UNIVERSITI PUTRA MALAYSIA

MOLECULAR DETECTION OF AFRICAN SWINE FEVER VIRUS AND CLASSICAL SWINE FEVER VIRUS FROM DOMESTIC PIGS AND WILD BOARS IN SELANGOR

CLARENCE LAU YEO DEK

MOLECULAR DETECTION OF AFRICAN SWINE FEVER VIRUS AND CLASSICAL SWINE FEVER VIRUS FROM DOMESTIC PIGS AND WILD BOARS IN SELANGOR

CLARENCE LAU YEO DEK

A project paper submitted to the
Faculty of Veterinary Medicine, Universiti Putra Malaysia
In partial fulfilment of the requirement for the
DEGREE OF DOCTOR OF VETERINARY MEDICINE
Universiti Putra Malaysia
Serdang, Selangor Darul Ehsan.

2023/2024

CERTIFICATION

It is hereby certified that we have read this project paper entitled “Molecular Detection of African Swine Fever Virus (ASFV) and Classical Swine Fever Virus (CSFV) from Domestic Pigs and Wild Boar in Selangor”, by Clarence Lau Yeo Dek and in our opinion it is satisfactory in terms of scope, quality, and presentation as partial fulfilment of the requirement for the course VPD 49999 – Final Year Project.

DR. NURUL IZZATI UDA ZAHLI

DVM (UPM), PhD (Miyazaki, Japan)

Lecturer,

Faculty of Veterinary Medicine

Universiti Putra Malaysia

(Supervisor)

ASSOCIATE PROFESSOR DR. OOI PECK TOUNG
DVM (UPM), PhD (Glasglow)
Associate Professor,
Faculty of Veterinary Medicine,
Universiti Putra Malaysia,
(Co-Supervisor)

ASSOCIATE PROFESSOR DR. NOR YASMIN ABD RAHMAN
DVM (UPM), PhD (UPM)
Associate Professor,
Faculty of Veterinary Medicine,
Universiti Putra Malaysia,
(Co-Supervisor)

DR. AZLAN CHE' AMAT

DVM (UPM), PhD (Universidad de Castilla La-Mancha)

Lecturer,

Faculty of Veterinary Medicine,

Universiti Putra Malaysia,

(Co-Supervisor)

DR. MICHELLE FONG WAI CHENG

DVM (UPM), PhD (UPM)

Lecturer,

Faculty of Veterinary Medicine,

Universiti Putra Malaysia,

(Co-Supervisor)

ACKNOWLEDGMENT

First and foremost, I extend my deepest appreciation to my project supervisor, Dr. Izzati for her invaluable guidance, unwavering support, and constructive feedback throughout the entire process. She was always there to back up and help me whenever I faced difficulties. Her expertise and encouragement have been instrumental in shaping the direction of this project.

I am also thankful to Dr. Ooi, Dr. Michelle and Dr. Yasmin whose insights and suggestions have significantly enriched the quality of this work.

I want to acknowledge the support and understanding of my FYP mates, Phan and Joey who stood by me during the ups and downs of this academic journey. Their encouragement provided the emotional strength needed to overcome challenges and persevere.

Special thanks are due to Natasha and Faiz from the virology lab whose collaboration and contributions have played a crucial role in the success of this project. The collective effort and teamwork have truly enhanced the outcome.

This project would not have been possible without the support of all these individuals and resources. Thank you for being an integral part of this academic endeavour.

Table of Content

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

APPV	Atypical Porcine Pestivirus
ASF	African Swine Fever
ASFV	African Swine Fever Virus
BDV	Border Disease Virus
BVDV-1	Bovine Viral Diarrhoea Type 1
BVDV-2	Bovine Viral Diarrhoea Type 2
CSF	Classical Swine Fever
CSFV	Classical Swine Fever Virus
DDH2O	Deionised Distilled Water
ELISA	Enzyme Linked Immunosorbent Assay
FAT	Fluorescent Antibody Test
HAD	Hemadsorption Test
IBT	Immunobead Test
IFAT	Immunofluorescent Antibody Test
IPT	Immunoperoxidase Test
mRT-PCR	Multiplex Reverse Transcriptase Polymerase Chain Reaction
nRT-PCR	Nested Reverse Transcriptase Polymerase Chain Reaction

PBS	Phosphate Buffer Reaction
PCR	Polymerase Chain Reaction
PCV	Porcine Circovirus
PDCoV	Porcine Deltacoronavirus
PEDV	Porcine Epidemic Diarrhea Virus
PPV	Porcine Parvovirus
PRRSV	Porcine Reproductive and Respiratory Virus
PRV	Pseudorabies Virus
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
TGEV	Transmissible Gastroenteritis Virus
VNT	Virus Neutralisation Test

LIST OF APPENDICES

ABSTRAK

Abstrak daripada kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD 4999 –Projek Tahun Akhir.

DETEKSI MOLEKULER VIRUS DEMAM BABI AFRIKA DAN VIRUS DEMAM BABI KLASIK PADA BABI DOMESTIK DAN BABI HUTAN DI SELANGOR

Oleh

Clarence Lau Yeo Dek

2023

Penyelia: Dr. Nurul Izzati Uda Zahli

Penyelia bersama: Profesor Madya Dr. Ooi Peck Toung, Profesor Madya Dr. Nor Yasmin Abd Rahman, Dr. Michelle Fong Wai Cheng, Dr. Azlan Che' Amat

Demam babi Afrika (ASF), penyakit virus yang sangat mudah berjangkit dan menyebabkan pendarahan pada babi domestik, pada mulanya bebas dari Asia sehingga tahun 2018. Selepas kes ASF pertama dilaporkan di China pada tahun 2019, penyakit ini merebak ke negara-negara Asia lain termasuk Malaysia, menyebabkan kematian tinggi di ladang-ladang yang terjejas. Virus demam babi Afrika (ASFV) adalah virus DNA dalam genus Asfivirus dan keluarga Asfaviridae. Demam babi klasik (CSF) pula adalah penyakit berjangkit di rantau Malaysia, menyebabkan prestasi rendah dan kehilangan berat badan pada babi. Virus demam babi klasik (CSFV) adalah virus RNA dalam genus Pestivirus dan keluarga Flaviviridae. Kedua-dua penyakit ini memberi impak ekonomi yang signifikan kepada industri babi tempatan kita. Kajian ini bertujuan untuk mengesan virus demam babi Afrika (ASFV) dan virus demam babi klasik (CSFV) dari babi domestik dan babi hutan dengan menggunakan Polymerase Chain Reaction (PCR). Sampel tisu termasuk nod limfa, tonsil, dan limpa daripada 12 ekor babi domestik dan 3 ekor babi hutan dikumpul dan dihomogenkan. “Formalin-Fixed Paraffin Embedded (FFPE) tissue” dari noda limfa, tonsil, dan limpa daripada 5 ekor babi domestik lain dihiris dan dihomogenkan. Ekstraksi DNA dan RNA dilakukan pada sampel yang dihomogenkan. “Reverse transcriptase PCR, uniplex conventional PCR dan Multiplex PCR” dijalankan pada semua sampel, diikuti dengan elektroforesis gel. PCR menunjukkan 5/15 (33%) sampel positif dengan CSFV dan 0/20 (0%) sampel positif dengan ASF. Sampel disyorkan untuk dihantar untuk penjujukan bagi menentukan strain dan genotip virus. PCR adalah kaedah diagnosis yang cepat (kurang dari 5 jam) dengan sensitiviti dan spesifisiti yang tinggi.

