnetic Bead Kit (IDEXX) (Appendix III). All the samples were thawed and kept at 18 - 26 °C prior to lysis. The lysis working solution was prepared by mixing 176 µL per sample of lysis buffer with 4 µL per sample of carrier RNA and 20 µL per sample of Proteinase K. 2 µL per sample of internal positive control containing pig DNA was added to the lysis working solution too. The solution was mixed thoroughly with vortex mixer. Next, 200 µL (± 5 µL) of lysis working solution was added to the microcentrifuge tubes containing 200 µL of sample materials and mixed gently by vortexing them several times. Subsequently, the mixed sample materials were incubated at 18-26 °C for 15 minutes.

Next, the bead solution was prepared by mixing 600 µL per sample of binding buffer with 20µL per sample of magnetic beads. The bead solution was mixed well by gently vortexing it multiple times. 620 µL (± 5 µL) of beads solution was added to the sample materials tubes. The tubes were then centrifuged at 1000 rpm for 1 second to

briefly settle the contents. In the beads washing process, the magnetic beads were separated by placing the tubes on the magnetic separator for 2 minutes to allow all the beads to be attracted to the magnets (Appendix III). Then, the supernatant was removed by pipetting, without disturbing the magnetic beads. Once the tube was removed from the magnetic rack, 600 µL (± 20 µL) of Wash 1 was added to the tubes and mixed thoroughly via vortexing and were then centrifuged at 1000 rpm for 1 second to briefly settle the contents. After that, the tubes were placed on the magnetic separator again for 2 minutes and the supernatant was removed as previously done. The beads-washing process was repeated with 600 µL (± 20 µL) of Wash 2 and 600 µL (± 20 µL) of 80% ethanol respectively. After the removal of 80% ethanol, the tubes were left to dry for 10 minutes at 18-26 °C.

Then, 100 µL per sample of elution buffer was added to the tubes and mixed well via vortex mixer several times and centrifuged at 1000 rpm for 1 second. Next, the tubes were placed on the magnetic separator for 2 minutes once again to separate the DNA from the beads. The supernatant containing purified nucleic acid was transferred to a new 1.5 ml microcentrifuge tube. 50 µL of the purified nucleic acid was pipetted into another set of 1.5 ml microcentrifuge tube and was stored at -18 °C.

3.6 DNA Amplification and Quantification – Realtime Polymerase Chain Reaction (qPCR)

All qPCRs were performed using the CFX Opus 96 RealTime System (Bio-Rad) (Appendix III). Real-time PCR results were recorded as quantification cycle (Ct) values as determined by the CFX Maestro Security Edition Software (Bio-Rad).

IDEXX RealPCR ASFV DNA test kit was used in this study (Appendix III). The PCR Mix was prepared by adding 10 µL per sample of ASFV DNA Mix with 10 µL per sample of DNA MMx into a sterile 1.5 ml microcentrifuge tube. The solution was gently mixed by vortex mixer multiple times. Next, 20 µL of the PCR Mix was slowly pipetted into the required labelled PCR multiwell plates with two extra wells which acts as the positive and negative control. Next, 5 µL of PCR negative control (PCR Grade Water) was added into the negative control well filled with PCR Mix and covered to prevent cross-contamination from samples or positive control. Then, 5 µL of sample DNA was added into each well with the final reaction volume adding up to 25 µL. Finally, 5 µL of PCR positive control was added to the positive control well. All wells were covered and mixed with vortex and centrifuged briefly at 4000 rpm for 10 seconds to settle contents and remove air bubbles. All these were carried out in biosafety cabinet class 2.

The thermal cycler was set up with IDEXX RealPCR Standard DNA/RNA Cycling Program with the IDEXX ASF Standard DNA-RNA Cycling Protocol. The cycling conditions were as shown in Table 3. The probe used for fluorescence detection were HEX and FAM. HEX (hexachlorofluorescein) detects the internal control in the samples whereas the FAM (fluorescein) detects the presence of ASFV. The validity criteria of the results obtained was shown in Table 4 whereas the interpretation of the results was shown in Table 5. A manual adjustment of the threshold of FAM curve was done by using the value of 10% of positive control Ct value.

Table 3: Cycling conditions of IDEXX qPCR for detection of ASFV.

Step	Time	Temperature	Cycle(s)
Reverse transcriptase (RT)	15 mins	50°C	1
Denaturation	1 min	95°C	1
Amplification	15 sec	95°C	45
(Annealing & Extension)	30 sec	60°C	
Fluorescence detection	Channels FAM and HEX		

Table 4: Validity criteria for IDEXX RealPCR ASFV DNA test kit.

	FAM Ct Value	HEX Ct value
Positive Control	< 38	< 38
qPCR Negative Control	No signal	No signal

Table 5: Interpretation of results for IDEXX RealPCR ASFV DNA test kit.

Sample Result	FAM Signal	HEX Signal
ASFV DNA Detected	Yes	Yes
ASFV DNA Not Detected	No	Yes
Invalid	No	No

3.7 Statistical Analysis

Statistical analysis was done using IBM SPSS statistical software. The data to be analysed was the number of flies between each location of the farms. First, a test of normality was performed using Shapiro-Wilk's Test, as the sample size is small (50) and the data showed a p-value of less than 0.05, indicating that the data was not normally distributed. Next, non-parametric test – Kruskal-Wallis Test was performed to determine if there are statistically significant difference in mean ranks between groups. With the confidence interval of 95%, if $p > 0.05$, the samples were considered as not significantly different; if $p < 0.05$, the samples were considered as significantly different.

4.0 RESULTS

4.1 Flies identification and classification.

In this study, three types of flies were being identified. They were being categorized as *Musca* sp. (Figure 4), *Chrysomya* sp. (Figure 5) and *Stomoxys* sp. (Figure 6) which falls under the suborder group of Cyclorrhapha. They have similar wing venation and antennae structure. They were classified into different genus based on their unique body morphology.

Musca sp. (Family: Muscidae)

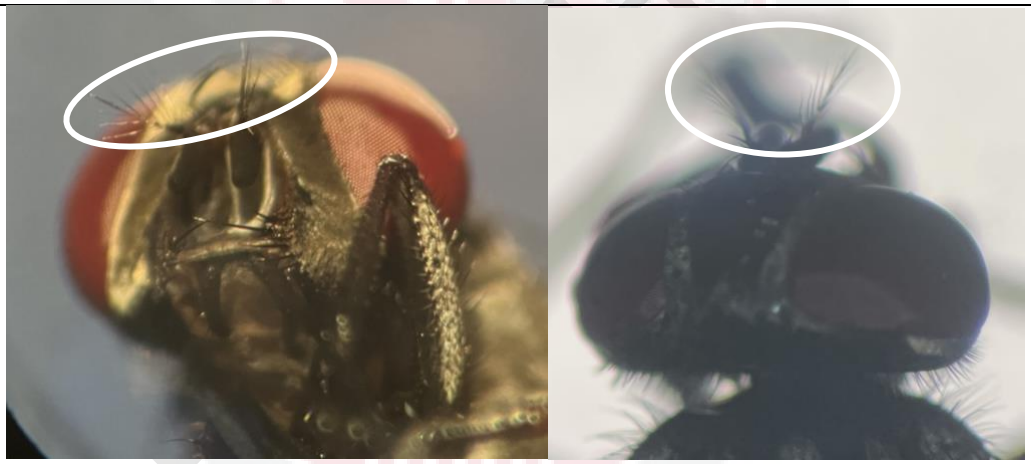


Image 1 & 2: The third segment of the antennae has an aristae that are bilaterally plumose (feather-like) to the tip. (white circle) [Magnification: 35x]

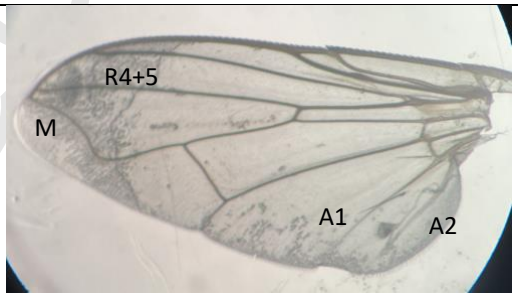


Image 3: Wings with vein A1 not reaching the wing edge; A2 not strongly curved. Strongly bent vein M ending close to R4+5.
[Magnification: 25x]

	
Image 4: White arrow shows that the thorax is not dorsoventrally flattened. It is grey with four dark longitudinal stripes. [Magnification: 20x]	Image 5: The abdomen has a yellowish-brown background colour with black median longitudinal stripe. [Magnification: 20x]

Figure 4: Images 1 to 5 shows *Musca* under stereomicroscope for identification and classification.

