UNIVERSITI PUTRA MALAYSIA

DETECTION OF AFRICAN SWINE FEVER VIRUS IN RATS

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DETECTION OF AFRICAN SWINE FEVER VIRUS IN RATS

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CERTIFICATION

It is hereby certified that we have read this project paper entitled "Detection of African swine fever virus in rats" by Poo Yuen Xun and in our opinion it is satisfactory in terms of scope, quality and presentation as partial fulfilment of the requirement for the course VPD4999 – Final Year Project.

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DEDICATION

This thesis is especially dedicated to:

My loving parents

Poo Chong Tek and Chang Sui Geuk

My supportive supervisors

Assoc Prof Dr Ooi Peck Toung

Dr Michelle Fong Wai Cheng

And

My loving partner

Dylon Wong Chung Yee

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DETEKSI AFRICAN SWINE FEVER VIRUS DALAM TIKUS

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Tikus merupakan perosak utama di dalam ladang ternakan babi dan tikus mempunyai peranan yang penting dalam penghantaran beberapa penyakit kepada babi. Peranan tikus dalam penghantaran mekanikal African swine fever virus (ASFV) kepada babi masih belum difahami dengan sepenuhnya. Oleh itu, perkara ini perlulah disiasat kerana wabak African swine fever (ASF) telah menyebabkan banyak kerugian kepada industri babi di seluruh dunia. Tujuan projek ini adalah untuk menentukan bahawa tikus ialah vektor mekanikal ASFV. Projek ini telah dilakukan dengan mengumpulkan sampel swab oronasal, toraks dan kaki dari 15 tikus di dalam lima ladang ternakan babi. Ini telah diikuti oleh langkah memproseskan sampel, melakukan ekstraksi DNA dengan meggunakan kit manik magnet IDEXX RealPCR^{TM} DNA/RNA (IDEXX Montpellier, Perancis) dan melaksanakn PCR masa

nyata dengan menggunakan ujian IDEXX RealPCR ASFV DNA (IDEXX Montpellier, Perancis) untuk deteksi ASFV di dalam sampel swab yang dikumpulkan dari tikus. Satu tikus telah disahkan sebagai ASFV-positif dan sampel yang positif tersebut ialah swab toraks. Tiada ASFV yang wujud dalam swab oronasal dan kaki. Keputusan positif swab toraks mungkin adalah disebabkan kawasan permukaan kawasan toraks yang besar berbanding dengan kawasan oronasal dan kaki. Seks tikus mungkin berkaitan dengan keputusan ASFV-positif tetapi panjang badan dan berat badan tikus mungkin tidak berkaitan dengan keputusan tersebut. Walau bagaimanapun, andaian di atas hanya dibuat berdasarkan saiz sampel yang terhad sahaja. Secara amnya, tikus merupakan vektor mekanikal ASFV dan penternak babi seharusnya mengawal populasi tikus di dalam ladang ternakan babi supaya ASF dapat dielakkan.

Kata kunci: *African Swine Fever Virus (ASFV), babi, tikus, Malaysia, PCR masa nyata*

ABSTRACT

An abstract of the project paper presented to the Faculty of Veterinary Medicine in partial fulfilment of the course VPD4999 - Final Year Project.

DETECTION OF AFRICAN SWINE FEVER VIRUS IN RATS

By

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Rats are major pests in pig farms that play a potential role in transmitting several diseases to pigs. The role of rats in transmitting African swine fever virus (ASFV) mechanically to pigs has not been completely understood, and this should be investigated as African swine fever (ASF) outbreaks had caused significant losses to the pig industries worldwide. The purpose of the study was to determine rats as mechanical vectors of ASFV. This study was done by collecting oronasal, thoracic and paw swab samples from 15 rats in five farms, conducting sample processing followed by DNA extraction using IDEXX RealPCR^{TM} DNA/RNA magnetic bead kit (IDEXX Montpellier, France), and carrying out real-time PCR using IDEXX RealPCR ASFV DNA test (IDEXX Montpellier, France) on the swab samples to detect the presence of ASFV. One rat was detected as ASFV-positive and the positive sample was a thoracic swab. No ASFV was detected in the oronasal and paw swab samples.

Positive result of thoracic swab might be due to the large surface area of thoracic region of rats compared to the oronasal region and paws. Sex of rats might be associated with ASFV-positivity but body length and body weight of rats might not be associated. However, the assumptions made were only based on the limited sample size of this study. Generally, rats are mechanical vectors of ASFV and the findings highlight the need for farmers to control the rat population in their farms.

Key words: *African Swine Fever Virus (ASFV), swine, rats, Malaysia, real-time PCR*

1.0 INTRODUCTION

African swine fever (ASF) is a disease that occurs in domestic and wild pigs of all ages (World Organisation for Animal Health [WOAH], 2019) caused by African swine fever virus (ASFV), which is an enveloped, large, double-stranded DNA virus that belongs to the Asfarviridae family as stated by Alonso et al. (2018). It is a highly deleterious and contagious disease of swine species (Galindo and Alonso, 2017) that can cause significant losses of pig industries worldwide. The clinical signs of ASF can be classified into peracute, acute, subacute and chronic and this largely depends on the virulence of the virus (WOAH, 2019).

In August 2018, the first case of ASF in Asia-Pacific was reported in Liaoning province in China (Ge et al., 2018). Then, it started to spread rapidly to other East Asia countries such as Hong Kong, South Korea, Mongolia and North Korea, along with Southeast Asia countries like Cambodia, Philippines, Laos, Vietnam, Indonesia, Myanmar and Timor-Leste (FAO, 2023). Besides that, Melanesia (Papua New Guinea) and South Asia countries, in particular, India were also affected (FAO, 2023). In February 2021, it was confirmed that backyard pigs from many locations in Sabah were infected with ASF through usage of real-time PCR (Khoo et al., 2021). This was the first outbreak of ASF in Malaysia. The ASF outbreak is still occurring at this point of time and the stamping out policy was implemented by Department of Veterinary Services Malaysia to eliminate ASF (Khoo et al., 2021).

African swine fever (ASF) can be transmitted among pigs through direct contact and indirect contact. Direct contact involves infection through skin abrasions or the

oronasal route due to contact with excretions and secretions of infected pigs (Dixon et al., 2019). On the other hand, indirect contact involves fomites like equipment and transport vehicles containing ASFV particles (Olesen et al., 2017). Soft ticks of the *Ornithodoros* genus also play an important part in the indirect transmission of ASF as mentioned by Lv et al. (2022). The three main transmission cycles of ASF are the sylvatic cycle, tick-pig cycle and domestic cycle in between pigs to pigs (Costard et al., 2013).

Besides that, an article stated that rats might be a low-risk vector for ASF (Sundberg, 2021). Although the samples collected showed a negative result for ASF (Sundberg, 2021), there is no evidence at all to support this finding. Recently, there are raising concern among farmers on the role of rats in ASF transmission. Thus, the role of rats in transmitting ASF should be investigated as ASF outbreak is still a huge global problem that causes great losses to the swine industry. Aside from that, rats are able to carry various types of pathogens that can infect pigs such as porcine circovirus type 2 (PCV2), encephalomyocarditis virus (EMCV), *Brachyspira* spp., *Leptospira* spp., *Lawsonia* spp., *Salmonella* spp., *Campylobacter* spp. and influenza A virus. (Backhans and Fellström, 2012; Cummings et al., 2019; TRUONG et al., 2013; M. Zhao et al., 2023). Since rats are vectors of many infectious diseases in pigs, there is a chance that ASFV might be one of it. In this study, detection of ASFV in rats will be conducted. This will be done by swabbing the mouth and paws of each rat and carrying out PCR by using the samples obtained. This study is of utmost importance as pest control measures can be taken immediately to control ASF outbreak if rats are proven to be mechanical vectors of ASFV.

Objective

1. To determine rats as mechanical vectors of African swine fever virus

Hypotheses

Null hypothesis: Rats are mechanical vectors of African swine fever virus

Alternative hypothesis: Rats are not mechanical vectors of African swine fever virus

2.0 LITERATURE REVIEW

2.1 Overview of African Swine Fever

African swine fever (ASF) is a viral illness that affects domestic pigs and results in a significant mortality rate (Galindo and Alonso, 2017; Parker et al., 1969; Thomson et al., 1980). This disease causes substantial economic losses, and without the availability of an effective vaccine, these losses are inevitable (Galindo and Alonso, 2017). Aetiological agent of ASF is ASFV, which is a double-stranded DNA virus characterized by a complex molecular structure (Galindo and Alonso, 2017). It is the sole representative of the Asfarviridae family (Galindo and Alonso, 2017), and it is the only DNA virus that is disseminated via arthropods, specifically soft ticks belonging to the *Ornithodoros* genus (Anderson et al., 1998; Galindo and Alonso, 2017; Kleiboeker et al., 1999). *Ornithodoros moubata* play a role in the sylvatic transmission cycle of the virus in Africa, while in Europe, the soft tick involved is *O. erraticus* (Galindo and Alonso, 2017).