Kata kunci: Virus Demam Babi Afrika, Virus Demam Babi Klasik, Reverse transcriptase PCR, Conventional PCR, Multiplex PCR

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.

MOLECULAR DETECTION OF AFRICAN SWINE FEVER VIRUS AND CLASSICAL SWINE FEVER VIRUS FROM DOMESTIC PIGS AND WILD BOARS AT SELANGOR

by

Clarence Lau Yeo Dek

2023

Supervisor: Dr. Nurul Izzati Uda Zahli

Co-supervisors: Assoc. Prof. Dr. Ooi Peck Toung, Assoc. Prof. Dr. Nor Yasmin Abd Rahman, Dr. Michelle Fong Wai Cheng, Dr. Azlan Che' Amat

African swine fever (ASF), a viral disease characterised by high contagion and haemorrhaging in domestic pigs, remained absent from Asia until 2018. After the first ASF case reported in China in 2019, the disease spread rapidly to other Asian countries including Malaysia, resulting in high mortality in affected farms. The African swine fever virus (ASFV) is a DNA virus classified within the Asfaviridae family and Asfivirus genus. Classical swine fever (CSF) is another fatal infectious disease currently endemic in Malaysia, responsible for low sow performance and weight loss in pigs. The classical swine fever virus (CSFV) is an RNA virus belonging to the Pestivirus genus and the Flaviviridae family. Both diseases result in a significant economic impact on our local pig industry. The study aimed to detect ASFV and CSFV from domestic pigs and wild boars by using Polymerase Chain Reaction (PCR). Fresh tissue samples including lymph nodes, tonsils and spleen from 12 domestic pigs and 3 wild boars are homogenised into pool samples respectively. Formalin-fixed paraffin-embedded tissue (FFPE) samples of lymph nodes, tonsils and spleen from another 5 domestic pigs are sliced and homogenised. DNA and RNA extraction are done on the homogenised samples. Uniplex reverse transcriptase PCR, uniplex conventional PCR and multiplex PCR are carried out on all samples, followed by gel electrophoresis. PCR revealed 5/15 (33%) samples are positive with CSFV and 0/20 (0%) samples are positive with ASF. The samples are recommended to be sent for sequencing to determine the strain and genetic characterization of the viruses. PCR is a rapid diagnostic method (less than 5 hours) with high sensitivity and specificity.

Keywords: African Swine Fever Virus, Classical Swine Fever Virus, Reverse-transcriptase PCR, Conventional PCR, Multiplex PCR

1.0 Introduction

1.1 Classical Swine Fever

Classical swine fever, formerly recognized as hog cholera, is a highly contagious disease of global significance, imposing a substantial economic burden on both local and international pork industries (Edwards et al., 2000). Designated as one of the OIE-listed diseases, the emergence of CSF leads to economic losses in swine industries, attributed to the virus's high morbidity and mortality rates, coupled with stringent trading restrictions imposed on pork or pork-based products (Saatkamp et al., 2000).

1.1.1 Classical Swine Fever Virus

Classical swine fever virus is classified within the genus of pestiviruses and the family Flaviviridae. Within the pestivirus genus, four well-recognized species are currently acknowledged, which include bovine viral diarrhoea viruses 1 and 2 (BVDV-1 and BVDV-2), CSFV, and border disease virus (BDV) (Jeffrey et al., 2019). The genome of CSFV is a single-stranded, positive-sense RNA, measuring 12.3 kb in size. It comprises a singular large open reading frame (ORF) that encodes a polyprotein encompassing all the structural components (E1, E2, Erns, and C), non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A, NS5B, Npro, and p7), and untranslated regions (UTR) located at its 5′ and 3′ ends (Ngoc et al., 2021). E2 glycoprotein is often used for genetic typing due to abundant sequence data (Jeffrey et al., 2019).

1.1.2 Classical swine fever in Malaysia

Classical swine fever has been endemic in Asian countries, including Malaysia. The initial case of CSF in Malaysia was documented in Perak in 1895. This occurrence might be attributed to the importation of pigs from foreign countries. The current status of CSF in Malaysia is still unknown due to the reluctance of pig farmers to report

diseases and the lack of studies on the issues. However, several sporadic cases have been noticed, particularly in areas with a high density of pig farms (Protocol Veterinary Malaysia, 2010). Outbreaks in Malaysia are usually due to low virulence strain of the virus, resulting in the chronic or sub-chronic form of the disease.

1.2 African Swine Fever

African swine fever is a viral haemorrhagic disease in pigs. It is endemic in sub-Saharan Africa. In 2007, the highly virulent ASF genotype II spread from Africa to Georgia, followed by subsequent outbreaks in countries over Asia, Europe, America, and Southeast Asia. The disease is contagious with high morbidity and also able to cause high case fatality in domestic pigs (Emad et al., 2022). ASF is not infectious to humans and does not cause public health. However, it is classified as a notifiable disease by the World Organisation for Health, due to its capability to cause serious economic and social impacts on pig trading, pig-by products, and food security (Jeffrey et al., 2019).

1.2.1 African Swine Fever Virus

African swine fever is a sizable, icosahedral, linear double-stranded DNA virus, representing the sole member of the family Asfaviridae and the genus Asfivirus (Jeffrey et al., 2019). It carries a genome of 170–194 kb encoding for more than 150 proteins of which more than 50 are structural proteins. The genome contains 150 to 170 open reading frames (ORFs). At least 16 proteins have been implicated in virus assembly, including the major capsid component p72 (encoded by the B646L gene) and the minor protein p49 (encoded by the B438L gene). These two genes can be used for OIE – recommended PCR diagnostic method and phylogenetic analysis (Yang et al, 2021).

1.2.2 African Swine Fever in Malaysia

Malaysia is currently suffering from an acute form of the disease, resulting in a rapid drop in our sufficiency level from 95% to 40% and a significant increase in our farm price from RM 800/kg to RM 1600/kg. In East Malaysia, outbreaks first started in farms in Sabah in February 2021 involving both domestic pigs and wild boars. Over 33 outbreaks had been reported in Sabah, involving culling and death of more than 1100 domestic pigs and wild boars. Gene sequencing and phylogenetic sequencing were done, suggesting the outbreaks were due to ASF genotype II which is responsible for ASF epizootic in Asia and Europe. Diseases are highly suggestive of being introduced by neighbouring countries by comparing the genotype, timeline, and Asia and Pacific ASF epidemiology studies. However, the route of introduction of the disease into Sabah remains unclear. (Khoo et al., 2021). In Peninsular Malaysia, the first outbreak reported was in December 2021. Many states have been attacked by the virus including Penang, Perak, Negeri Sembilan, Malacca, and Johor. ASF was also detected in wild boars in Pahang and Negeri Sembilan. There are no reports of outbreaks in the Selangor area. Unfortunately, there are no available vaccines for ASF. The policies we have in Malaysia include stamping out policy, cleaning and disinfection, zoning, and movement restriction. (Khoo et al., 2021).