***Chrysomya* sp. (Family: Calliphoridae)**

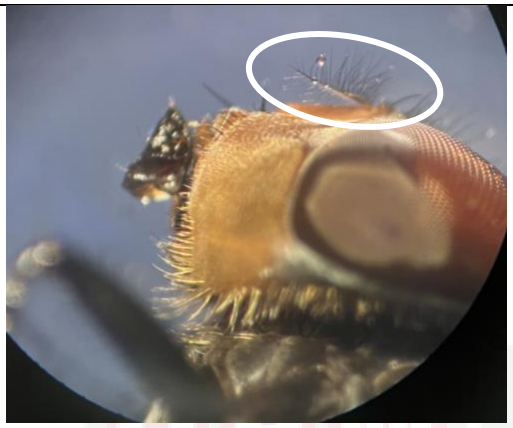


Image 6: The third segment of the antennae has an aristae that are bilaterally plumose (feather-like) to the tip. (white circle) [Magnification: 35x]

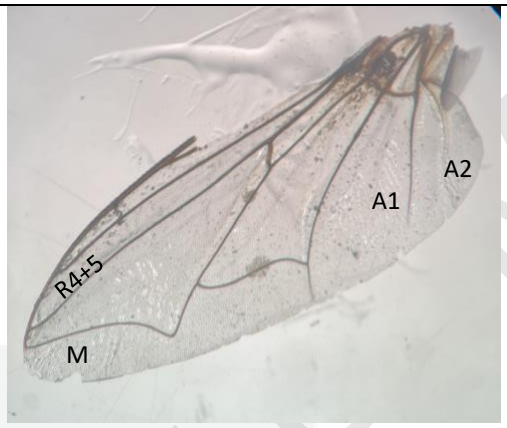


Image 7: Wings with vein A1 not reaching the wing edge; A2 not strongly curved. Strongly bent vein M ending close to R4+5. Slightly narrower than *Musca*. [Magnification: 25x]

Image 8 & 9: No dark stripes on the thorax and abdomen, uniformly coloured, has metallic, iridescent appearance (green). [Magnification: 20x;15x]

Figure 5: Images 6 to 9 shows *Chrysomya* under stereomicroscope for identification and classification.

***Stomoxys* sp. (Family: Muscidae)**

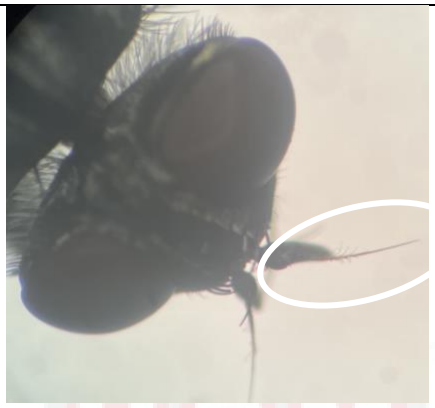


Image 10: The third segment of the antennae has an aristae that are bilaterally plumose. (white circle) [Magnification: 35x]

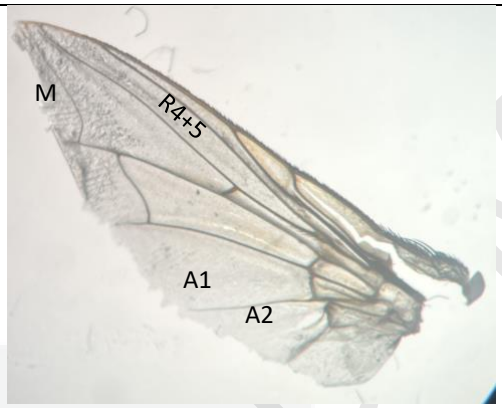


Image 11: Wings with vein A1 not reaching the wing edge; A2 not strongly curved. [Magnification: 25x]

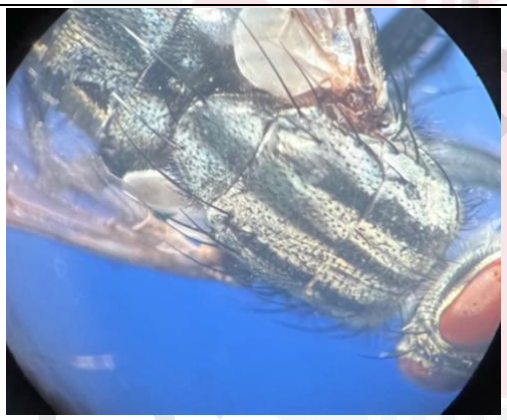


Image 12 & 13: The body is generally grey in colour with four longitudinal dark stripes on the thorax; the abdomen is grey with dark spots on the second and third abdominal segments. [Magnification: 20x]

Figure 6: Images 10 to 13 shows *Stomoxys* under stereomicroscope for identification and classification.

4.2 Flies collected in swine farms

A total of 2254 flies were sampled from the sewage pool, feed house, finisher pen and sow pen of four farms (Table 6). After identification and classification, there were 2218 *Musca* sp. (98%), 22 *Chrysomya* sp. (1%), and 14 *Stomoxys* sp. (1%) obtained from the field sampling (Figure 7).

Table 6: Number of Musca sp., Chrysomya sp., and Stomoxys sp. collected from swine farms in Peninsular Malaysia

Farm	Houses	*Musca* sp.	*Chrysomya* sp.	*Stomoxys* sp.
1	Sewage Pool	13	0	0
	Feed House	7	0	0
	Finisher Pen	122	0	0
	Sow Pen	259	2	0
2	Sewage Pool	7	8	10
	Feed House	12	0	2
	Finisher Pen	18	2	0
	Sow Pen	6	1	1
3	Sewage Pool	54	0	0
	Feed House	1088	9	1
	Finisher Pen	55	0	0
	Sow Pen	253	0	0
4	Sewage Pool	249	0	0
	Feed House	71	0	0
	Finisher Pen	3	0	0
	Sow Pen	11	0	0
	Total	2218	22	14