During the early 1900s, ASF emerged in East Africa, presenting itself as an acute haemorrhagic fever that resulted in an extremely high fatality rate among infected domestic pigs (Dixon et al., 2019). The infection source was traced back to a virus, which originated from an ancient sylvatic cycle (Dixon et al., 2019; Eustace Montgomery, 1921; Plowright et al., 1969). Since then, ASFV gradually spread to most of the sub-Saharan African nations (Dixon et al., 2019; Mulumba-Mfumu et al., 2019). The transcontinental spread of ASFV initially occurred in Portugal in 1957 and 1960. From there, it spread to various countries in Europe, the Caribbean, and Brazil (Dixon et al., 2019). By the mid-1990s, eradication efforts proved to be successful in

most affected areas, except for Sardinia, where the disease remained endemic (Dixon et al., 2019).

In 2007, ASFV was introduced to Georgia in the Caucasus region (Dixon et al., 2019). From Georgia, ASF was rapidly transmitted to the Russian Federation, Ukraine, and Belarus, and in 2014, it reached the EU Baltic States and Poland (Dixon et al., 2019). By 2018, ASF had also spread to Belgium, Hungary, Romania, the Czech Republic, Slovakia, Bulgaria and Serbia (Dixon et al., 2019; EFSA Panel on Animal Health and Welfare [AHAW], 2015; European Food Safety Authority [EFSA] et al., 2017). In 2018, the situation became worse when ASFV was identified in China, a country consisting of half of the world's swine population (Dixon et al., 2019). Its widespread prevalence in China was soon followed by its spread to neighboring countries, including Mongolia, Cambodia, Vietnam, North Korea, Laos, Myanmar and the Philippines (Dixon et al., 2019).

The first outbreak of ASF in Malaysia occurred in February 2021, which the disease was detected in backyard pigs in Sabah through the usage of real-time PCR (Khoo et al., 2021). After the outbreak, the Malaysia government planned to cull 3000 wild and domestic pigs (Reuters, 2021). African swine fever (ASF) had also been found in wild boars in Sungkai and Bidor in Perak, and Jerantut in Pahang, as reported by the Department of Veterinary Services (DVS) on 18th of December, 2021 (The Star, 2021a). On 25th of December 2021, the DVS officially declared an ASF outbreak on seven commercial pig farms located in Paya Mengkuang at Masjid Tanah, Melaka (The Star, 2021b).

Following that, on 10th of January 2022, Sarawak has identified ASF in three backyard pig farms located in Durin, within the Sibu division (Pei, 2022). Another ASF outbreak was confirmed by DVS at two commercial pig farms in the Kerian district of Perak on 17th and 19th of September 2022 (The Star, 2022). Subsequently, the affected farms were prevented from transporting of any pigs and vehicle movement was prohibited (The Star, 2022). In Peninsular Malaysia, ASF was also detected in Negeri Sembilan, Terengganu, Kelantan and Johor (FAO, 2023). During the beginning of 2023, ASF outbreak was reported in Seberang Perai District, Pulau Pinang, along with Kinta and Hilir Perak districts in Perak (FAO, 2023).

African swine fever virus (ASFV) can give rise to a spectrum of syndromes, ranging from peracute and acute diseases to chronic conditions, and even subclinical infections (WOAH, 2019). Typically, the incubation period for ASF is 4 to 19 days (WOAH, 2019). In the presence of more virulent strains, infected pigs may develop a peracute or acute haemorrhagic disease (WOAH, 2019). This is characterized by clinical symptoms such as high fever, reduced appetite, presence of haemorrhages in the skin and internal organs, and even death within 4 to 10 days (WOAH, 2019). In some cases, pigs may succumb to the disease even before the first clinical sign appears. The case fatality rates in such instances can be significantly high, reaching up to 100% (WOAH, 2019). Less virulent strains of ASF typically result in milder clinical signs in infected pigs (WOAH, 2019). These signs include slight fever, diminished appetite, and a sense of depression (WOAH, 2019). Interestingly, these clinical symptoms can easily be mistaken for other pig-related diseases, which may not give rise to suspicion of ASF (WOAH, 2019).

2.2 Diagnostic Methods for African Swine Fever

The diagnosis of African swine fever (ASF) involves identifying animals that are currently or have been previously infected with the ASFV (Oura et al., 2013). A positive diagnosis involves detecting and identifying ASFV-specific antibodies, antigens or DNA in diagnostic samples collected from pigs using virus isolation, serological tests, or molecular assays (Oura et al., 2013). Laboratory diagnostic methods for ASF can be categorized into two groups, which are virological and serological tests. These assays include the fluorescent antibody test (FAT), virus isolation, polymerase chain reaction (PCR), enzyme-linked immunosorbent assay (ELISA) and indirect immunoperoxidase test (IPT) (WOAH, 2019). Nonetheless, carrying out such diagnostic procedures in many countries where ASFV is currently circulating may not be possible due to limited access to molecular diagnostic tools and the challenges in carrying out virus isolation (Oura et al., 2013).

The cheaper assays for detecting ASF antigen like ELISA and FAT are available (Oura et al.,2013). However, it's important to note that these assays may have a lower sensitivity compared to PCR (Oura et al., 2013). For example, although FAT is highly sensitive in detecting acute ASF cases, its sensitivity significantly decreases in subacute and chronic cases of ASF. This may be due to the formation of antigen-antibody complexes in infected pig tissues, which prevents the ASFV antigen from interacting with ASF conjugate (Oura et al., 2013). Similar to the FAT test, the antigen ELISA also exhibits greatly reduced sensitivity in cases of subacute and chronic disease (Oura et al., 2013). Thus, it is recommended to perform ELISA in parallel with

antigen testing so that the sensitivity and specificity of the results can be increased (Sánchez-Vizcaíno et al., 2015).

Next, the IPT is considered as the best test to test fluids, blood or exudate tissue samples due to its great performance and sensitivity (Gallardo et al., 2015; WOAH, 2019). Besides that, this test also serves as a valuable serological technique for the early detection of ASF, even in cases where only limited number of antibodies are detectable (Gallardo et al., 2015). However, it is important to note that IPT is a time-consuming method that needs individual microscopic examination of each sample and the interpretation of results may differ according to the examiner (Gallardo et al., 2015). Subsequently, the haemadsorption (HAD) test, which is a type of virus isolation test that relies on the fact that pig erythrocytes can bind to the surface of pig monocyte or macrophage cells that have been infected with ASFV can also be used in ASFV detection (de León et al., 2013). A key limitation of HAD is that it needs primary cell cultures, and this extends the time required to confirm a negative outcome to six days (Oura et al., 2013).

PCR has been developed utilizing primers from a highly conserved region of the genome in order to detect and identify many isolates of all the known virus genotypes, including viruses that do not induce haemadsorption and low-virulence isolates (WOAH, 2019). Real-time PCR (rPCR) is considered to be the most powerful method for detection of viruses in field samples, including ASFV at the present time (Truong et al., 2020). Aside from that, PCR is the recommended method for both screening and confirmation of suspected cases due to its great sensitivity and specificity, along with its potential for a high throughput application (WOAH, 2019). Currently, rPCR is

considered to be the gold standard test for ASFV genome detection, and this test is employed in all the OIE regional reference laboratories. This is because PCR allows for the rapid diagnosis of ASF within hours of receiving the sample, enabling a quicker implementation or removal of control measures compared to virus isolation (Oura et al., 2013).

Several conventional and real-time PCR methods have been documented (Fernández-Pinero et al., 2013; Tignon et al., 2011; WOAH, 2019) and various commercial PCR kits are available for detection of ASFV. For example, the QIAamp® DNA Mini kit, VetMAX™ African Swine Fever Virus Detection Kit, ID Gene African Swine Fever Duplex and POBGEN™ African Swine Fever Virus Detection qPCR kit are real-time PCR kits that can be used to detect ASFV (Lee et al., 2022). The following PCR tests have also been proved to be fit for detection of ASFV: INgene q PPA (Ingenasa), virotype ASFV (Indical Bioscience, formerly Qiagen Leipzig), RealPCR ASFV (IDEXX), ID Gene ASF Duplex (IDvet), SwineFever combi (gerbion), ViroReal Kit ASF Virus (Ingenetix), VetMAX African Swine Fever Virus Detection Kit (Thermo Fisher Scientific) and Kylt ASF Real-Time PCR (Anicon) (Blome et al., 2020). Duplex RT-PCR techniques have also been described for the simultaneous and differential detection of both ASFV and CSFV (Agüero et al., 2004; Haines et al., 2013; WOAH, 2019). In addition, several multiplex qRT-PCR assays have been developed to detect ASFV, classical swine fever virus (CSFV) and porcine reproductive and respiratory syndrome virus (PRRSV) (Chen et al., 2021), ASFV, CSFV and atypical porcine pestivirus (APPV) (H. Liu et al., 2021), or ASFV, CSFV and *Erysipelothrix rhusiopathiae* (L. Zhao et al., 2023) simultaneously. For DNA

extraction before carrying out PCR, the assays that can be used include the IDEXX RealPCR™ DNA/RNA magnetic bead extraction assay and the MagMax Core DNA/RNA extraction assay (Havas et al., 2022).