1.3 Objective

The study was conducted with the following objectives:

1. To detect CSFV and ASFV from domestic pigs and wild boar by PCR method
2. To compare between multiplex PCR and uniplex PCR

1.4 Hypothesis

Null hypothesis 1: Absent of CSFV in the domestic pigs and wild boars in Selangor.

Alternative hypothesis 1: Presence of CSFV in the domestic pigs and wild boars in Selangor.

Null hypothesis 2: Absent of ASFV in the domestic pigs and wild boars in Selangor.

Alternative hypothesis 2: Presence of ASFV in the domestic pigs and wild boars in Selangor.

1.5 Justification

There is currently no study done on molecular detection and genotyping of CSFV in Malaysia. There is currently no study done on molecular detection and genotyping of ASFV in peninsular Malaysia. It is very hard to distinguish CSF and ASF based on clinical signs. There is still a lack of data for epidemiological studies of CSF and ASF in Malaysia.

2.0 Literature Review

2.1 Classical Swine Fever and African Swine Fever

Classical swine fever virus is a small, enveloped, roughly spherical, positive-sense, single-stranded RNA virus (Jeffrey et al., 2019). CSFV has been classified into three genotypes and several subgenotypes, based on the genetic code of 5′-NTR, E2, and NS5B as these regions can discriminate between isolates and segregate viruses into genetic groups (Paton et al.,2000). Genotype 1 contains most of the historical vaccine strains. G2 contains most of the currently prevalent strains. G3 contains most of the historical strains distributed in separate geographic regions (Beer et al., 2015, Paton et al., 2000, Sakoda et al., 1999). Previously known as hog cholera, CSF is characterised by fever, haemorrhage, leukopenia, and disseminated intravascular coagulation. The impact of the virus varies depending on both the virulence of the virus itself and the immune response of the susceptible host. This can lead to acute outbreaks characterised by high mortality rates, or alternatively, result in subacute and chronic infections. Suckling and weaning pigs are mainly the victims of CSF which could be due to stress factors. Infected sows may be asymptomatic and become major transmission sources. Another factor contributing to the complexity of this disease is that the genetic typing of the disease does not correlate with antigenic typing. Recent studies found that genetically closely related CSFV isolates show different clinical manifestations and virulence (Gong et al., 2019).

Since reported, there are several “mutant isolated ASFV in the field” which could be due to long-term coexistence, an adaptation of the virus with its host, and evolution of the virus, leading to chronic or asymptomatic clinical signs (Wang et al., 2022). After more than hundreds of years of evolution, there are currently 24 genotypes and at least 8 serotypes of the virus (Wang et al., 2022). P72 protein encoded by the B646

gene is the major capsid protein, showing high antigenicity and reactogenicity, and a suitable candidate to divide ASFV into different genotypes. (Emad et al., 2022). On the other hand, P30 and P54 proteins are important candidates for the pathogenesis of ASFV and therefore, are excellent targets for the detection of ASFV. These proteins are also the main studies of subunit vaccine development which respectively help in virus attachment and internalisation, resulting in the development of neutralising antibodies (Emad et al., 2022). Depending on the virulence of the virus strain, the effect on farms could be different. Virus isolates such as classical strain BA71 and Georgia 2007/1 can cause 100% mortality in infected pigs. Strain such as Estonia 2014 shows lower virulence which causes lower mortality (Nah et al., 2022).

2.2 The Role of Wild Boar in Spreading Classical Swine Fever and African Swine Fever

According to Jeffrey et al. (2019), wild boars and domestic pigs are the exclusive natural hosts of CSF. Wild boars are one of the potential carriers and reservoirs of CSFV in the wild. Transmission of disease from wild boars to domestic pigs is responsible for 52% of disease outbreaks (Jeffrey et al., 2019). Oral vaccination has been implemented by European communities since the 1990s with success in controlling the spread of the virus. Recent research in Japan shows that pre-baiting and repeated vaccination events can help to enhance the ingestion of oral vaccines by wild boars (Takashi et.al., 2022).

In places of Africa that are endemic to ASF, there is usually the presence of wild boars (warthogs and bush pigs) and soft tick vectors (Francis M. & Kevin JD, 2023). Soft ticks are of the genus of Ornithodoros and serve as biological reservoirs. In Africa, the roles of soft ticks and wild boars are important to maintain the virus in a sylvatic cycle without causing clinical symptoms. However, there is no role of ticks in the

current European and Asia epidemic. In Asia, the virus is still maintained in wildlife through the role of wild boars and potentially causes an outbreak in pig farms through livestock–wildlife interaction. Farm size, presence of wild boars near the herd, and frequency of outbreaks occurrence were the major risk factors of transmission. But still, current epidemiological studies amplify the roles of human behaviour, transportation of contaminated products, and selling of suspected disease animals in spreading the disease (Emad, 2022).

2.3 Vaccination

The C-strain live attenuated vaccine which is developed based on the genotype 1 strain is the most common vaccine used in pig industries against the disease (Madiha et al, 2021). In Malaysia, the vaccine efficacy can only go as high as 95%. In China, the reported circulating strains of ASFV have been identified as subgenotypes 1.1, 2.1, 2.2, and 2.3. Notably, subgenotype 2.1b has emerged as predominant over the last decade, while subgenotype 2.1d has been observed circulating in herds vaccinated with the C-strain (Madiha F. et al., 2021). Despite the compulsory vaccination of CSF in China, there are still sporadic outbreaks with mild clinical signs in certain areas. This raises the issue of virulence and pathogenicity of subgenotype 2.1 and the efficacy of the vaccine in cross-protecting against different subgenotypes. The study still confirms that C-strain vaccines provide both clinical and virological protection against the genotype 1 Shimen strain and cross-protection against the prevalent genotype 2 field strain (Qin et al., 2021). Contrarily, another study has demonstrated that C-strain vaccination does not offer cross-protection against the 2.1d strain concerning both pathological and virological aspects. Nevertheless, it has been observed that the pathogenicity of the 2.1d strain is lower in comparison to the Shimen strain, which belongs to genotype 1 (Luo Y. et al., 2017). There is always

concern regarding the efficacy of the C-strain vaccine, given the genetic shift occurring in CSFV (Wang et al., 2022). In Malaysia, the vaccination efficacy can only go as high as 95% despite all the hard work to improve the vaccination quality. A vaccination process executed under poor conditions, improper vaccination dose, and failure to establish a sterilising condition in the pig herd can result in the re-emergence of the disease (Coronado et al., 2021).