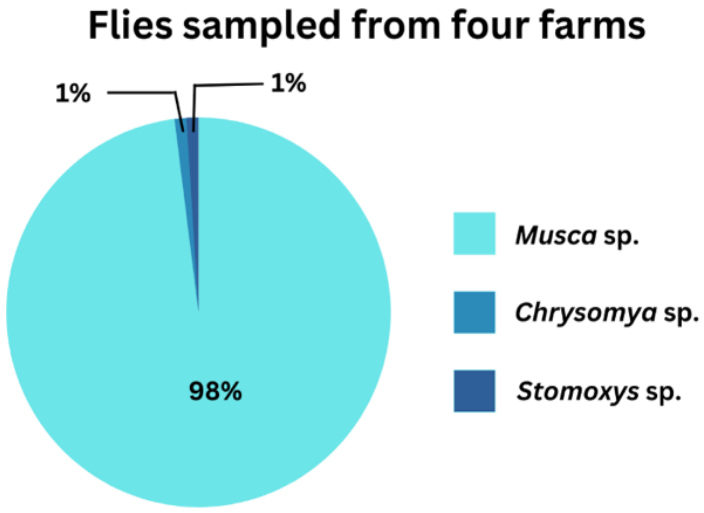
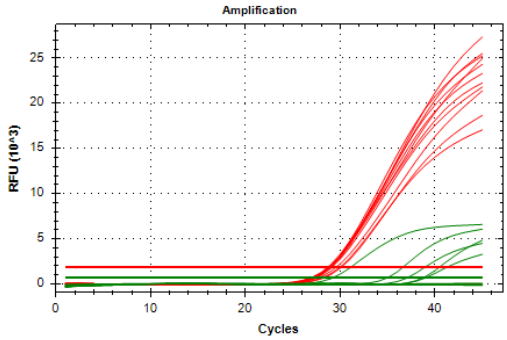
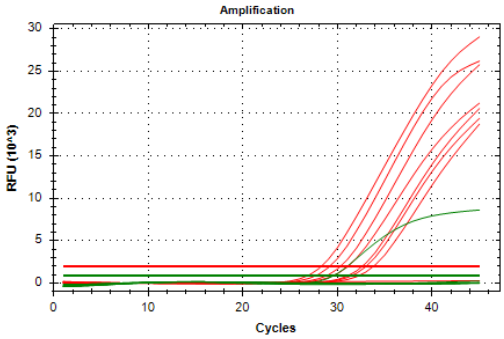


Figure 7: Flies sampled from four pig farms in Peninsular Malaysia consisted of 98% *Musca* sp., 1% *Chrysomya* sp. and 1% *Stomoxys* sp.

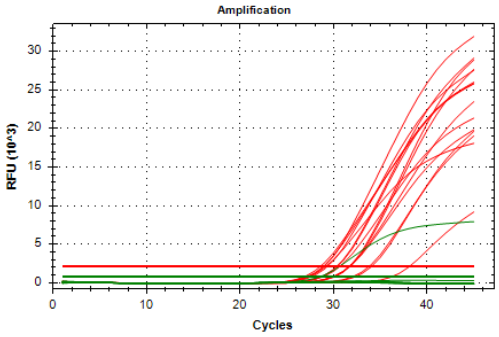
4.3 Realtime-PCR



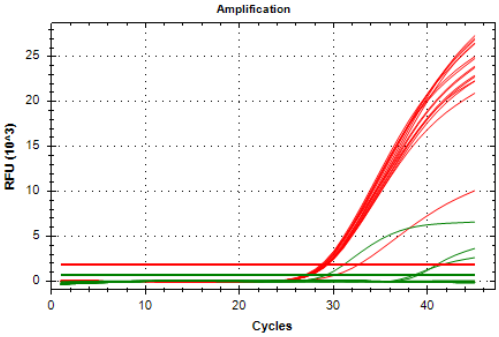
FARM 1



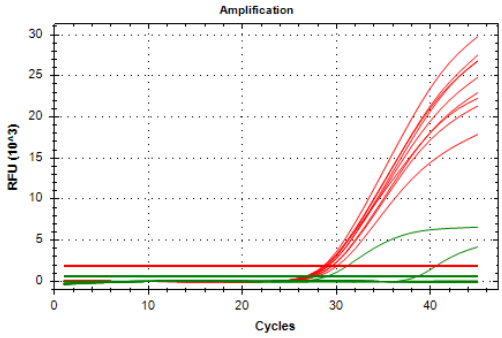
FARM 2(a)



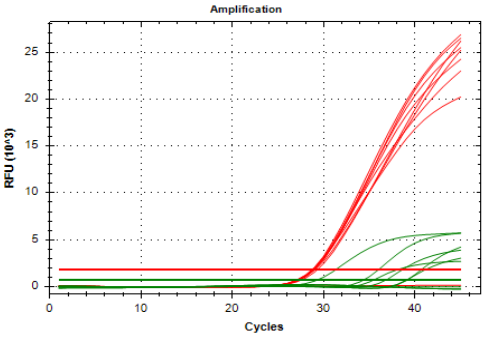
FARM 2(b)



FARM 3



FARM 4



FARM 1, 3, 4 – positive samples

Figure 8: Realtime PCR amplification with FAM-curve (green) and HEX-curve (red) on fly samples from Farm 1, Farm 2, Farm 3 and Farm 4.

Table 7: Ct values on FAM-curve (ASFV) using qPCR.

Farm	Location	Flies	Type of sample	Ct value (FAM-curve: ASFV)
			Negative control	-
			Positive control	29.21
1	Sewage Pool	*Musca* sp.	Head	35.39
			Body	-
	Feed House	*Musca* sp.	Head	-
			Body	-
	Finisher Pen	*Musca* sp.	Head	-
			Body	-
	Sow Pen	*Musca* sp.	Head	-
			Body	-
		Chrysomya sp.	Head	-
			Body	-
2	Sewage Pool	*Musca* sp.	Head	-
			Body	36.77
		Chrysomya sp.	Head	-
			Body	-
		Stomoxys sp.	Head	-
			Body	-
	Feed House	*Musca* sp.	Head	-
			Body	-
		Stomoxys sp.	Head	-
			Body	-
	Finisher Pen	*Musca* sp.	Head	-
			Body	-
		Chrysomya sp.	Head	-
			Body	-
	Sow Pen	*Musca* sp.	Head	-
			Body	-
		Chrysomya sp.	Head	-
			Body	-
		Stomoxys sp.	Head	-
			Body	-
3	Sewage Pool	*Musca* sp.	Head	-
			Body	-
	Feed House	*Musca* sp.	Head	-
			Body	36.73
		Chrysomya sp.	Head	-
			Body	-
		Stomoxys sp.	Head	-
			Body	-
	Finisher Pen	*Musca* sp.	Head	-

			Body	-
	Sow Pen	*Musca* sp.	Head	-
			Body	-
4	Sewage Pool	*Musca* sp.	Head	-
			Body	-
	Feed House	*Musca* sp.	Head	-
			Body	-
	Finisher Pen	*Musca* sp.	Head	-
			Body	-
	Sow Pen	*Musca* sp.	Head	34.29
			Body	-

A total of four flies were identified as positive ASFV with the Ct-value from FAM-curve of < 38. The value used was an in-house laboratory standard guideline for detection of ASFV via qPCR.