2.3 Information about Rats

Rats belong to the kingdom Animalia, phylum Chordata, class Mammalia and order Rodentia (Nur-Fazila, 2023). Rodentia comes from the Latin word "rodere", which means gnaw (Legendre, 2003). Therefore, all rodents have the notable feature of having two pairs of continually growing incisors that they use for gnawing (Legendre, 2003). Rodentia is the largest order of mammals and there are five families within this order, namely Muridae, Echymyidae, Sciuridae, Dipodidae and Heteromyidae (Hoffman and Cawthorn, 2014). Rats are classified within the family Muridae, where it consists of approximately 66 % of all of the rodent taxa (Hoffman and Cawthorn, 2014).

Rats are frequently used as laboratory animals and are bred for commercial purposes in the pet trade (Tully, 2009). In contrast to mice, rats exhibit excellent pet qualities, characterized by a charming and personable demeanour, along with high intelligence (Tully, 2009). The common rat species kept as a companion animal is *Rattus norvegicus*, with the white rat and hooded rat being the most common variations (Tully, 2009). The birth weight of a rat is 5 to 6 g, and an adult male rat has a body weight of 450 to 520 g. At the same time, an adult female rat has a body weight of 250 to 320 g (Tully, 2009). Rats have a weaning age of three weeks, and they can reach sexual maturity at six to eight weeks, with an oestrus cycle of four to five days (Nur-Fazila, 2023). The breeding life for both male and female rats is one year, and

they can live up to one and a half to three years (Nur-Fazila, 2023). Next, rats have a gestation period of 22 to 23 days (Hain, 1936), and a litter size of eight to 12 (Nur-Fazila, 2023).

In Malaysia, the most common rat species include *Rattus norvegicus*, which is the Norway rat or brown rat and *Rattus rattus diardii*, known as the common house rat or black rat (Mohd-Qawiem et al., 2022). There are some differences in between these two rat species. In terms of body weight, adult brown rats have a body weight of 340 to 567 g while adult black rats have a body weight of 230 to 304 g (Koehler et al., 2023). Next, brown rats have a blunt nose while black rats have a more pointed nose (Nur-Fazila, 2023). Additionally, brown rats have a smaller ear then black rats, with a heavy and thick body in comparison to the light and slender body of the black rat (Nur-Fazila, 2023). Brown rats also have a tail which is shorter than the head and the body while black rats have a tail which is longer than the head and the body (Nur-Fazila, 2023). For the fur colour, brown rats usually have coarse brown fur with some scattered black hairs while black rats have fur colour ranging from brownish grey to grey or solid black (Koehler et al., 2023).

Mice also belongs to the order Rodentia and family Muridae as in rats (Ellenberger, 1993), but they are different from rats in some aspects. For example, mice have a body weight of 20 to 40 g while rats have a body weight of 300 to 500 g (Nur-Fazila, 2023). Other than that, mice have a gallbladder but rats do not (Nur-Fazila, 2023). Mice need to consume 4 to 5 g of food per day while rats need to consume 15 to 20 g of food per day (Nur-Fazila, 2023). At the same time, mice require 6 to 7 ml of water per day while rats require 30 to 50 ml of water per day (Nur-Fazila, 2023).

However, rats and mice are similar in a way that both of them are nocturnal animals where they feed at night (Penicaud et al., 2013). They are also considered as social animals with body temperature of 36.5 °C (Nur-Fazila, 2023).

2.4 Pathogens in Pigs with Rats as Vectors

Rats are classified under the order Rodentia. Rodentia is considered as one of the most varied mammalian orders in this world (Islam et al., 2021; Solari and Baker, 2007). The 2552 known species within this order make up around 39.3% of mammals and they are the crucial elements of numerous terrestrial ecosystems (Islam et al., 2021). Rats have some beneficial roles in nature, such as insect control and soil aeration (Burgin et al., 2018; Islam et al., 2021; Rabiee et al., 2018). Nonetheless, rats also serve as reservoirs for several swine pathogens (Le Moine et al., 1987; TRUONG et al., 2013). The close contact and proximity of rats to pigs raise the potential for the transmission of infections (Le Moine et al., 1987; TRUONG et al., 2013). Consequently, it is crucial to conduct screenings of rats for swine pathogens (TRUONG et al., 2013). This screening is vital for assessing the risk of microbial transmission within swine farms (TRUONG et al., 2013).

Diseases carried by rats can be classified into two primary categories (Rabiee et al., 2018). This includes those that are transmitted directly and those that are transmitted indirectly (Rabiee et al., 2018). In the first category, diseases spread through bites or inhalation of germs found in rat faeces, while in the second category, infection in human or animals can occur after consuming food and water tainted by rat faeces or urine (Rabiee et al., 2018). Similarly, rats might serve as amplifying hosts when it comes to diseases that can be transmitted through arthropod vectors (Rabiee

et al., 2018). Additionally, if rats are accidentally consumed by livestock and these livestock products are not adequately treated before being consumed by human or animals, they could facilitate the transmission of diseases (Meerburg et al., 2009; Rabiee et al., 2018).

Viruses in pigs that can be transmitted biologically through rats include porcine circovirus type 2 (PCV2) and encephalomyocarditis virus (EMCV). First of all, PCV2 is a small, non-enveloped virus containing single-stranded circular DNA (ssDNA) that has the potential to induce PCV-associated diseases (PCVADs) (Zhao et al., 2023). The amplification and characterization of novel PCV2 strains in wild rats which were captured far away from pig farms showed that among the 14 tissue samples, half of them were tested positive for PCV2 (Zhao et al., 2023). In another study, PCV2 presence was identified in various organs of rats using PCR, showing an overall prevalence of 14.7% (15 out of 102 samples) (TRUONG et al., 2013). These organs include the tonsils, lymph nodes, spleen, lungs, liver, heart, and kidneys (TRUONG et al., 2013).

Secondly, EMCV is a small, non-enveloped ssRNA (Carocci and Bakkali-Kassimi, 2012) cardiovirus of the family Picornaviridae that will lead to sudden deaths and acute form of myocarditis in growing pigs (Backhans and Fellström, 2012; Koenen et al., 1999; Murnane et al., 1960). It will also cause reproductive issues in sows such as birth of weak piglets or abortion (Backhans and Fellström, 2012; Dea et al., 1991; Koenen et al., 1994). It has been confirmed that wild rats are natural reservoir of EMCV (Backhans and Fellström, 2012; Spyrou et al., 2004), where the virus will be shed in the faeces (Backhans and Fellström, 2012; Psalla et al., 2006).

Next, 3.9% of the rats collected was positive for EMCV, and the virus were present in the kidneys, lungs, liver, heart and spleen (TRUONG et al., 2013)).

Subsequently, bacterial infections that can be transmitted biologically by rats and infect pigs include swine dysentery, yersiniosis, leptospirosis, salmonellosis and campylobacteriosis. (Backhans and Fellström, 2012; TRUONG et al., 2013). *Brachyspira hyodysenteriae* serves as the causative agent of swine dysentery (Backhans & Fellström, 2012; Taylor & Alexander, 1971), a condition in pigs characterized by intense mucohaemorrhagic diarrhea. This disease can affect pigs of all age groups, excluding newborns (Backhans & Fellström, 2012). Microscopic observation has revealed the presence of intestinal spiral-shaped bacteria in both rats captured from natural habitats and those within laboratory settings, where some of them demonstrated the morphological characteristics of *Brachyspira* spp. (Backhans & Fellström, 2012; Lee & Phillips, 1978). Rat and mouse populations within pig herds can carry *B. hyodysenteriae* (Backhans & Fellström, 2012; Claes et al., 2004; Trott et al., 1996).

Yersiniosis caused by *Yersinia enterocolitica* is widely reported across several countries and it is the third most prevalent zoonosis in the European Union (Thong et al., 2018). Pigs are the primary host for *Yersinia enterocolitica* (Bhaduri et al., 2005; Korte et al., 2004; Virtanen et al., 2012). Next, *Yersinia pseudotuberculosis* has also been isolated from wild boars (Backhans and Fellström, 2012; Fredriksson-Ahomaa et al., 2009) and domestic pigs (Backhans and Fellström, 2012; Niskanen et al., 2002; Shiozawa et al., 1988). Additionally, *Y. pseudotuberculosis* has been detected in mice, moles, and barn rats (Backhans and Fellström, 2012; Fukushima et al., 1990).