P72 is an important part of the viral capsid and antigen which is highly recognized by the immune system to provoke a response during the late-stage expression of the infection (Adekunle et al., 2023). It is also a key for cellular invasion. Therefore, antibodies against the p72 protein can prevent the virus invasion into cells, thereby stopping viral replication (Wang et al., 2021). Studies have demonstrated that immunisation of pigs with eight viral – vectored ASF genes including B602L, B646L, CP204L, E183L, E199L, EP153R, F317L, and MGF505-5R can protect pigs from virulent ASFV (Goatley et al., 2020). The complexity of the virus in interfering with immunomodulation, safety, and side effects of the live attenuated vaccine and the unclear roles of antibodies make vaccine development to be difficult (Revilla et al., 2018). Another limitation of an effective vaccine design is the limited knowledge of an antigen that provides cross-protection between varying strains of ASFV (World Organization for Animal Health (OIE), 2021). Therefore, for a vaccine to be effective, it must be derived from the strain causing the current outbreak as the virulence of the virus is different between strains.

2.4 Molecular detection and sequencing of Classical Swine Fever Virus and African Swine Fever Virus

The three regions of the virus genome that are usually used for genetic characterization of new CFSV isolates include 150 nucleotides of the 5’ nontranslated

region (NTR), the 3' end of the polymerase gene (NS5B), and 190 nucleotides of the gene coding E2 glycoprotein. The sequences of the untranslated region show a high degree of conservation among the Pestiviruses including CSFV, BVDV, and BDV (Hsu et al., 2014). Therefore, this region is a suitable candidate for molecular detection of pestiviruses but further genotyping must be done to differentiate between viruses. The method of genetic typing based on E2 glycoprotein is commonly used due to the established abundant sequence data. (Jeffrey et al., 2019). In today's diagnostic procedure, NTR and E2 genomes are commonly used to characterise individual isolates. However, the short sequence length of these fragments has limitations in differentiating closely related isolates with minimal variables. (Postel et al, 2012). Available technologies capable of sequencing the whole genome CSFV are costly and only limited to several laboratories. (Postel et al, 2012). Reasons such as an increase in public databases regarding the full length of the E2 encoding genome and the roles of E2 and E^{rns} protein as one of the important immunogens make publications have interest in this genomic region. (Postel et al, 2012).

The ASFV genotypes can be distinguished into up to 24 genotypes through molecular detection and sequencing of the 3' terminal end of the B646L open reading frame, which encodes the p72 protein major capsid protein (Achenbach et al., 2017; Boshoff et al., 2007; Quembo et al., 2018). Additionally, closely related ASFV strains can be further differentiated into subgroups by analysing tandem repeat sequences (TRS) in the central variable region (CVR) within the B602L gene and in the intergenic region between the I73R and I329L genes, situated at the right end of the genome (Gallardo et al., 2009; Gallardo et al., 2014). Other gene regions, such as E183L (encoding p54 protein), CP204L (encoding p30 protein), and the EP402R gene (CD2v), have also proven to be valuable tools for analysing ASFVs from different locations and tracking the spread of the virus.

2.5 Quantitative Nested Polymerase Chain Reaction (PCR)

Nested PCR is a modified version of conventional PCR which involves two amplification reactions. Nested PCR is used to increase the sensitivity and specificity of PCR especially when amplifying a specific part of a polymorphic gene family or cDNA from a specific mRNA which present in low abundance in the samples (Michael RG & Joseph S, 2019). A study from (Liu et al., 1991) shows that conventional PCR may fail to detect CSFV if the virus load is too low. Nested PCR usually involves two steps which involve two different pairs of primers. The product from the first amplification reaction is used as a template for the second amplification, where the second set of primers target prime oligonucleotides which are located internal to the first pair (Michael RG & Joseph S, 2019). To reduce the amount of post-PCR processing, a combination of conventional PCR and Taqman can be done to simplify PCR diagnosis. TaqMan assay is able to detect specific templates through the inclusion of a fluorogenic-labelled probe during the PCR reaction. Cleavage of the probe during amplification of a specific fragment will release a reporter dye and the fluorescent can be monitored (McGoldrick A, et al, 1998).

2.6 Reverse Transcriptase Polymerase Chain Reaction (RT – PCR)

Conventional PCR is used to amplify a small template of the genome of a DNA virus. RT – PCR involves the use of reverse transcriptase enzyme and DNA polymerase to convert the single-strand RNA into a double-strand cDNA. From there, the product is used for the amplification process (Vilcek et al., 1994). RT-PCR can be done with single or double assays. One-step RT-PCR combines both reverse transcription and PCR in one tube.

2.7 Multiplex Polymerase Chain Reaction

Malaysia is still endemic with CSF while being hit hard by ASF in the year 2021. Farms in countries such as China are prevalent with CSF, ASF, and APPV, and are common to have concurrent two or three viral infections. Pigs infected with CSFV and ASFV show similar clinical signs and pathological lesions, making clinical manifestation and post-mortem results unreliable to be used for diagnosis (Brown & Bevins, 2018). Piglets infected by CSF and APPV will show signs of congenital tremor (Malik et al., 2020). Besides, virus isolation followed by serological testing is time-consuming, complex, and laboratory-specific (Song et al., 2023). Multiplex PCR can detect multiple pathogens. Multiplex real-time PCR is relatively fast with higher detection capability where the limit of detection can be as low as 1 copy μL^{-1} with higher sensitivity, specificity, and reproducibility (Song et al., 2023). Another study developed multiplex nRT-PCR which was shown to have high sensitivity and specificity for rapid detection and differentiation of CSFV (Yan L et al., 2007). However, the development of a multiplex PCR is more difficult due to the usage of multiple primers and even more complex when involving the use of probes in multiplex real-time PCR.

2.8 Diagnostic method

There are many similarities between ASF and CSF in terms of clinical signs and post-mortem lesions.

2.8.1 Diagnostic Method for African Swine Fever

Serological assays are common, less expensive and simple tests used to diagnose ASF. Besides, currently, there are no available commercial vaccines against the disease. The presence of antibodies indicates infection. The immune system responds to produce antibodies as soon as 7-10 days after infection and persists for

several months or even years. However, a virulent strain usually causes death in pigs before the antibodies are produced. The commonly used serology test is ELISA, which can be used on both serum and blood samples. Samples testing positive in ELISA should be validated using an alternative test, such as IFAT, IPT, or immunoblotting (Gallardo et al., 2015). In areas where ASF is endemic, a comprehensive approach involves a standard serological test (ELISA), complemented by additional serological tests (IFAT, IPT, IBT), and an antigen-detection test to confirm positive cases. In some countries, over 95% of positive cases have been identified using a combination of IFATs and FAT (OIE Terrestrial Manual, 2019). The Hemadsorption (HAD) test can be used as erythrocytes bind to the surface of monocytes or macrophages infected with ASFV. Additionally, a majority of viruses exhibit the HAD phenotype (Gallardo et al., 2015).