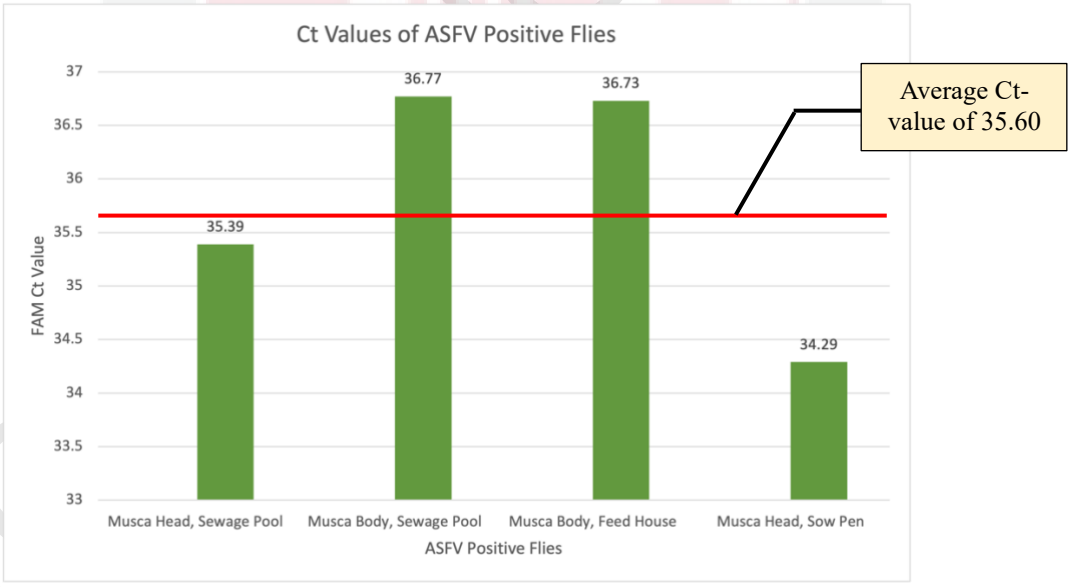


Figure 9: Range of ASFV positive fly samples FAM-curve Ct-values. The range of Ct-value falls between 34.29 to 37.00 with an average positive Ct-value of 35.60 (red line)

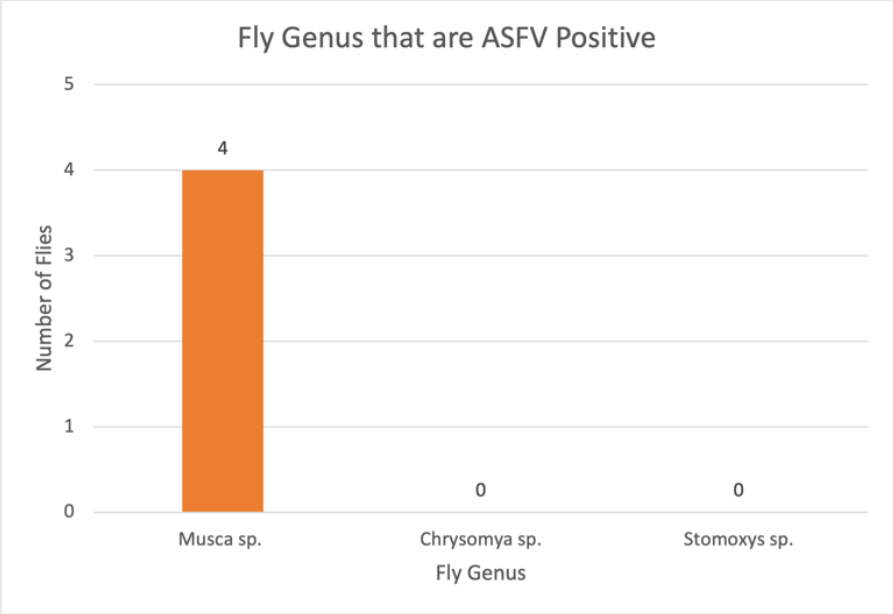


Figure 10: The ASFV positive flies were all from the *Musca* sp.

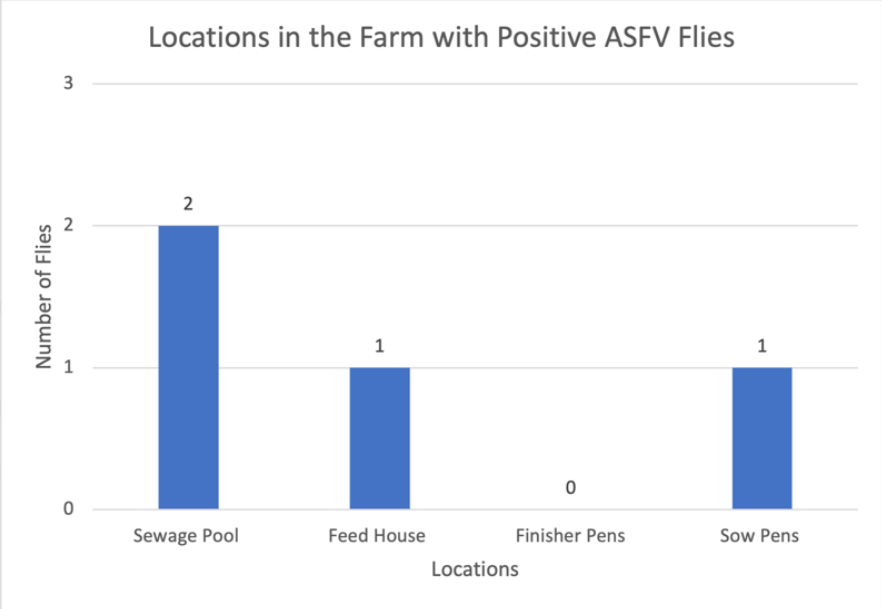


Figure 11: ASFV positive flies were detected from the sewage pool, feed house and sow pens.

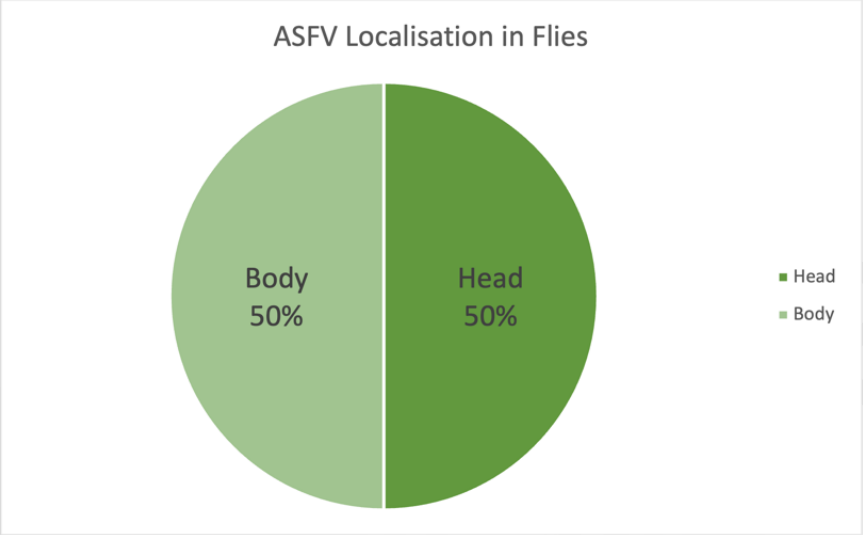


Figure 12: Out of the four positive samples, 50% of detected on the head of the flies, another 50% was detected on the body of the flies.

4.4 Statistical Analysis

The p-value from Shapiro-Wilk's Test was referred for the test of normality as the sample size was less than 50. As $p < 0.05$, the data obtained was not normally distributed. Non-parametric test of Kruskal-Wallis Test with $p > 0.05$ indicated that there was no significant difference in the densities of flies in different areas of the farm.

4.4.1 Test of normality

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	House_allsp	Statistic	df	Sig.	Statistic	df	Sig.
No_of_flies2	Sewage Pool	.416	12	.000	.446	12	.000
	Feed House	.452	12	.000	.362	12	.000
	Finisher Pen	.393	12	.000	.538	12	.000
	Sow Pen	.465	12	.000	.489	12	.000

a. Lilliefors Significance Correction

Figure 13: Test of normality with Kolmogorov-Smirnov and Shapiro-Wilk's Test showed p < 0.05, indicating that the data was not normally distributed.