Leptospirosis is a globally widespread zoonotic illness that gives rise to a spectrum of subclinical to severe cases of icteric leptospirosis accompanied by renal failure, and it is commonly referred to as Weil's disease (Backhans and Fellström, 2012; Levett, 2001). *Leptospira* spp. can be found in 63.7% of the rodents collected from areas surrounding nine pig farms in northeast South Korea (TRUONG et al., 2013). Altogether, 37 rats (14%) tested positive through at least one of the diagnostic tests, which is contrary to the frequently cited prevalence rates of 50-70% for wild rats in the UK (Webster et al., 1995). Lastly, *Leptospira* spp. has also been identified in numerous pigs experiencing reproductive issues (Yung et al., 2008).

Salmonellosis, an infectious disease affecting both humans and animals, is caused by the bacteria belonging to the genus *Salmonella* (WOAH, 2018). In a study done to investigate rats as a source of *Salmonella* contamination in wet markets located in Thailand, a total of 110 rats which includes *Rattus norvegicus* and *Rattus exulans* were examined (Ribas et al., 2016). The overall prevalence of *Salmonella* in rats was 49.10 %, with a variation between 0 % and 73.3 % among different wet markets (Ribas et al., 2016). (Ribas et al., 2016) *Salmonella Typhimurium* typically originates from sources such as pig, poultry, or bovine meat (Backhans and Fellström, 2012). Affected pigs often serve as subclinical carriers of zoonotic Salmonella, though certain serovars can lead to disease in the pig population (Backhans and Fellström, 2012).

Lastly, for campylobacteriosis, a particular study concluded that the presence of rats emerged is a risk factor contributing to elevated *Campylobacter* prevalence within broiler chicken flocks (Backhans and Fellström, 2012; Berndtson et al., 1996). Pigs, especially those in the growing stage, frequently exhibit colonization by

Campylobacter spp., primarily by *C. coli*, and to a lesser extent by *C. jejuni*. However, findings derived from genotyping investigations reveal that genetic distinctions between isolates from pigs and those from humans are more pronounced than the genetic variations seen between isolates from pigs and poultry (Backhans and Fellström, 2012; Denis et al., 2009).

The only pathogen that was proved to be mechanically transmitted by rats is the influenza A virus (IAV). Influenza A virus belongs to the family Orthomyxoviridae with a characteristic of segmented, negative-strand RNA genomes (Bouvier and Palese, 2008). In swine, influenza A viruses will lead to acute respiratory infections, causing substantial economic losses in the global pig production (Mancera Gracia et al., 2020). In a study done to investigate presence of Influenza A in Norway rats (*Rattus norvegicus*), it was found that 18 among 163 rats captured were IAV-positive in the RT-PCR carried out (Cummings et al., 2019).

2.5 Sample Collection Methods for Rat-Borne Diseases

Due to their non-toxic nature, traps are classified as one of the safest approaches for eliminating rats (Koehler et al., 2023). Besides that, dead rats found in rat traps can easily be located and disposed of than those rats that are killed by poisons (Koehler et al., 2023). There are a variety of rat traps that can be used to catch rats (Herbreteau et al., 2011). The traps include live-traps, kill-traps (Herbreteau et al., 2011) and glue boards (Koehler et al., 2023). Live-traps include cage-traps and box-traps, and they are important in situations where animals need to be released or when specific tissue samples or blood are needed (Herbreteau et al., 2011). On the other hand, kill-traps include snap traps and snares, and they are proved to be more effective in capturing

numerous species, offering the advantages of being small and cheap (Herbreteau et al., 2011). In addition, glue boards, also known as sticky traps, are disposable cardboard or plastic units coated with a thick layer of sticky paste (Herbreteau et al., 2011). Glue boards are non-toxic, cost-effective and convenient without need of any specialized application equipment (Williams, 2013).

The standard set of samples that can be collected from rats for a PCR Rodent Infectious Agent (PRIA) testing include oral swabs, faecal pellets and body swabs (Henderson and Clifford, 2013). A body swab can be used to detect *Corynebacterium bovis* pinworms and fur mites (Henderson and Clifford, 2013). It can be collected by swabbing the entire body against the direction of hair growth by using a "sticky" swab (Henderson and Clifford, 2013). Oral swabs can be collected to detect several types of respiratory bacteria and viruses (Henderson and Clifford, 2013). For oral swab collection, a dry non-alginate swab can be used to swab the oral cavity in a circular motion (Henderson and Clifford, 2013). For oronasal swabs, the samples were taken by first swabbing the external nares, then inserting the swab into the throat until it reaches the junction of the oropharynx and nasopharynx (Cummings et al., 2019). Most of the pathogens like parvovirus, murine norovirus, *Helicobacter* spp. and *Citrobacter rodentium* can be found from faecal pellets of rodents (Henderson and Clifford, 2013). Besides oral swab, oronasal swab and body swab, paw swabs can also be collected to detect diseases such as influenza A virus (IAV) in rats (Cummings et al., 2019). In this study, oronasal swab, thoracic swab and paw swabs were collected by swabbing the rats' oral cavity and external nares, surface of thoracic area and surface of paws respectively.

2.6 Justification

There is a significant apprehension within the farming community regarding the potential of rats as disease vectors. The risk of pathogens being spread to production animals like pigs by rats on farms is readily apparent, primarily because it's challenging to prevent rats from accessing the animal housing facilities (Backhans & Fellström, 2012). Several studies have also specifically investigated rats as potential carriers of various pathogens (Backhans & Fellström, 2012). This concern is particularly pronounced in the current agricultural set-up in Malaysian farmers situation. In these regions, the farms are closely situated due to high population density and insufficient land, increasing the risk of rat-borne diseases outbreak. Malaysian farmers also noticed that the ASF outbreaks in their farms did not start from regions near the main door or main road. Instead, it started from the internal parts of the farms. This has raised the farmers' concern as they are worried that the main reason causing ASF outbreak in their farms are mechanical vectors such as rats.

3.0 MATERIALS AND METHODS

3.1 Sample Collection

In this study, a total of 60 swab samples were taken from 15 rats originating from five pig farms in Peninsular Malaysia. Table 3.1-1 shows the number of rats collected from five pig farms. Sample collections were done in the morning of 4/9/23, 13/9/23, 19/9/23 and 21/9/23 within the pig farms. Four swab samples (oronasal swab, thoracic swab, forelimbs' paw swab and hindlimbs' paw swab) were collected from each rat. The forelimbs' paw swab sample and hindlimbs' paw swab sample from each rat were pooled afterwards during sample processing to form 1 sample. Thus, there were three samples per rat after sample processing and the total number of samples for DNA extraction became 45 for 15 rats. The sampling method is considered as convenience sampling.

Table 3.1-1: Number of rats collected from each farm.

Farm	1	2	3	4	5	**Total**
No. of rats	6	1	1	2	5	15

Before departing for sampling, zip-lock plastic bags, sterile swab sticks, cold boxes, ice, marker pen, gloves and 70% alcohol were prepared. Just before sample collection, gloves were worn and the zip-lock plastic bags were labelled as "farm X, rat X" by using marker pen. Besides that, the sterile swab sticks were also labelled as "farm X, rat X, O (oronasal swab)/ T (thoracic swab)/ FL (forelimbs' paw swab)/ HL (hindlimbs' paw swab)" using marker pen. Before swabbing the thoracic area,

forelimbs' paws and hindlimbs' paws of rats, the swab tips were immersed in mineral water first. Four sterile swab sticks were used to roll over the oronasal area, thoracic area, forelimbs' paws and hindlimbs' paws of each rat. The swab sticks were then inserted back into their original tubes. After completing the swabbing process, each rat was placed into one labelled zip-lock plastic bag and all of the bags were placed into an ice box containing several ice packs. The swab samples were also temporarily stored in the same ice box. The rats' carcasses and swab samples were immediately transported back to Virology Lab, Faculty of Veterinary Medicine, Universiti Putra Malaysia. The time between sample collection and sample processing ranged from 2 to 5 hours due to different locations of the farms.