The PCR is a highly sensitive test which requires primers that are able to bind to high conservative regions of the virus genome. It amplifies the genome and can detect a broad spectrum of isolates encompassing all known virus genotypes. PCR is suitable for samples that are unsuitable for virus isolation or antigen detection due to factors such as poor samples, putrefaction and virus inactivation. Due to its high sensitivity and specificity, PCR is recommended for both screening and confirming suspected cases (OIE Terrestrial Manual, 2019).

2.8.2 Diagnostic Method for Classical Swine Fever

Serological assays must be used for the diagnosis of suspicious cases. Serology tests can also be used for surveillance purposes and epidemiological studies. Antibodies against CSFV can be detected 2–3 weeks post-infection and can persist for a lifetime. During the terminal stage of the disease, presence of neutralising antibodies might mask a positive result. Detection of antibodies indicates previous infection and

vaccination. For the detection of the immune response, antibody ELISAs and virus neutralisation tests (VNT) are commonly used. False positive results might be obtained when using serological diagnosis due to cross-reactivity with ruminant pestiviruses (BVDV and BDV). Inaccuracy can be due to unidentified circulating BDV strains, closely related BDV strains with no available test and false positive or false negative reactions in ELISA kits due to poor samples (OIE Terrestrial Manual, 2019). Due to the immunosuppressive effects of CSFV, the reliable detection of antibodies is not possible until at least 21 days post-infection. For the purpose of eradication, serological investigations targeting residual infection foci, particularly in breeding herds, become valuable during the terminal phase. Antibody titers provide important epidemiological ideas and can aid in determining the virus's entry route (OIE Terrestrial Manual, 2019).

The RT-PCR can detect viruses even during the incubation period and long period after the pigs recover. Due to its rapidity and enhanced sensitivity, RT-PCR has become a preferred method for both screening and confirming suspected cases of disease, gaining acceptance in multiple countries (OIE Manual, 2019). Numerous conventional and real-time PCR protocols have been outlined by researchers (Hoffmann et al., 2005; McGoldrick et al., 1998; Paton et al., 2000b; Risatti et al., 2003; 2005). The outcomes can be analysed either through agarose gel electrophoresis, a standard method for traditional RT-PCR, or by real-time techniques, specifically RT-qPCR.

2.9 Comparison of sensitivity between Polymerase Chain Reaction (PCR) and other diagnostic methods

Several promising antigen–ELISA have been developed but are limited in differentiating CSFV from BVDV, BDV, and wild-strain of CSFV, in addition to

laborious and time-consuming. (Yan et al., 2007). A study from (Rissati et al., 2005) showed that real time RT-PCR has higher sensitivity (100% to 72%) with slightly slower specificity (98.9% to 100%) than virus isolation when using nasal swabs. The study also shows that RT-nPCR is better for early detection of CSFV than virus isolation and antigen – ELISA (Dewulf et al., 2004).

PCR is recommended by WOAH for screening and confirmation of a suspected ASF case due to its high sensitivity and specificity (WOAH Manual, 2019). PCR is more recommended for many purposes of agent identification such as confirmation of cases, the prevalence of infection, population freedom and eradication policies, in comparison to other diagnostic methods such as virus isolation, FAT, and ELISA for antigen detection (WOAH Manual, 2019). The sensitivity of the antigen ELISA was lower when compared to PCR, even in the case of predominantly field-derived samples with a high virus load.

3.0 METHODOLOGY

3.1 Samples

Samples collected include the formalin-fixed paraffin-embedded tissue samples including spleen, tonsils and lymph nodes from 5 domestic pigs which were 2021 onwards, fresh tissue samples including spleen, tonsils and lymph nodes from 12 domestic pigs and fresh tissue samples including spleen tonsils and lymph nodes from 3 wild boars captured by perhilitan.

3.2 Homogenization of Samples

3.2.1 Materials

Homogenizer, dissection scissor, forceps, autoclaved deionized distilled water (ddH_2O), autoclaved PBS solution, tissue paper, falcon tubes, waste beaker, 1000 µl pipette, 1000 µl tips, 2 ml tubes.

3.2.2 Procedure

1. Four falcon tubes were prepared and labelled as alcohol 1, alcohol 2, ddH_2O and PBS. 15ml of the solutions were added into respective falcon tubes.
2. Four pieces of tissue samples were prepared and labelled as alcohol 1, alcohol 2, ddH_2O and PBS respectively.
3. Homogenizer was cleaned with alcohol two times, followed by ddH_2O and PBS.
4. Scissor and forceps were rinsed with water and Dettol and wiped dry.
5. The scissor and forceps were then sprayed with 70% alcohol and wiped dry.
6. 0.2g of spleen, tonsils and lymph nodes from the same animal source were cut and placed in a 2ml tube with 800 µl of PBS solutions to prepare 10% suspension of tissue in PBS buffer

7. The organs were cut into smaller pieces inside the 2 ml tube by using scissors.
8. The organs were then homogenised.
9. Step 4 was repeated.
10. Step 5 to 7 were repeated on another three organs from the same animal source.
11. Step 3 was repeated, followed by step 8 with a new homogenizer.
12. All the homogenised samples were centrifuged at 8000 rpm for 1 min.

3.3 DNA & RNA Extraction from Tissue Samples

3.3.1 Materials

innuPREP Virus DNA/RNA kit was used. Materials include Lysis solution CBV, Carrier mix, Proteinase K, Binding solution SBS, Washing Solution LS, Washing Solution HS, RNase – free water, Spin filter, Receiver tubes, Elution tubes, 1.5 ml reaction tube, 1000 µl pipette, 100 µl pipette, 10 µl pipette

3.3.2 Procedure

1. A 10% suspension of tissue in the PBS buffer was prepared.
2. The suspension was centrifuged at maximum speed for 2 minutes to remove particles. The clear particle – free supernatant was used for further processing.
3. 200 µl Lysis Solution CBV/Carrier Mix was added into a 1.5ML reaction tube. 200 µl of the sample and 20 µl Proteinase K were mixed vigorously by pulsed vortexing for 10 seconds. The sample was then incubated at 70°C for 10 minutes. After lysis, the reaction tube was centrifuged shortly to remove condensate from the lid of the tube.
4. 400 µl Binding Solution SBS was applied to the lysed sample and mixed by pipetting up and down several times.