4.4.2 Kruskal-Wallis Test

Kruskal-Wallis Test

Ranks

	House_allsp	N	Mean Rank
No_of_flies2	Sewage Pool	12	24.96
	Feed House	12	25.79
	Finisher Pen	12	22.33
	Sow Pen	12	24.92
	Total	48	

Test Statistics[a,b]

	No_of_flies2
Kruskal-Wallis H	.464
df	3
Asymp. Sig.	.927

a. Kruskal Wallis Test

b. Grouping Variable: House_allsp

Figure 14: Kruskal-Wallis Test showed p > 0.05, indicating that there was no significant difference in the densities of flies in different areas of the farm.

5.0 Discussion

The *Musca* genus comprises of about 60 species including the housefly, *Musca domestica*. They have an average flight range of 3 km with some reaching up to 30 km (Baldacchino *et al.,* 2018). The common housefly is known for its epidemiological role in the transmission of pathogens which causes significant diseases in both livestock and humans (Issa, 2019; Fila & Woźniakowski, 2020; Yin *et al.,* 2022; Hornok *et al*., 2023). The life cycle for housefly ranges from several days to two months as it is greatly dependent on the environmental temperature, the higher the ambient temperature, the shorter the development time of the larvae (Fila & Woźniakowski, 2020). For instance, at 25°C, the growth of larvae to adult takes about two weeks. The adult female flies live up to 15 to 25 days with high egg deposition of five batches of 75 to 150 eggs (Meerburg *et al.*, 2007). Housefly feeds on blood, sweat, tears, saliva and other body fluids or environmental fluids via their sponging mouthparts (Issa, 2019). They are known to be mechanical vectors for porcine reproductive and respiratory syndrome (PRRS) (Meerburg *et al.,* 2007; Pitkin *et al.,* 2009), *Toxoplasma. gondii*, *Cryptosporidium. parvum*, *Clostridium. jejuni*, transmissible gastroenteritis virus (TGE), *Yersinia. enterocolitica*, and *Escherlichia. coli* O157 (Meerburg *et al.,* 2007).

The *Chrysomya* genus flies of the family Calliphoridae, also known as blowflies, are usually the first insect to be associated to dead body remains (Siddiki & Sp, 2017). The adults and larvae either feed on carrion or decaying tissue of a vertebrae host (Taylor *et al.,* 2015). These necrophagous flies have a fly range of 2-3 km per day (Braack & De Vos, 1990) and have the ability to smell carcasses from up to 16 km (Siddiki & Sp, 2017). The adult female blowfly deposits about 150-200 eggs in a batch

for 10-13 times in her entire life and lives up to 3-4 weeks depending on the species (Shah *et al*., 2016). *Chrysomya megacephala* has been reported to be a possible mechanical vector for pathogenic bacteria such as *Salmonella* sp. and *E.coli* O157 (Chaiwong *et al.,* 2014) yet there was limited research or experimental studies on blowflies as mechanical vectors for swine-related pathogens.

Stomoxys sp. are biting flies where the adults have an average lifespan between two to four weeks. These hematophagous flies feed on animal blood with a blood meal size that ranges from 10-15 µl (Baldacchino *et al.,* 2013) and can travel up to 5 km in search of a new host to feed on (Bonnet *et al.,* 2020). Painful bites which result in interrupted feeding aids the mechanical transmission of pathogens where the residual blood present on the mouthparts is transferred to new animals (Baldacchino *et al.,* 2013). A possible reason for *Stomoxys* to be the least abundant flies found in pig farms in Malaysia is the implementation of closed house farming system with adequate ventilation, where it reduces the fly densities within the buildings.

A total of 2254 flies were sampled from four different locations (sewage pool, feed house, finisher pen and sow pen) of four pig farms in Peninsular Malaysia over 24 hours. *Musca* sp. (Muscidae) was the highest sampled flies with 2218 flies (98.40%) followed by *Chrysomya* sp. (Calliphoridae) with 22 flies (0.68%) and finally *Stomoxys* sp. (Muscidae) with 14 flies (0.62%). *Musca* sp has been reported to be the most regularly found flies in pig farms (Meerburg *et al.,* 2007; Yoon *et al*., 2021; *Hornok et al*., 2023). Moreover, there is positive correlation between the *Musca* sp. with the relative humidity and total rainfall (Jin & Jaal, 2009). Malaysia's climate which has high humidity and heavy rainfall provides ideal breeding environment for the *Musca* sp.

Based on the non-parametric Kruskal-Wallis Test, there was no significant difference in the fly densities in different areas of the farm, indicating that all possibilities could contribute to the variation in numbers. The four farms have mixed status of being abandoned farms or farms with pigs. The presence of pigs as well as sanitation level of the farm could greatly affect the number of flies in the locations within the farms. Ideally, sewage pool should have higher densities of flies compared to feed house, finisher pens and sow pens. With the presence of pigs, there will be an increased in the manure and waste production daily. Not only will the concentrated manure which produces odour in the sewage pool attract the flies, but also serve as ideal breeding sites for the flies (Hughes & Walker, 1969).

All the four ASFV positive fly samples were of the *Musca* sp. Currently, there is no research study in southeast Asia that investigates the *Musca* sp. as mechanical vectors of ASFV. However, based on the findings in this study, *Musca* sp. is a mechanical vector of ASFV. This could probably be because *Musca* sp. was the most abundantly sampled flies in this study, attributing to 98.40% of the total flies sampled, hence, increasing the possibility of detecting ASFV in them. Besides, the feeding nature of the *Musca* sp. which feeds on liquid manure or environmental fluids could also possibly be the reason for the flies to harbour ASFV (Issa, 2019). The presence of ASFV positive *Musca* sp. indicates that there is an environmental contamination of ASFV in the farms which acts as source of mechanical transmission to the flies. The possible sources of environmental contamination of ASFV are liquid manure in sewage pools or wastewaters from the daily cleaning processes of the pens.

Out of the four pooled fly samples which were detected ASFV positive, two were head samples and two were body samples. The small number of positive samples

are not conclusive to draw any speculations on the location on the flies where the ASFV DNA could be detected. In general, ASFV can attach to the mouthparts, body, leg hairs or food pads of the flies when they land on ASF contaminated areas (Baldacchino *et al.,* 2013). ASF survivability in excretion is largely dependent on temperature where the virus remained infectious in faeces for 8 days at 4 °C and 3-4 days at 37 °C; in urine, the virus was viable up to 15 days at 4 °C and 2-3 days at 37 °C. On the other hand, the survivability of ASF in feed and water stored at room temperature was at one day and 50 days respectively (Mazur-Panasiuk *et al.,* 2019).

The ASFV positive pooled fly samples were found in the sewage pool, feed house and sow pens. As the *Musca* sp. have an average fly range of about 3 km (Baldacchino *et al.,* 2018), the flies could have obtained the ASFV from surrounding areas before being sampled at the location. This thought could be alarming to farmers as it warrants an increased biosecurity measure on fly control to reduce the risk of ASF transmission from surrounding farms. However, it is also undeniable that there is a possibility where the flies could have obtained the ASFV from the locations where they were sampled. For example, positive ASFV flies in the sewage pool could suggest that there is presence of ASFV remain or contamination sources in the sewage. ASFV can be detected in slurry for over 100 days depending on environmental temperature (Sánchez-Vizcaíno *et al.* 2009)

There are few possibilities which could explain the high number of ASF negative pooled fly samples (46 out of 50). Firstly, the time of fly sampling has exceeded the window of transmission as the last ASF positive case in the post-ASF outbreak sampled farms had been more than a year. The levels of ASFV DNA that

remained detectable in flies reduces with time (Olesen *et al.*, 2018b). The flies could have harboured ASFV at a point of time, but the virus no longer persist on the flies in the absence of a susceptible host for virus replication. Besides, the short life cycle of the *Musca* sp. which overall has a lifespan shorter than a month (Meerburg *et al.,* 2007) reduces the chances of ASFV to remain on the flies, breaking the cycle of transmission and reducing the probability of positive ASFV detection.