3.2 Sample Processing

During sample processing, appropriate number of 1.5 ml microcentrifuge tubes were first prepared according to the number of swab sticks present. The tubes were labelled as "Farm X, Rat X, O (oronasal swab)/ T (thoracic swab)/ FL (forelimbs' paws swab)/ HL (hindlimbs' paws swab)". Then, 800 µl of PBS was added into each microcentrifuge tube. Each swab stick was immersed in one tube and the swab tip was swirled in the PBS against the walls of the extraction tubes. Next, the upper part of the swab stick was cut off so that the stick will only be as long as the tube. The tube was closed to remove as much dirt as possible from the swab tip. After 10 minutes, each tube was vortexed for 5 to 10 seconds. A second set of 1.5 ml microcentrifuge tubes was prepared and labelled in the same way as the first set of microcentrifuge tubes to pool the paw swabs from the rats' forelimbs with the paw swabs from the rats' hindlimbs. For the oronasal swabs and thoracic swabs, 200 µl of PBS was removed

from the first set of tubes and added in the second set of tubes. For the paw swabs, 100 µl of PBS containing dirt from the forelimbs' paws and 100µl of PBS containing debris from the hindlimbs' paws were removed from the first set of tubes and mixed within the second set of tubes. The samples were then stored at –20°C so that an optimum DNA yield and quality can be obtained. All the steps during sample processing were done in Virology Lab, Faculty of Veterinary Medicine, Universiti Putra Malaysia.

3.3 DNA Extraction

The DNA was extracted by using the IDEXX RealPCR^{TM} DNA/RNA magnetic bead kit (IDEXX Montpellier, France). All of the samples were thawed and kept at a temperature of 18 to 26 °C before lysis. The amount of Lysis Working Solution required was calculated. An additional 10% of Lysis Working Solution was prepared to allow for pipetting loss. Preparation of the Lysis Working Solution was done by mixing 193.6 µl of Lysis Buffer (LB), 4.4 µl of Carrier RNA (RNA), 22 µl of Proteinase K (PK) and 2.2 µl of internal positive control per sample. The total amount needed for each reagent was obtained by multiplying amount of reagent needed per sample with total number of samples. The Lysis Working Solution was thoroughly mixed through vortex method. 200 µl of Lysis Working Solution was added into each 2 ml microcentrifuge tube containing 200 µl of sample material. Each tube was then vortexed for 5 to 10 seconds and centrifuged at 1000 rpm for 1 second. Incubation was done for 15 minutes at 18 to 26 °C.

The next step is preparation of the Bead Solution, followed by addition of the beads. Firstly, the amount of Bead Solution required was calculated. An additional 10% of Bead Solution was prepared to allow for pipetting loss. The Bead Solution was

prepared by mixing 660 µl of Binding Buffer (BB) and 22 µl of Magnetic Beads (MB) per sample. The total amount needed for each reagent was obtained by multiplying amount of reagent needed per sample with total number of samples. Following that, the Bead Solution was mixed well by vortexing it gently. 620 µl of Bead Solution was added into each tube consisting of a mixture of the Lysis Working Solution and the sample material. Subsequently, the tubes were vortexed gently to mix the solution well and centrifuged briefly.

To wash the beads, the microcentrifuge tubes were placed on the magnetic separator (step 1). After 2 minutes, the supernatant was carefully removed without disturbing the magnetic beads by using pipetting or aspiration technique (step 2). The tubes were then removed from the magnetic separator (step 3). 600 µL (±20 µL) of Wash 1 was added into each tube. The tubes were vortexed until the beads were resuspended completely (step 4). After that, the tubes were centrifuged at 2000 rpm for 1 second (step 5). Step 1 and 2 were repeated to remove Wash 1. Then, step 3 was done and 600 µL (±20 µL) of Wash 2 was added into each tube. Step 4 and 5 were carried out for all the tubes. Step 1 and 2 were repeated to remove Wash 2. Step 3 was carried out and 600 µL (±20 µL) of 80% ethanol was added into each tube. Step 4 and 5 were repeated, then, step 1 and 2 were carried out to wash off the 80% ethanol. The beads were dried for 10 minutes at 18 to 26 °C.

For elution of DNA or RNA from the beads, 100 µL of Elution Buffer was added into each microcentrifuge tube. Next, the tubes were vortexed gently and centrifuged briefly. The magnetic beads were separated by placing the tubes on the magnetic separator. After at least 2 minutes, all the beads were attracted to the magnets. Then,

the supernatant containing purified nucleic acid was transferred to a new set of labelled 2ml microcentrifuge tubes for immediate use or storage. Lastly, the purified nucleic acid was stored at a temperature below 0°C for storage. All of the procedures during DNA extraction were done in Virology lab, Faculty of Veterinary Medicine, Universiti Putra Malaysia.

3.4 Real-time PCR

Real-time PCR (rPCR) was carried out by using IDEXX RealPCR ASFV DNA test (IDEXX Montpellier, France) and CFX Opus 96 RealTime System (Bio-Rad, the United States). Before carrying out PCR, materials and equipment were prepared and disinfected with UV for 30 minutes. During UV disinfection, the processed samples, RealPCR ASFV DNA Mix (ASFV DNA Mix, IDEXX Montpellier, France), RealPCR DNA Master Mix (DNA MMx), positive control (PC) and negative control (NC) were thawed. After completion of UV disinfection, preparation of PCR mix was carried out by mixing 10 µl of ASFV DNA Mix and 10 µl of DNA MMx per sample in an empty 1.5 ml microcentrifuge tube. The total amount of each reagent needed was calculated by multiplying amount of reagent needed per sample with total number of samples. Then, the solution was gently vortexed to ensure that the components were mixed well. After that, the PCR strip tubes were labelled using a marker pen. Numbers were written to represent each sample, while PC was labelled as "+" and NC was labelled as "-". 20 µl of PCR Mix was pipetted into each PCR strip tube. 5 µl of NC (PCR Grade Water) was added into the tube labelled with "-" and the tube was covered with a cap. Next, 5 µl of sample DNA was pipetted into each tube labelled with a number and 5 µl of PC was pipetted into the tube labelled "+". All of the PCR strip tubes were

covered by using the caps provided. Following that, the strip tubes were centrifuged at 4000 rpm for 10 seconds to settle the contents and remove air bubbles.

A thermal cycler was set up with IDEXX RealPCR Standard DNA/ RNA Cycling Program. The program was started with one cycle of reverse transcription (RT) at 50 °C for 15 minutes, followed by one cycle of denaturation at 95 °C for 1 minute to separate the DNA strands. Subsequently, annealing at 95 °C for 15 seconds occurred. Next, extension at 60 °C for 30 seconds took place to allow DNA polymerase to synthesize new DNA strands. The processes of annealing and extension were repeated for 44 times. In total, there were 45 cycles of amplification (annealing and extension). Fluorescence detection were done using channels fluorescein amidites (FAM), hexachloro-fluorescein (HEX) and carboxy-X-rhodamine (ROX). The whole rPCR process was carried out at Virology Lab, Faculty of Veterinary Medicine, Universiti Putra Malaysia.

Table 3.4-1: Validity criteria of real-time PCR for detection of ASFV.

Type of Control	**FAM Ct Value**	**HEX (VIC) Ct Value**
Positive Control	<38	<38
Negative Control	No signal	No signal

According to Table 3.4-1, for the real-time PCR result to be valid, the FAM and HEX Ct values of the positive control should be less than 38. Besides that, the negative control should not have any signal for both FAM and HEX.

Table 3.4-2: Interpretation of real-time PCR results.

FAM Signal	HEX (VIC) Signal	Ct Value (FAM Curve)	Sample Result
Yes	Yes/ No	<38	ASFV DNA is detected & result is positive
		>38	ASFV DNA is detected & result is negative
No	Yes	-	ASFV DNA is not detected
No	No	-	Invalid

As shown in Table 3.4-2, the interpretation of real-time PCR result is based on the FAM and HEX signal. If the sample has a positive FAM signal, it means that ASFV DNA is detected in the sample regardless of the HEX signal. Samples with positive FAM signal and a FAM Ct Value of less than 38 will be considered as positive for ASFV, while samples with positive FAM signal and a FAM Ct value which is more than 38 will be considered as negative for ASFV. If FAM signal is absent but HEX signal is present, ASFV DNA is not detected in the sample. Lastly, if the sample does not have both the FAM and HEX signals, the result is invalid.

4.0 RESULT

4.1 Real-time PCR Result

4.1.1 Real-time PCR Amplification Curves

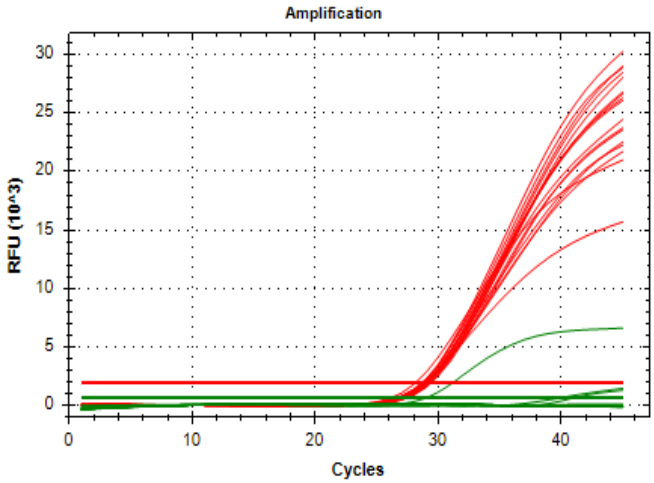

Figure 4.1.1-1: Real-time PCR amplification FAM and HEX curves showing results of thoracic and paw swab samples collected from rat 2 in farm 1, negative control and positive control.