5. The sample was introduced into the Spin Filter situated within a 2.0 ml Receiver Tube. The cap was sealed, and the setup was subjected to centrifugation at 10,000 x g (approximately -12,000 rpm) for a duration of 1 minute.
6. The receiver tube containing the filtrate was discarded, and the spin filter was subsequently transferred to a new 2.0 ml receiver tube.
7. 500 µl Washing Solution HS was added into the spin filter and centrifuged at 10.000 x g (-12.000 rpm) for 1 minute. The receiver tube with filtrate was discarded and the spin filter was placed into a new 2.0 ml receiver tube.
8. 650 µl Washing Solution HS was added into the spin filter and centrifuged at 10.000 x g (-12.000 rpm) for 1 minute. The receiver tube with filtrate was discarded and the spin filter was placed into a new 2.0 ml receiver tube.
9. 650 µl Washing Solution LS was introduced into the spin filter, followed by centrifugation at 10,000 x g (-12,000 rpm) for 1 minute. The receiver tube containing the filtrate was then discarded, and the spin filter was transferred to a new 2.0 ml receiver tube.
10. The spin filter was centrifuged at 10.000 x g (-12.000 rpm) for 5 minutes to remove all traces of ethanol. The 2.0 ml receiver tube was discarded.
11. The spin filter was placed into a 1.5 ml Elution Tube. The cap of the filter was opened carefully and 60 µl pre-heated RNase-free water (70°C) was added. The product was incubated at room temperature for 2 minutes and followed by centrifugation at 8.000 x g (-10.000 rpm) for 1 minute. (Two elution steps with equal volumes of RNase-free water (e.g.f. 30ml + 30 µl) might increase the yield of extracted viral DNA/RNA.

12. The extracted viral DNA/RNA were stored at +4°C. (For long-term storage, the product is recommended to be placed at -22°C to -18°C).

3.4 RNA Isolation from Formalin-Fixed Paraffin Embedded (FFPE) Archived Samples

3.4.1 Materials

innuPREP FFPE total RNA kit was used. Materials include Lysis Solution MA, Proteinase K, Washing Solution C, Washing Solution BS, RNase – free water, Spin filter, Receiver tubes, Elution tubes, 1.5 ml reaction tube, 1000 µl pipette, 100 µl pipette, 10 µl pipette

3.4.2 Procedure

1. The FFPE material was placed into a 1.5 ml reaction tube and centrifuged at maximum speed for 1 minute.
2. The reaction tube was opened, and 400 µl of Lysis Solution MA and 40 µl of Proteinase K were introduced into the sample. The mixture was thoroughly blended by pulsed vortexing for 10 seconds. (It's crucial to ensure that the FFPE material is entirely covered by Lysis Solution MA, as fragments not covered may not be fully lysed, potentially impacting the quality and purity of the extracted RNA.)
3. The reaction tube underwent a 30-minute incubation at 65°C in a thermal mixer with continuous shaking at 1,000 rpm.
4. Following the lysis step, the sample underwent centrifugation at maximum speed for 3 minutes. Subsequently, the tube was unsealed, and the supernatant was carefully transferred into a new 1.5 ml reaction tube, ensuring it was RNase-free and a Safe Lock tube.
5. The reaction tube underwent a 30-minute incubation at 80°C in a thermal mixer with continuous shaking at 1,000 rpm.

6. The sample was centrifuged at maximum speed for 3 minutes and the supernatant was transferred into a new 1.5 ml reaction tube.
7. 600 µl of ethanol absolute (96-99%) was added to the sample and mixed vigorously by pipetting up and down several times.
8. 600 µl of the sample was placed onto a Spin Filter situated in a 2.0 ml Receiver Tube. The cap was sealed, and the assembly was centrifuged at 10,000 x g (-12,000 rpm) for 1 minute. Following centrifugation, the receiver tube containing the filtrate was discarded, and the spin filter was transferred to a new 2.0 ml Receiver Tube.
9. The residual sample was moved onto the Spin Filter situated in a 2.0 ml Receiver Tube. After closing the cap, the setup was centrifuged at 10,000 x g (-12,000 rpm) for 1 minute. Subsequently, the receiver tube containing the filtrate was discarded, and the spin filter was transferred to a new 2.0 ml Receiver Tube.
10. The spin filter was opened, and 500 µl of Washing Solution C was introduced. After closing the cap, the system was centrifuged at 10,000 x g (approximately -12,000 rpm) for 1 minute. The resulting filtrate was then discarded, and the spin filter was reinserted into the 2.0 ml Receiver Tube.
11. The spin filter was unsealed, and 650 µl of Washing Solution BS was applied. After closing the cap, the system underwent centrifugation at 10,000 x g (-12,000 rpm) for 1 minute. The resulting filtrate was then discarded, and the spin filter was repositioned into a new 2.0 ml Receiver Tube.
12. The spin filter was opened, and 650 µl of absolute ethanol (96-99%) was introduced. After closing the cap, the system was centrifuged at 10,000 x g (-12,000 rpm) for 1 minute. The resulting filtrate was then discarded, and the spin filter was reinserted into a new 2.0 ml Receiver Tube.

13. The spin filter was subjected to centrifugation at maximum speed for 3 minutes to eliminate all remnants of ethanol. Subsequently, the 2.0 ml Receiver Tube was discarded.
14. The spin filter was inserted into a 1.5 ml Elution Tube. The cap of the spin filter was gently opened, and 50 µl of RNase-free water was introduced. The blend was then left to incubate at room temperature for 1 minute, after which it underwent centrifugation at 10,000 x g (approximately -12,000 rpm) for 1 minute. (Performing a second elution step is recommended to enhance the extracted RNA yield.)

3.5 DNA Isolation from Formalin-Fixed Paraffin Embedded (FFPE) Archived Samples

3.5.1 Materials

blackPREP FFPE DNA kit was used. Materials include Lysis Solution MA, Proteinase K, Washing Solution C, Washing Solution BS, El, Spin filter, Elution buffer, Receiver tubes, Elution tubes, 1.5 ml reaction tube, 1000 µl pipette, 100 µl pipette, 10 µl pipette

3.5.2 Procedure

1. The FFPE material was placed into a 1.5 ml reaction tube and centrifuged at maximum speed for 1 minute.
2. The reaction tube was opened, and 400 µl of Lysis Solution MA along with 40 µl of Proteinase K were introduced into the sample. The mixture underwent thorough mixing by pulsed vortexing for 10 seconds. (It is crucial to ensure that the FFPE material is entirely covered by Lysis Solution MA, as fragments not covered may not be fully lysed, potentially affecting the quality and purity of the extracted RNA.)
3. The reaction tube underwent a 1-hour incubation at 90°C in a thermal mixer with continuous shaking at 1,000 rpm.

4. The samples were incubated at room temperature for 5 minutes.
5. The sample was subjected to centrifugation at maximum speed for 2 minutes. Subsequently, the tube was unsealed, and the supernatant was carefully transferred into a new 1.5 ml reaction tube.
6. 400 µl of ethanol absolute (96-99%) was added to the sample and mixed vigorously by pipetting up and down several times.
7. The sample was placed onto a Spin Filter within a 2.0 ml Receiver Tube. After sealing the cap, the system was centrifuged at 10,000 x g (-12,000 rpm) for 1 minute. Subsequently, the receiver tube containing the filtrate was discarded, and the spin filter was transferred to a new 2.0 ml Receiver Tube.
8. The spin filter was unsealed, and 500 µl of Washing Solution C was introduced. After closing the cap, the system was centrifuged at 10,000 x g (-12,000 rpm) for 1 minute. The resulting filtrate was then discarded, and the spin filter was reinserted into the 2.0 ml Receiver Tube.
9. The spin filter was unsealed, and 650 µl of Washing Solution BS was applied. After closing the cap, the system underwent centrifugation at 10,000 x g (-12,000 rpm) for 1 minute. The resulting filtrate was then discarded, and the spin filter was repositioned into a new 2.0 ml Receiver Tube.
10. The spin filter was opened, and 650 µl of absolute ethanol (96-99%) was introduced. After closing the cap, the system was centrifuged at 10,000 x g (-12,000 rpm) for 1 minute. The resulting filtrate was then discarded, and the spin filter was reinserted into a new 2.0 ml Receiver Tube.
11. The spin filter was subjected to centrifugation at maximum speed for 3 minutes to eliminate any remaining traces of ethanol. Following this, the 2.0 ml Receiver Tube was discarded.