The sewage pool and sow pens ideally possess a higher chance of obtaining ASF flies. The faeces, urine and water used to wash and clean the houses daily are drained and accumulated into the sewage pool. The excreta from the ASF pigs could contain the virus and be mechanically transmitted to the flies attracted via the strong odour emitted from the waste. Besides, majority of swine farming practices in Malaysia keep sows for about 7-8 litters, which is about 4 years in comparison to the finisher pigs which will be slaughtered at 6 months. The longer duration of sows in the farms increases the chance for ASF transmission cycle via possible mechanical vectors to take place.

Based on the findings in this study, it is established that *Musca* sp. are mechanical vectors of ASFV. However, the chances of the ASFV positive flies detected in this study to reinfect the pigs are relatively low. Based on experimental studies, 30 to 57 ASF infected *Stomoxys calcitrans* were required to feed on naïve pigs to cause the disease (Mellor *et al.,* 1987), whereas pigs were infected after ingestion of 20 ASF positive *S. calcitrans* (Olesen *et al.,* 2018a). However, it seems impractical for ingestion of 20 flies to take place in a natural setting (Olesen *et al.,* 2018a). Nevertheless, a calculated dose of $5\log_{10}$ TCID in 20 flies was required to establish an infection via oral ingestion in the study (Olesen *et al.,* 2018a).

Although the risk of ASFV reinfection from flies to pigs is comparatively low, fly control is still crucial in reducing the risk of ASFV transmission and outbreak in swine farms in Malaysia. Hence, there is a need to reassess the biosecurity practices to prevent and control the population of flies in pig farms in Malaysia. Control measures can be divided into a few categories such as environmental control, biological control, chemical control, and mechanical control. The basic and gold standard management is to practise year-round sanitation. For example, daily removal of manure from pig pens, daily cleaning of feed troughs, regular cleaning of feed house, proper drainage of excreta and wash waste reduces breeding sites for flies. The eggs, larvae and pupae of a fly requires to live in faeces, moist hay, spilled silage etc for about 10 to 21 days. Elimination of these breeding sites helps to break the life cycle of flies and acts as an effective fly control (Kaufman *et al.,* 2000).

Installation of ventilation filters in closed house system greatly reduces the number of flies that enters the house too. The high wind velocity paired with the filter inhibits flight entry and activities, limiting the chances of fly contact with pigs or feed (Baldacchino *et al.,* 2018). Insecticides is another routinely used fly control measure. The effectiveness of insecticides is dependent on the active ingredient and formulation, application method and setting, resistance level, fly species and fly densities (Baldacchino *et al.,* 2018). Chemical control of flies comes in space sprays, baits, larvicides, residual premise sprays, and whole-animal sprays (Kaufman *et al.,* 2000). In the usage of larvicide which controls fly immature stages, it is essential to understand the life cycle of flies and the insecticide mode of action.

6.0 CONCLUSION

In conclusion, *Musca* sp. was the most abundant found flies in the pig farms in Peninsular Malaysia followed by *Chrysomya* sp. and *Stomoxys* sp. With the positive detection of ASFV in the flies, flies are considered as mechanical vectors of ASFV. Besides, the difference in densities of flies in the sewage pool, feed house, finisher pen and sow pen of four farms were not significant. Since flies has the possibility of transmitting the virus, pig farms in Malaysia must invest and implement cost-effective fly control program to improve farm biosecurity. Screening of flies can also be included as one of the surveillance tools for ASFV outbreak. A thorough cleaning and disinfection protocol for ASF outbreak farms must be implemented to ensure that the ASFV present in the environment are completely eradicated before any repopulation work proceeds.

7.0 LIMITATIONS AND RECOMMENDATIONS

The limitation in this study is the restricted information from the farms that were sampled. Data such as farm size, herd size, farm layout, pesticide control and possible breeding sites of flies were not disclosed due to privacy and confidentiality. Without the records, tracing back for the source of ASF infection in flies was not possible. Besides, cost constrain has also limited the number of samples that could be processed.

For similar projects in the future, it is recommended that the sample size is increased to at least six pooled samples of the same genus from each location within the farms. Moreover, new fly traps could be placed for at least three days to a week daily for sample collection.

REFERENCES

Alikhan, M. A., Ghamdi, K. A., Mahyoub, J. A., & Alanazi, N. (2018). Public health and veterinary important flies (order: Diptera) prevalent in Jeddah Saudi Arabia with their dominant characteristics and identification key. *Saudi Journal of Biological Sciences*, *25*(8), 1648–1663. https://doi.org/10.1016/j.sjbs.2016.08.014

Baldacchino, F., Muenworn, V., Desquesnes, M., Desoli, F., Charoenviriyaphap, T., & Duvallet, G. (2013). Transmission of pathogens by *Stomoxys*flies (Diptera, Muscidae): a review. *Parasite*, *20*, 26. https://doi.org/10.1051/parasite/2013026

Baldacchino, F., Desquesnes, M., Duvallet, G., Lysyk, T. J., & Mihok, S. (2018). 3. Veterinary importance and integrated management of Brachycera flies in dairy farms. In *Ecology and control of vector-borne diseases* (pp. 55–90). https://doi.org/10.3920/978-90-8686-863-6_3

Barradale, F., Sinha, K., & Lebestky, T. (2017). Quantification of drosophila grooming behavior. *Journal of Visualized Experiments*, *125*. https://doi.org/10.3791/55231

Bonnet, S., Bouhsira, E., De Regge, N., Fite, J., Etore, F., Garigliany, M., Jori, F., Lempereur, L., Potier, M. L., Quillery, E., Saegerman, C., Vergne, T., & Vial, L. (2020). Putative role of arthropod vectors in African swine fever virus transmission in relation to their Bio-Ecological Properties. *Viruses*, *12*(7), 778. https://doi.org/10.3390/v12070778

Braack, L. E., & De Vos, V. (1990). Feeding habits and flight range of blow-flies (Chrysomyia spp.) in relation to anthrax transmission in the Kruger National Park, South Africa. *The Onderstepoort journal of veterinary research*, *57*(2), 141–142.

Catangui, M. (2020, September 24). *Fly Control for Biosecurity in Swine Farms*. AmerisourceBergen MWI Animal Health. https://www.mwiah.com/our-insights/fly-control-for-biosecurity-in-swine-farms

Chaiwong, T., Srivoramas, T., Sueabsamran, P., Sukontason, K., Sanford, M. R., & Sukontason, K. L. (2014). The blow fly, Chrysomya megacephala, and the house fly, Musca domestica, as mechanical vectors of pathogenic bacteria in Northeast Thailand. *Tropical biomedicine*, *31*(2), 336–346.

Erwanas, A. I., Masrin, A., Amnah, O., Chandrawathani, P., J, Premaalatha, B., & Ramlan, M. (2015). Vectors Of Veterinary Importance In Malaysia: A Survey Of Biting Flies In Relation To Trypanosomiasis In Perak. *Malaysian Journal Of Veterinary Research*, *6* (January 2015), 89–96.

European Food Safety Authority. (2023). Vector-borne diseases. *European Food Safety Authority*. https://www.efsa.europa.eu/en/topics/topic/vector-borne-diseases

Fila, M., & Woźniakowski, G. (2020). African swine fever virus – the possible role of flies and other insects in virus transmission. *Journal of Veterinary Research*, *64*(1), 1–7. https://doi.org/10.2478/jvetres-2020-0001

Förster M., Klimpel S., Sievert K. (2009). The house fly (*Musca domestica*) as a potential vector of metazoan parasites caught in a pig-pen in Germany. Vet Parasitol 2009, 160, 163–167.