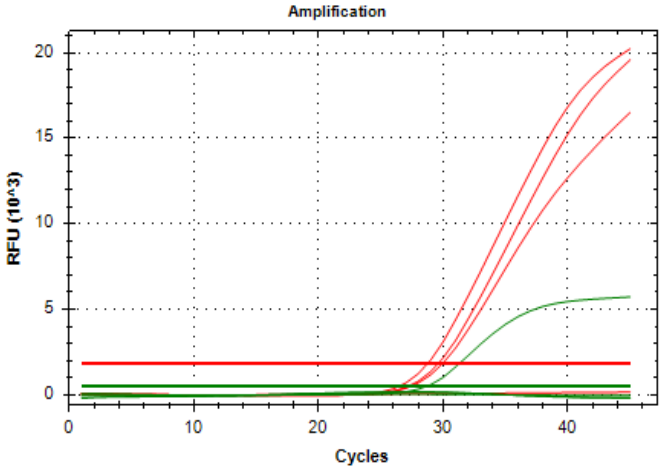

Figure 4.1.1-2: Real-time PCR amplification FAM and HEX curves showing results of oronasal swab samples collected from rat 1 in farm 2, negative control and positive control.

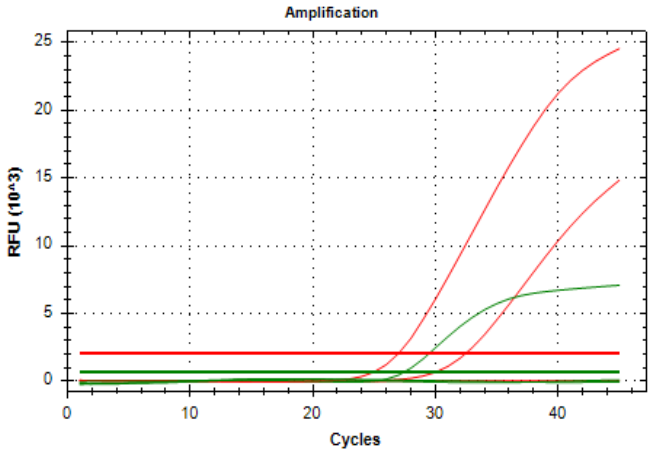

Figure 4.1.1-3: Real-time PCR amplification FAM and HEX curves showing results of 16 swab samples collected from six rats, negative control and positive control.

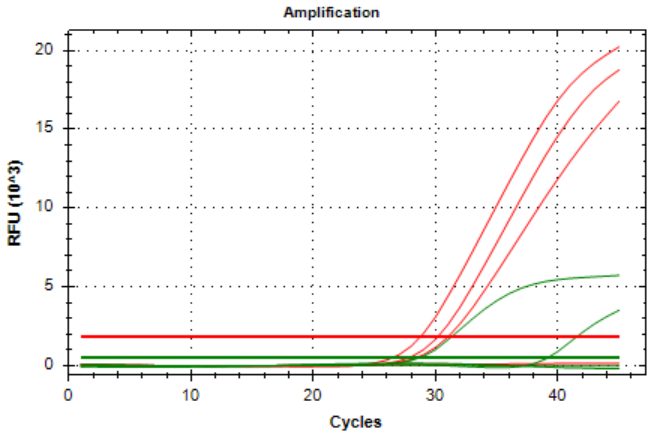

Figure 4.1.1-4: Real-time PCR amplification FAM and HEX curves showing results of thoracic and paw swab samples collected from rat 1 in farm 2, negative control and positive control.

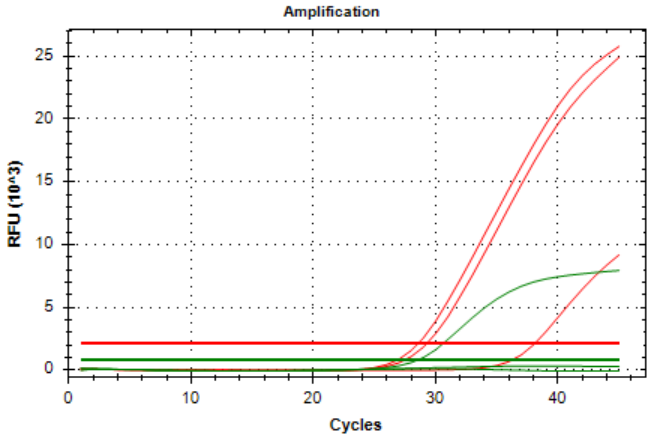

Figure 4.1.1-5: Real-time PCR amplification FAM and HEX curves showing results of oronasal swab sample collected from rat 1 in farm 3, negative control and positive control.

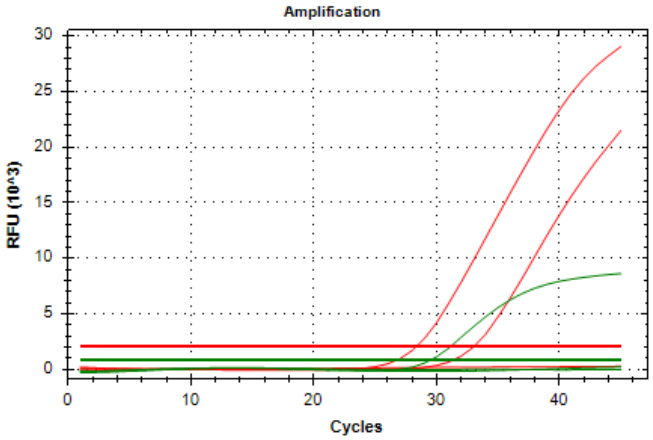

Figure 4.1.1-6: Real-time PCR amplification FAM and HEX curves showing results of paw swab sample collected from rat 1 in farm 3, negative control and positive control.

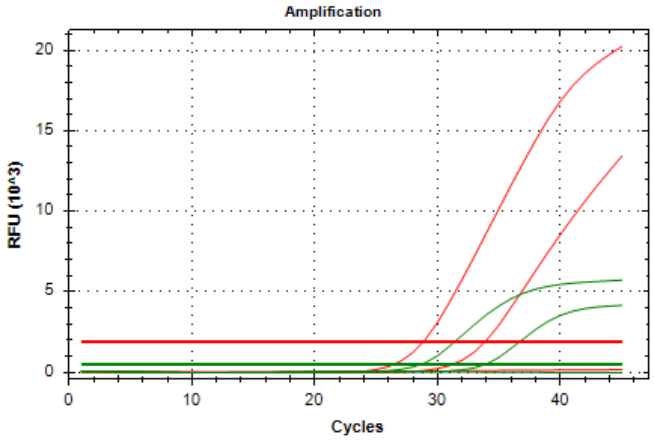

Figure 4.1.1-7: Real-time PCR amplification FAM and HEX curves showing results of thoracic swab sample collected from rat 1 in farm 3, negative control and positive control.

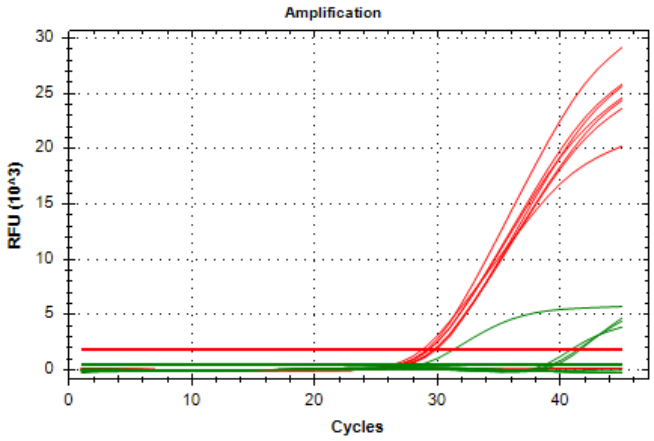

Figure 4.1.1-8: Real-time PCR amplification FAM and HEX curves showing results of 6 swab samples collected from 2 rats in farm 4, negative control and positive control.