12. The spin filter was inserted into a 1.5 ml Elution Tube. The cap of the spin filter was carefully opened and a 50 µl elution buffer was added. The mixture was incubated at room temperature for 2 minutes, followed by centrifugation at 10.000 x g (-12.000 rpm) for 1 minute. A second elution step was done to increase the yield of extracted DNA.

3.6 One-step Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

3.6.1 Materials

My Taq ™ One-Step RT-PCR Kit was used. Reagents include My Taq One-Step Mix, Forward primers, Reverse primers, reverse transcriptase, Ribosafe RNase inhibitor, Autoclaved DDH_2O. Apparatus include micropipette (1000 µl, 100 µl, 20 µl, 2.5 µl), filter tips (1000 µl, 100 µl, 10 µl), PCR tube, 1.5 ml tube, racks, waste bottle, freezer rack, ice packs, vortex mixer and centrifuge.

3.6.2 Primers

Forward primer 5'- ATGCCCWTAGTAGGACTAGCA-3' and reverse primer 5'-TCAACTCCATGTGCCATGTAC-3' were used. (Vilcek et al.,1994). The primers targeted a non-translated region of the genome and the expected length of the base pair is 280 bp.

3.6.3 Procedure

1. Mastermix was prepared based on the number of reactions.
2. The volume of reagents for 1x reactions include 12.5 µl My Taq One Step Mix, 4.75 µl of DDH_2O, 1 µl of 10 µM of Forward primer, 1 µl of 10 µM of reverse Primer, 0.50 µl of RNAse inhibitor and 0.25 µl of RT-enzyme.
3. The prepared master mix was vortexed and centrifuged for 10 seconds.
4. All the PCR tubes were opened.
5. 20 µl of master mix was added for each PCR tube and the tube was closed at prompt.
6. 5 µl of DDH_2O was added in the negative control PCR tube.

7. 5 µl of samples were added into sample PCR tubes respectively.
8. A new glove was changed.
9. 5 µl of extracted CSF vaccine was added in the positive control PCR tube.
10. Each tube was vortexed for 10 seconds.
11. Each tube was centrifuged for 10 seconds.
12. The PCR tube was put into the thermocycler.
13. The reaction mixture was treated as follows: (i) reverse transcription process for 20 min at 45°C, followed by polymerase activation for 1 min at 95°C; (ii) subjected to 40 cycles of PCR, with 1 cycle consisting of 1 min at 95°C, 1 min at 59°C, and 30 s at 72°C; and (iii) incubated for 10 min at 72°C.

3.7 One step Conventional Polymerase Chain Reaction (RT-PCR)

3.7.1 Materials

My Taq ™ One-Step RT-PCR Kit was used. Reagents include My Taq One-Step Mix, Forward primers, Reverse primers, and Autoclaved DDH_2O. Apparatus include micropipette (1000 µl, 100 µl, 20 µl, 2.5 µl), filter tips (1000 µl, 100 µl, 10 µl), PCR tube, 1.5 ml tube, racks, waste bottle, freezer rack, ice packs, vortex mixer and centrifuge.

3.7.2 Primers

Forward primer 5'-GGCACAAGTTCGGACATGT-3' and reverse primer 5'-GTACTGTAACGCAGCACAG-3' were used. (Bastos et al., 2003). The primers targeted B646L gene which encodes for P72 protein and the expected length of base pair was 478 bp.

3.7.3 Procedure

1. Mastermix was prepared based on the number of reactions.
2. The volume of reagents for 1x reactions include 12.5 µl My Taq One Step Mix, 5.50 µl of DDH_2O, 1 µl of 10 µM of Forward primer, 1 µl of 10 µM of reverse Primer.
3. The prepared master mix was vortexed and centrifuged for 10 seconds.

4. All the PCR tubes were opened.
5. 20 µl of master mix was added for each PCR tube and the tube was closed at prompt.
6. 5 µl of DDH_2O was added in the negative control PCR tube.
7. 5 µl of samples were added into sample PCR tubes respectively.
8. A new glove was changed.
9. 5 µl of extracted DNA from the positive sample was added in the positive control PCR tube.
10. Each tube was vortexed for 10 seconds.
11. Each tube was centrifuged for 10 seconds.
12. The PCR tube was put into the thermocycler.
13. The reaction mixture underwent the following treatment: (i) an initial denaturation for 1 minute at 95°C; (ii) it was then subjected to 35 cycles of PCR, with each cycle comprising 15 seconds at 95°C, 20 seconds at 50°C, and 10 seconds at 72°C; and (iii) finally, it was incubated for 5 minutes at 72°C.

3.8 Multiplex Reverse Transcriptase Polymerase Chain Reaction (mRT-PCR)

3.8.1 Materials

My Taq ™ One-Step RT-PCR Kit was used. Reagents include My Taq One-Step Mix, two pairs of Forward primers and Reverse primers, Reverse transcriptase, Ribosafe RNase inhibitor, Autoclaved DDH_2O. Apparatus include micropipette (1000 µl, 100 µl, 20 µl, 2.5 µl), filter tips (1000 µl, 100 µl, 10 µl), PCR tube, 1.5 ml tube, racks, waste bottle, freezer rack, ice packs, vortex mixer and centrifuge.

3.8.2 Primers

Two pairs of primers were used to simultaneously detect both CSF and ASF genome. The pair of forward primer 5'-GGACTAGCAAACGGAGGGACT-3' and reverse primer 5'-TCGAGGTGGGCTTCTGCTCACG-3' was used to detect non translated region of

CSF genome with expected length of base pair was 108 bp. Another pair of primers with forward primer 5'-AGTTATGGGAAACCCGACCC-3' and reverse primers 5'-CCCTGAATCGGAGCATCCT-3' was used to detect B646L gene encoding for VP73 protein with expected length of base pair was 257 bp. (Agüero et al., 2004).