Forth, J. H., Amendt, J., Blome, S., Depner, K., & Kampen, H. (2017). Evaluation of blowfly larvae (Diptera: Calliphoridae) as possible reservoirs and mechanical vectors of African swine fever virus. *Transboundary and Emerging Diseases*, *65*(1), e210–e213. https://doi.org/10.1111/tbed.12688

Garfias, C. R. B., Acosta, F. T., & Ramírez, G. s. C. (2021). Fly Borne Diseases in Animals. *International Journal of Veterinary Science*, *Chapter 1*. https://doi.org/10.47278/book.vpph

Howey, E. B., O'Donnell, V., De Carvalho Ferreira, H. C., Borca, M. V., & Arzt, J. (2013). Pathogenesis of highly virulent African swine fever virus in domestic pigs exposed via intraoropharyngeal, intranasopharyngeal, and intramuscular inoculation, and by direct contact with infected pigs. *Virus Research*, *178*(2), 328–339. https://doi.org/10.1016/j.virusres.2013.09.024

Hornok, S., Bruno, A. M. C., Takács, N., Keve, G., Sándor, A. D., & Kontschán, J. (2023). An update on the occurrence of flies (Diptera: Muscidae, Calliphoridae) and sucking lice (Phthiraptera: Anoplura) of veterinary importance in Malta: First record of Lucilia cuprina and Linognathus africanus. *Frontiers in Veterinary Science*, *10*. https://doi.org/10.3389/fvets.2023.1143800

Issa, R. (2019). Musca domestica acts as transport vector hosts. *Bulletin of the National Research Centre*, *43*(1). https://doi.org/10.1186/s42269-019-0111-0

Jin, B. L., & Jaal, Z. (2009). Temporal changes in the abundance of Musca domestica Linn (Diptera: Muscidae) in poultry farms in Penang, Malaysia. *PubMed*, *26*(2), 140–148. https://pubmed.ncbi.nlm.nih.gov/19901900

Kazim, A. R., Houssaini, J., Tappe, D., & Heo, C. C. (2022). A checklist of the ticks of Malaysia (Acari: Argasidae, Ixodidae), with lists of known associated hosts, geographical distribution, type localities, human infestations and pathogens. *Zootaxa*, *5190*(4), 485–530. https://doi.org/10.11646/zootaxa.5190.4.2

Kaufman, P., Rutz, D., & Pitts, C. (2000). Pest Management Recommendations for Sheep, Goats, and Swine. NYS IPM Type Livest. Fact Sheet. http://hdl.handle.net/1813/42388

Khoo, C. K., Norlina, D., Roshaslinda, D., Iti Suraya Hani M, M. S., Zunaida, B., Mohd Hasrul, A. H., Pauzi, N.A. S., Roslina, H., Aizah Hanim, M. S., & Leow, B. L. (2021). African swine fever in backyard pigs of Sabah state, East Malaysia, 2021. *Tropical Biomedicine*, *38*(4), 499–504. https://doi.org/10.47665/tb.38.4.095

Liu, Y., Zhang, X., Qi, W., Yang, Y., Liu, Z., An, T., Wu, X., & Chen, J. (2021). Prevention and Control Strategies of African Swine Fever and Progress on Pig Farm Repopulation in China. *Viruses*, *13*(12), 2552. https://doi.org/10.3390/v13122552

Lv, T., Xie, X., Song, N., Zhang, S., Ding, Y., Liu, K., Diao, L., Chen, X., Jiang, S., Li, T., Zhang, W., & Cao, Y. (2022). Expounding the role of tick in Africa swine fever virus transmission and seeking effective prevention measures: A review. *Frontiers in Immunology*, *13*. https://doi.org/10.3389/fimmu.2022.1093599

Machtinger, E. T., Gerry, A. C., Murillo, A. C., & Talley, J. L. (2021). Filth fly impacts to animal production in the United States and associated research and extension needs. *Journal of Integrated Pest Management*, *12*(1). https://doi.org/10.1093/jipm/pmab026

Mazur-Panasiuk, N., Zmudzki, J., & Woźniakowski, G. (2019). African swine fever virus – persistence in different environmental conditions and the possibility of its indirect transmission. *Journal of Veterinary Research*, *63*(3), 303–310. https://doi.org/10.2478/jvetres-2019-0058

Meerburg, B. G., Vermeer, H. M., & Kijlstra, A. (2007). Controlling risks of pathogen transmission by flies on organic pig farms: a review. *Outlook on AGRICULTURE*, *36*(3), 193-197.

Mellor, P. S., Kitching, R., & Wilkinson, P. (1987). Mechanical transmission of capripox virus and African swine fever virus by Stomoxys calcitrans. *Research in Veterinary Science*, *43*(1), 109–112. https://doi.org/10.1016/s0034-5288(18)30753-7

Nielsen, S. S., Alvarez, J., Bicout, D., Calistri, P., Depner, K., Drewe, J. A., Garin-Bastuji, B., Rojas, J. L. G., Schmidt, C. G., Herskin, M. S., Michel, V., Pasquali, P., Roberts, H., Sihvonen, L., Spoolder, H., Ståhl, K., Velarde, A., Winckler, C., Blome, S., Chueca, M. Á. M. (2021). Research priorities to fill knowledge gaps in the control of African swine fever: possible transmission of African swine fever virus by vectors. *EFSA Journal*, *19*(6). https://doi.org/10.2903/j.efsa.2021.6676

Ola-Fadunsin, S. D., Gimba, F. I., Abdullah, D. A., Abdullah, F. F. J., & Sani, R. A. (2020). Dataset on the diversity and distribution of biting flies collected from cattle farms in Peninsular Malaysia. *Data in Brief*. https://doi.org/10.1016/j.dib.2020.105315

Olesen, A. S., Hansen, M. F., Rasmussen, T. B., Belsham, G. J., Bødker, R., & Bøtner, A. (2018b). Survival and localization of African swine fever virus in stable flies (Stomoxys calcitrans) after feeding on viremic blood using a membrane feeder. *Veterinary Microbiology*, *222*, 25–29. https://doi.org/10.1016/j.vetmic.2018.06.010

Olesen, A. S., Lohse, L., Hansen, M. F., Boklund, A., Halasa, T. H. B., Belsham, G. J., Rasmussen, T. B., Bøtner, A., & Bødker, R. (2018a). Infection of pigs with African swine fever virus via ingestion of stable flies (*Stomoxys calcitrans*). *Transboundary and Emerging Diseases*, *65*(5), 1152–1157. https://doi.org/10.1111/tbed.12918

Onmaz, A. C., Beutel, R. G., Schneeberg, K., Păvăloiu, A. N., Komarek, A., & Van Den Hoven, R. (2012). Vectors and vector-borne diseases of horses. *Veterinary Research Communications*, *37*(1), 65–81. https://doi.org/10.1007/s11259-012-9537-7

Panei, C. J., Larsen, A. E., Fuentealba, N. A., Metz, G. E., Echeverría, M. G., Galosi, C. M., & Valera, A. R. (2019). Study of horn flies as vectors of bovine leukemia virus. *Open Veterinary Journal*, *9*(1), 33. https://doi.org/10.4314/ovj.v9i1.6

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

Qin, W., Gao, Z., Wu, S., & Bao, W. (2021). Preliminary analysis of whether mosquitoes can carry and transmit African swine fever. *BMC Veterinary Research*, *17*(1). https://doi.org/10.1186/s12917-021-02865-2

Sánchez-Vizcaíno, J. M., Martínez-López, B., Martínez-Avilés, M., Martins, C., Boinas, F., Vialc, L., Michaud, V., Jori, F., Etter, É., Albina, E., & Roger, F. (2009). Scientific review on African Swine Fever. *EFSA Supporting Publications*, *6*(8), p1-141. https://doi.org/10.2903/sp.efsa.2009.en-5

Shah, B., Khan, I. A., & Ahmed, N. (2016). Study on the biology of blowfly and the food consumption of blowfly maggots. *Journal of Entomology and Zoology Studies*, *3*(3), 321–323. https://doi.org/10.22271/j.ento

Showler, A. T., & Osbrink, W. L. A. (2020). Stable Fly, Stomoxys calcitrans (L.), Dispersal and Governing Factors. *International Journal of Insect Science*, *7*(1).