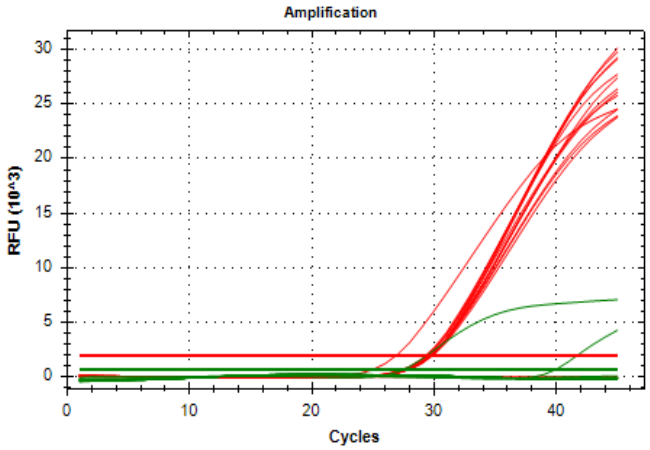

Figure 4.1.1-9: Real-time PCR amplification FAM and HEX curves showing results of 12 swab samples collected from five rats in farm 5, negative control and positive control.

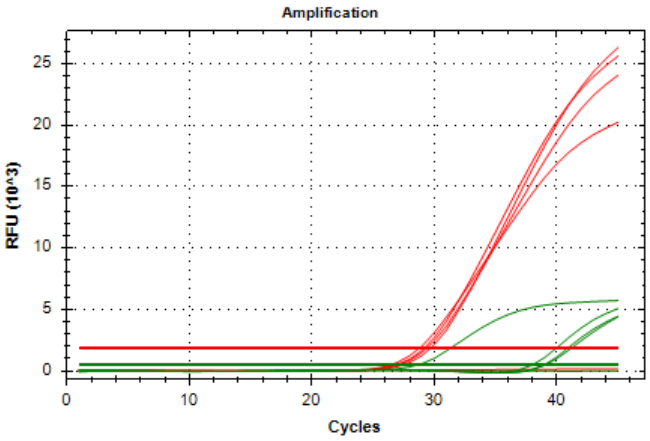

Figure 4.1.1-10: Real-time PCR amplification FAM and HEX curves showing results of oronasal and thoracic swab samples from rat 3, paw swab sample from rat 5, negative control and positive control.

A positive HEX or FAM signal can be confirmed when the HEX or FAM curve exceeds its own cycle threshold and has a sigmoid shape. From Figure 4.1.1-1, it can be seen that positive HEX signal was present for all of the swab samples and positive control, but it was absent for negative control. Thus, this test was valid. Among the 16 swab samples, only two had positive FAM signals. Figure 4.1.1-2 shows that both the thoracic and paw swab samples along with the positive control had positive HEX

signals, but this was not the case for the negative control. The two swab samples were negative for FAM.

In Figure 4.1.1-3, it is shown that the oronasal swab sample and positive control both had positive HEX signals, but the negative control had a negative HEX signal. Besides that, only the positive control showed a positive FAM signal. Following that, Figure 4.1.1-4 shows that between the thoracic and paw swab samples, only one showed a positive FAM signal. Except for the negative control, all of the swab samples and positive control had positive HEX signals.

Additionally, Figure 4.1.1-5 shows that the oronasal swab sample, positive control and negative control all showed positive HEX signals. The oronasal swab showed a negative FAM signal. Next, Figure 4.1.1-6 shows that the paw swab sample and positive control showed positive HEX signals but the negative control did not. The paw swab had a negative FAM signal, so it was negative for ASFV. From Figure 4.1.1-7, it can be observed that the thoracic swab sample and positive control showed positive HEX signals while the negative control showed a negative HEX signal. At the same time, the thoracic swab sample had a positive FAM signal.

From Figure 4.1.1-8, it can be seen that all of the swab samples and the positive control had positive HEX signals, but not the negative control, proving that the real-time PCR result was valid. Only three swab samples had positive FAM signals.

Figure 4.1.1-9 shows that except for the negative control, all of the swab samples and the positive control had positive HEX signals. Only 1 swab sample had a positive FAM signal. Lastly, Figure 4.1.1-10 shows that all of the swab samples and

the positive control had positive HEX signals but not the negative control. All of the swab samples had positive FAM signals.

4.1.2 Real-time PCR Ct Values

Table 4.1-1: Ct values and real-time PCR result of each swab sample collected from rats in five farms.

Farm	Rat	Sex	Weight (g)	Length (cm)	Type of Swab Samples	Ct Value (FAM Curve)	Real-time PCR Result (+/-)
1	1	Unidentified	19.17	8.4	Oronasal	-	-
					Thoracic	-	-
					Paw	-	-
	2	Female	19.26	7.5	Oronasal	-	-
					Thoracic	-	-
					Paw	-	-
	3	Male	19.64	7	Oronasal	-	-
					Thoracic	41.12	-
					Paw	-	-
	4	Male	205.85	16	Oronasal	-	-
					Thoracic	-	-
					Paw	-	-
	5	Female	84.46	13.5	Oronasal	-	-
					Thoracic	-	-
					Paw	-	-
	6	Male	22.21	8	Oronasal	40.07	-
					Thoracic	-	-
					Paw	-	-

2	1	Female	266.55	19	Oronasal	-	-
					Thoracic	-	-
					Paw	39.54	-
3	1	Female	114.14	15.5	Oronasal	-	-
					Thoracic	34.40	+
					Paw	-	-
4	1	Female	39.22	11.7	Oronasal	40.09	-
					Thoracic	-	-
					Paw	-	-
	2	Female	90.71	11	Oronasal	39.71	-
					Thoracic	39.16	-
					Paw	-	-
5	1	Male	142.2	14	Oronasal	-	-
					Thoracic	40.01	-
					Paw	-	-
	2	Male	113.82	13	Oronasal	-	-
					Thoracic	-	-
					Paw	-	-
	3	Male	46.68	9.5	Oronasal	38.34	-
					Thoracic	39.30	-
					Paw	-	-
	4	Female	22.01	8	Oronasal	-	-
					Thoracic	-	-
					Paw	-	-

	5	Female	152.29	12	Oronasal	-	-
					Thoracic	-	-
					Paw	39.41	-

Table 3.4-2 indicates that in order for a sample to be considered positive for ASFV, the FAM Ct value must be less than 38. From Table 4.1-1, it is shown that thoracic swab of rat 3 in farm 1 had a FAM Ct value of 41.12 while oronasal swab of rat 6 in farm 1 had a FAM Ct value of 40.07. The other samples from farm 1 had a FAM Ct values which exceeded the total cycles of real-time PCR, thus, the values were not shown. To summarize, all of the swab samples from farm 1 had FAM Ct values which were more than 38, which were considered negative for ASFV.

For farm 2, paw swab from rat 1 had a FAM Ct value of 39.54 while the FAM Ct values of oronasal and thoracic swab from the same rat were not shown. Therefore, all of the swab samples from farm 2 were ASFV-negative as the FAM Ct values were all greater than 38. In farm 3, the thoracic swab from rat 1 had a FAM Ct value of 34.40 while the oronasal and paw swabs from the same rat had Ct values that were not shown. Thus, the thoracic swab from rat 1 in farm 3 was positive for ASFV while the oronasal and paw swabs from the same rat were negative for ASFV.

For rat 1 in farm 4, its oronasal swab had a FAM Ct value of 40.09 while the thoracic swab and paw swabs from the same rat had FAM Ct values that were too large to be shown. For rat 2 in farm 4, its oronasal swab had a FAM Ct value of 39.71 while the thoracic swab had a FAM Ct value of 39.16. At the same time, the paw swab from

rat 2 in farm 4 had a FAM Ct value that was not shown. Hence, all of the swab samples were ASFV-negative as the FAM Ct values were all larger than 38.

In farm 5, there were a total of 5 rats, giving rise to 15 swab samples. Thoracic swab from rat 1 had a FAM Ct value of 40.01 while the FAM Ct values of oronasal and thoracic swab from rat 3 were 38.34 and 39.30 respectively. The FAM Ct value of paw swab from rat 5 was 39.41. The FAM Ct values of other swab samples from farm 5 were not shown. So, all of the swab samples from farm 5 were ASFV-negative due to their FAM Ct values which were more than 38.

5.0 DISCUSSION

From this study, it was found that rats are mechanical vectors of ASFV as one rat was tested positive for ASFV. African swine fever was more commonly found in pig farms with large population of wildlife, including birds, rats, and insects such as flies and mosquitoes (Lai et al., 2022). The two statements above contradict with a research project on ASF done in Vietnam, which discovered that rats around the farms with ongoing ASF outbreaks were negative for ASF in various sample types, indicating they are likely not significant vectors for ASFV (Sundberg, 2021). Besides that, experts from affected countries such as Estonia and Latvia also reported that there was no evidence or link between potential mechanical vectors and ASF (De La Torre et al., 2022). Thus, it can be concluded that rats can carry ASFV as many of the studies that stated that rats are not vectors of ASFV, there has been no evidence to support this particular finding.