Siddiki, S., & Sp, Z. (2017). Studies on Time Duration of Life Stages of Chrysomya megacephala and Chrysomya rufifacies (Diptera: Calliphoridae) during Different Seasons. *Journal of Forensic Research*, *08*(03). https://doi.org/10.4172/2157-7145.1000379

Sindryakova, I., Morgunov, Y., Chichikin, A., Газаев, И., Kudrjashov, D. A., & Tsybanov, S. (2016). The Influence Of Temperature On The Russian Isolate Of African Swine Fever Virus In Pork Products And Feed With Extrapolation To Natural Conditions. *Sel'skohozâjstvennaâ Biologiâ*, *51*(4), 467–474. https://doi.org/10.15389/agrobiology.2016.4.467eng

Taylor, M. A., Coop, R. L., & Wall, R. L. (2015). *Veterinary parasitology*. John Wiley & Sons.

Wall, R., & Shearer, D. (2001). *Veterinary ectoparasites: Biology, Pathology and Control*. Wiley-Blackwell.

Yin, J., Kelly, P. J., & Wang, C. (2022). Flies as vectors and potential sentinels for bacterial pathogens and antimicrobial resistance: a review. *Veterinary Sciences*, *9*(6), 300. https://doi.org/10.3390/vetsci9060300

Yoon, H., Hong, S., Lee, I., Choi, D., Lee, J., Lee, E., & Wee, S. (2021). Arthropods as potential vectors of African swine fever virus outbreaks in pig farms in the Republic of Korea. *Veterinary Medicine and Science*, *7*(5), 1841–1844. https://doi.org/10.1002/vms3.545

APPENDIX I

Sticky fly traps in the sewage pool

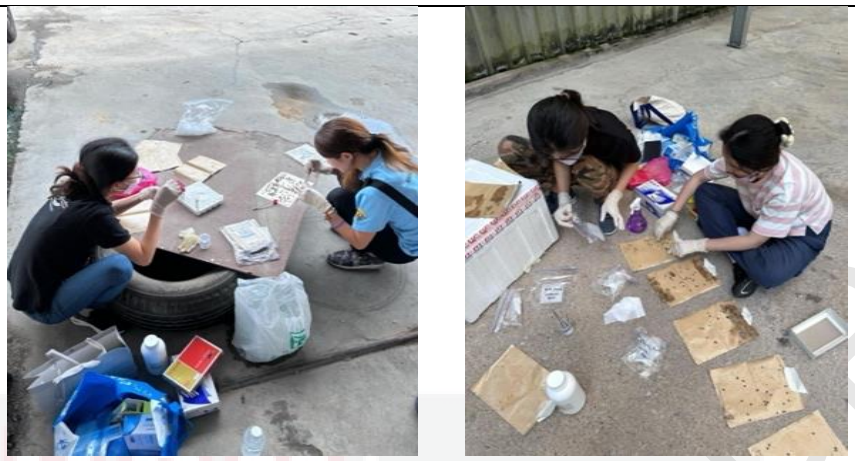
Sticky fly traps in the feed house

Sticky fly traps in the finisher pen

Sticky fly traps in the sow pen

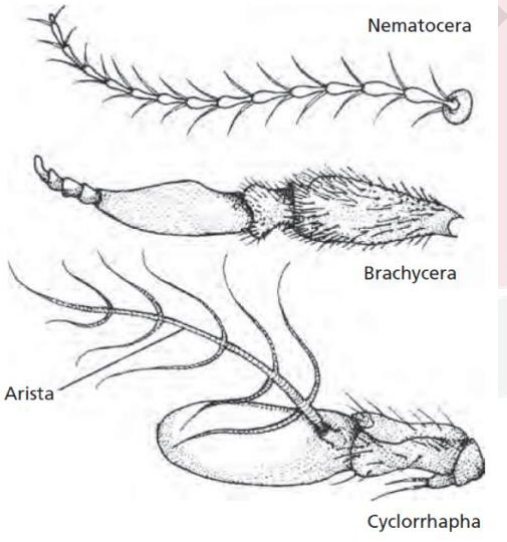
Flies collected on sticky fly traps

Sticky fly traps used for fly collection

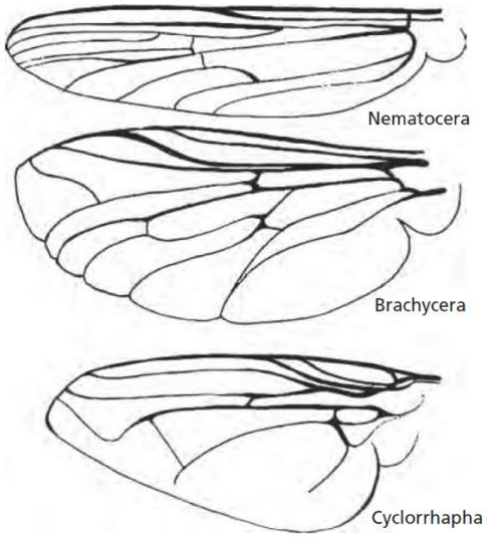


Field sampling of flies at the farm

APPENDIX II



Variation in antennae of three suborders of Diptera (Taylor et. al., 2015)

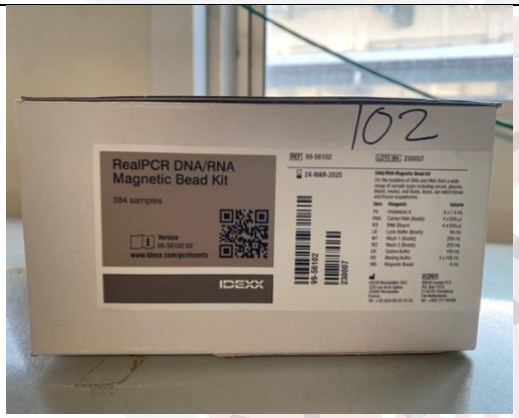


Variation in wing venation of three suborders of Diptera (Taylor *et al*., 2015)

APPENDIX III

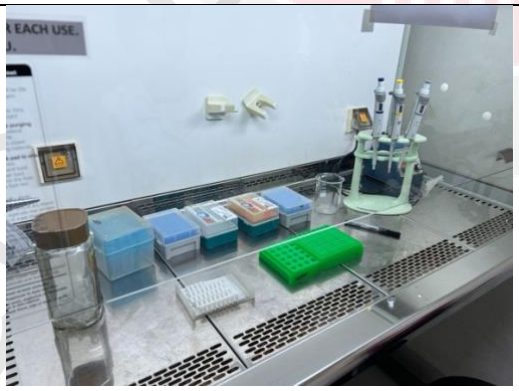
Homogeniser and materials for homogenisation

Magnetic separator rack for DNA extraction using magnetic beads kit



RealPCR DNA/RNA
Magnetic Bead Kit by IDEXX

RealPCR ASFV DNA Mix
by IDEXX



Materials needed for qPCR procedure

CFX Opus 96 RealTime System
(Bio-Rad)