Rats might be effective mechanical vectors of ASFV because of their long running distance. At the end of the 6-week period being housed with the wheels, rats were voluntarily running approximately 2.5 kilometres per day (Korsak et al., 2023). Additionally, rats act as biological vectors for numerous swine pathogens (Le Moine et al., 1987; TRUONG et al., 2013) such as PCV2 (M. Zhao et al., 2023), EMCV (Backhans and Fellström, 2012; Koenen et al., 1999; Murnane et al., 1960), *Yersinia* spp., *Leptospira* spp., *Campylobacter* spp. and *Brachyspira hyodysenteriae* (Backhans and Fellström, 2012; Taylor and Alexander, 1971). They also act as mechanical vectors of swine pathogens like influenza A virus (Cummings et al., 2019). Next, rats

have a wide territory in comparison to their size, where an area of roughly 500 m^2 was documented by Akande (2008).

Rats also have an explosive reproductive rates (Macdonald et al., 2015), where females of Norway Rats (*Rattus Norvegicus*) which is commonly seen in Malaysia, gave birth to a litter every 37 days in a study done (Panti-May et al., 2016). On average, sexually mature female Norway Rats produced one viable offspring every 4.6 days or 79 viable offspring per year (Panti-May et al., 2016). Next, rats display a highly inquisitive and exploratory behaviour (Animal Research Review Panel, 2007; Scharmann, 1991), and this enables them to pick up ASFV easily. Lastly, rats are major pests in pig farms, and they pose a biosecurity threat to farms by bridging the gap between wild animals and pigs, thus aiding in the transmission of pathogens (Makovska et al., 2023). In short, many research paper stated that rats can effectively transmit pathogens among wildlife, domestic animals, human and vectors (Strand and Lundkvist, 2019), proving that they are effective carriers of diseases.

There are many possible routes of transmission of ASFV from rats to pigs. The transmission cycle might start when rats come into contact with infected pig carcass, and fomites, environment or food materials contaminated with infectious blood, along with infectious excretions and secretions such as respiratory secretions, saliva, faeces and urine (United States Department of Agriculture [USDA], 2023) Then, pigs may be succumbed to ASF through ingestion of feed contaminated by ASFV-positive rats that has not been cooked properly (Liu et al., 2021), ingestion of water being contaminated by ASFV-positive rats or inhalation of contaminated air containing ASFV after ASFV-positive rats ran around in the pens (Howey et al., 2013). They

might also be infected with ASFV through contact with fomites or environment contaminated by ASFV-positive rats (Mazur-Panasiuk et al., 2019; Mur et al., 2012), or even the contaminated rats directly.

As mentioned in the previous paragraph, ASFV can be picked up by rats when they have contact with infected pig carcass, or environment and fomites contaminated with infectious secretions and excretions or infectious blood. Thus, the survivability of ASFV in these materials should be studied. Firstly, faecal samples from experimentally infected pigs remained infectious for 8 days at 4°C and 3-4 days at 37°C (Mazur-Panasiuk et al., 2019). However, ASFV can survive for 11 days in faeces at room temperature (Liu et al., 2021). Meanwhile, urine might contain viable virus for up to 15 days at 4°C, five days at 21°C, and two to three days at 37°C (Mazur-Panasiuk et al., 2019). The results of these studies were in contrast to a study done by Fischer et al. (2020) which stated that no infectious virus was recovered from urine and faeces after one week, regardless of the matrix. Next, it has been confirmed that ASFV can retain its infectivity in blood for up to 18 months at 4°C, and blood is able to remain infectious for over three months (Fischer et al., 2020).

For infected pig carcass, skin of infected pigs, when stored at -20°C, 4°C, and room temperature (RT), remained infectious for up to three months, six months, and three months, respectively (Fischer et al., 2020). Nonetheless, it was found that ASFV was able to survive in skin fat for 300 days, as mentioned by Conan et al. (2021). The survival time for ASFV in bone marrow, muscle (Conan et al., 2021; Zani et al., 2020), fat was 180 to 188 days (Conan et al., 2021; EFSA Panel on Animal Health and Welfare (AHAW), 2014), 90 to 183 days (Conan et al., 2021; Zani et al., 2020) and

123 days (Conan et al., 2021; McKercher et al., 1987) respectively. In oral fluid, ASFV DNA was detectable for 35 days at 4°C, 14 days at 12°C and 21°C, but none was detectable at 37°C (Davies et al., 2017). In a contaminated pen, ASFV was able to survive for one month (Liu et al., 2021). In short, there may be a high probability for rats to act as vectors of ASFV due to the long survivability of the virus.

Subsequently, ASFV is highly resistant to inactivation as it remains stable within the pH range of 4 to 10 and will not be affected by meat maturation. It also needs heating at 60°C for 20 minutes to be inactivated (Costard et al., 2013). To achieve ASFV-free status for smoked sausages and air-dried hams, they need to be smoked at 32°C and 49°C for up to 12 hours, and dried for 25 to 30 days (Coetzer et al., 1994; Costard et al., 2013). It has been proved that ASFV demonstrates high resistance to environmental conditions and can remain infectious during long storage periods at temperatures either below 0°C or at 4°C (Mazur-Panasiuk et al., 2019). Additionally, ASFV can withstand a lot of freeze-thaw cycles and survive a temperature of 56°C for more than an hour (Mazur-Panasiuk et al., 2019). The high resistance of ASFV means there may be a higher probability for the rats to pick up the virus from the environment and transmit it to pigs.

In this study, the thoracic area of one of the rats was tested positive for ASFV. Firstly, thoracic area has a larger surface area in comparison to the oronasal area and the paws of rats. Secondly, thoracic area be considered as one of the rats' body parts that has the greatest contact with the ground. Therefore, the fur at the thoracic area of rats can easily be contaminated by ASFV when the animals come into contact with infected surfaces or equipment (Domanska-Blicharz et al., 2023), facilitating the

transmission of ASFV to pigs. The paws of the rats were negative for ASFV probably due to highest contact frequency of the paws with contaminated ground or surfaces compared to other body parts. So, the ASFV carried on the paws might be diluted.

Following that, the only ASFV-positive rat had a body length of 15.5cm, which was larger than the average body length of 15 rats obtained in this study (11.61cm). At the same time, the rat weighed 114.14g, which was larger than the average body weight of 15 rats used in this study (90.55g). Larger body length means the rat might have a larger body surface area, promoting uptake of ASFV on the fur. Next, larger body weight means the rat might be larger in size with larger body surface area, leading to more ASFV being taken up by it. However, due to lack of samples, the association between positivity of ASFV with rats' body weight or body length cannot be confirmed.

Sex may or may not play a role in influencing the result of real-time PCR tested against ASFV. Female rats were found to be more active and exploratory than males (van Hest et al., 1987). More exploratory behaviour of female rats results in higher probability of rats' being contaminated by ASFV. However, intra-specific aggression in male rats seems to contribute to pathogen transmission (Himsworth et al., 2014). This statement can be supported by Hinson et al. (2004), who stated that aggression may be the main method of Seoul virus transmission among male Norway rats, but not in female Norway rats. Despite the contradictory statements, it is believed that females rats may be able to carry more ASFV and transmit them to pigs. Nevertheless, this statement cannot be confirmed due to limited sample size in this study.

6.0 CONCLUSION

In conclusion, rats are mechanical vectors of ASFV. Through real-time PCR, ASFV was found to be positive on the thoracic area of a rat, with a FAM Ct value of 34.40 which was lesser than 38. However, both the oronasal area and paws of the rats were ASFV-negative. This might be due to larger surface area of the thoracic area than the oronasal area and the paws, along with the fact that thoracic area is one of the body parts of rats that has the greatest contact with the ground. Besides that, the only ASFV-positive rat was a female with a body weight and body length which were greater than the average body weight and body length. This shows that sex, body weight and body length of the rats might influence the ASFV-positivity. However, the association between ASFV-positivity and these characteristics cannot be confirmed due to limited sample size in this study.

7.0 RECOMMENDATIONS

There are a few recommendations for improvement of this study. First of all, the sample size of this study should be increased to at least 20 rats to improve the validity of the real-time PCR result. Larger sample size will provide smaller margin of errors, leading to a more accurate result. In this study, only 15 rats were collected and swabbed to detect the presence of ASFV on rats.

Secondly, the time of monitoring rat collection for each farm should be increased from one week to one month. This is so that there would be more time for the farmers and workers to monitor whether there are any rat carcasses in the farm, thus increasing the sample size of the study.

Lastly, the number of farms should be increased so that there could be more rats collected for this study. This could improve the validity and accuracy of the result. In this study, there were only five farms for rat collection as very limited time was provided to persuade our commercial partners for collaboration.

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9.0 APPENDIX

Pictures of swab samples collection

	Collection of oronasal swab from a rat
	Collection of thoracic swab from a rat
	Collection of oronasal swab from a rat