3.8.3 Procedure

1. Mastermix was prepared based on the number of reactions.
2. The volume of reagents for 1x reactions include 12.5 µl My Taq One Step Mix, 2.75 µl of DDH_2O, 1 µl of 10 µM of Forward primer CSF, 1 µl of 10 µM of Reverse primer CSF, 1 µl of 10 µM of Forward primer ASF, 1 µl of 10 µM of Reverse primer ASF, 0.50 µl of RNAse inhibitor and 0.25 µl of RT-enzyme.
3. The prepared master mix was vortexed and centrifuged for 10 seconds.
4. All the PCR tubes were opened.
5. 20 µl of master mix was added for each PCR tube and the tube was closed at prompt.
6. 5 µl of DDH_2O was added in the negative control PCR tube.
7. 5 µl of samples were added into sample PCR tubes respectively.
8. A new glove was changed.
9. 2.5 µl of extracted DNA from the ASF positive sample and 2.5 µl of extracted RNA from CSF live attenuated vaccine were added into the positive control PCR tube.
10. Each tube was vortexed for 10 seconds.
11. Each tube was centrifuged for 10 seconds.
12. The PCR tube was put into the thermocycler and run for PCR.
13. The reaction mixture was treated as follows: (i) reverse transcription process for 20 min at 45°C, followed by polymerase activation for 1 min at 95°C; (ii)

subjected to 40 cycles of PCR, with 1 cycle consisting of 30 s at 94°C, 30 s at 59°C, and 30 s at 72°C; and (iii) incubated for 10 min at 72°C.

3.9 Agarose Gel electrophoresis

3.9.1 Materials

Agarose powder, 1X TAE buffer, Redsafe, Autoclaved DDH_2O, Loading dye, Ladder, PCR products, Gel tray with comb, power supply

3.9.2 Procedure

1. Appropriate mass of agarose powder was weighed and agarose gels were prepared using w/v percentage solution. (2% agarose gel was prepared for gel electrophoresis of uniplex CSF and ASF PCR products whereas 3% agarose gel was prepared for multiplex PCR products.
2. Mixture of agarose powder and 1X TAE buffer was heated until the powder completely dissolved.
3. The agarose solution was allowed to cool down to room temperature, followed by addition of redsafe.
4. The agarose solution was poured into a gel tray with appropriate comb, and allowed to solidify.
5. The gel tray, together with solidified agarose, was placed into a gel box.
6. The 1X TAE buffer was filled until the whole gel was covered by the solution.
7. A 3 µl molecular weight ladder was carefully loaded into the ladder well.
8. 5 µl of sample PCR products was mixed with 1 µl of loading dye, then carefully loaded into the respective sample well.
9. 5 µl of positive control PCR products was mixed with 1 µl of loading dye, then carefully loaded into the positive control well.

10. 5 µl of negative control PCR products was mixed with 1 µl of loading dye, then carefully loaded into the negative control well.
11. The gel box was run under 90 V; 400mA for 35 minutes.
12. The DNA fragments were visualised in a UV light transilluminator.

4.0 RESULTS

4.1 Uniplex RT-PCR to detect CSFV

A total of 3/5 archived tissue samples from domestic pigs of 2021 and onwards were positive with CSFV. All of the fresh tissue samples from domestic pigs and wild boar were negative from CSFV. (Figure 1.1 and figure 2.0).

4.2 Uniplex conventional PCR to detect ASFV

All of the fresh tissue samples from domestic pigs and wild boars and all of the archived samples from domestic pigs are negative from ASFV. (Figure

4.3 Multiplex RT-PCR to detect CSFV and ASFV

Positive control only shows one band instead of expected two bands. The result is not valid.

Figure 1.1 Uniplex RT-PCR for CSFV detection. C-: negative control; M: molecular weight ladder; 1-5 archived tissue samples from domestic pigs; C+: positive control (CSFV RNA).

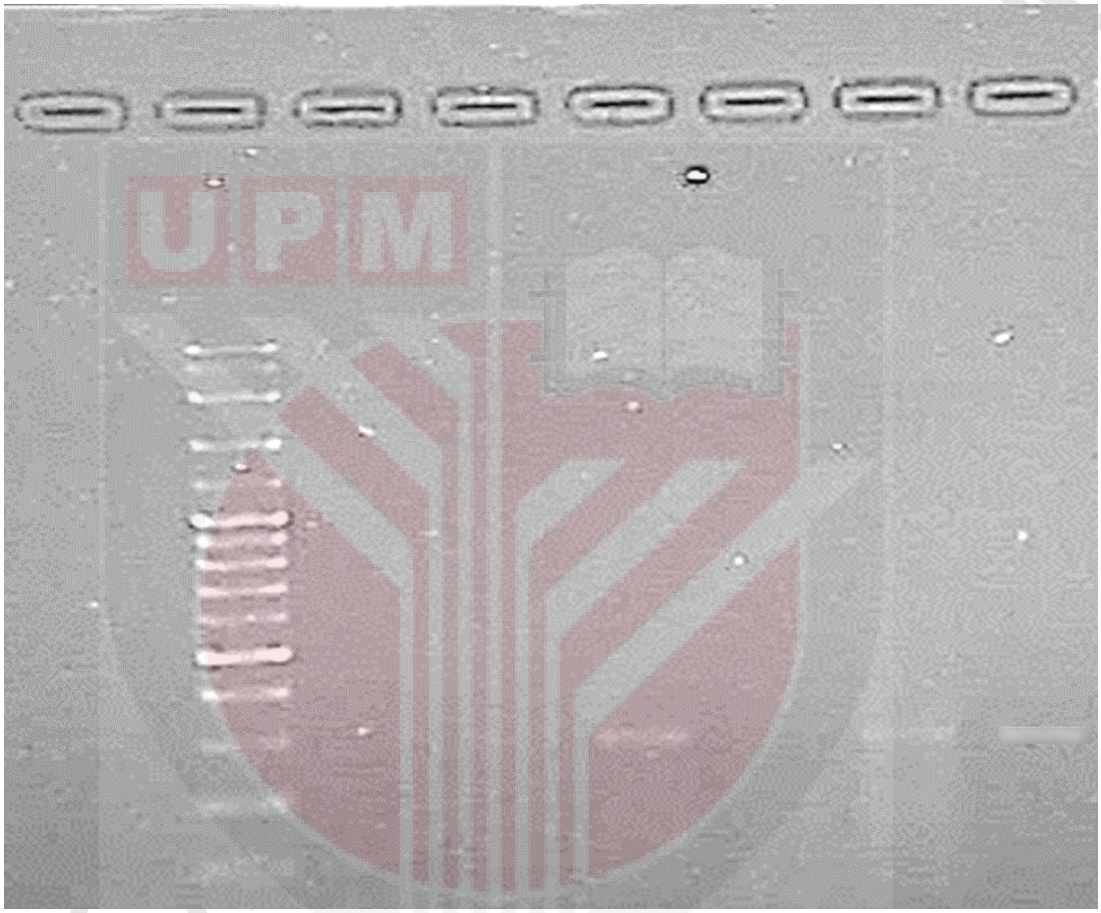


Figure 1.2 Uniplex RT-PCR for CSFV detection. C-: negative control; NTC: no template control; M: molecular weight ladder; 1-10 fresh tissue samples from domestic pigs; 11-13 fresh tissue samples from wild boar; C+: positive control (CSFV RNA).

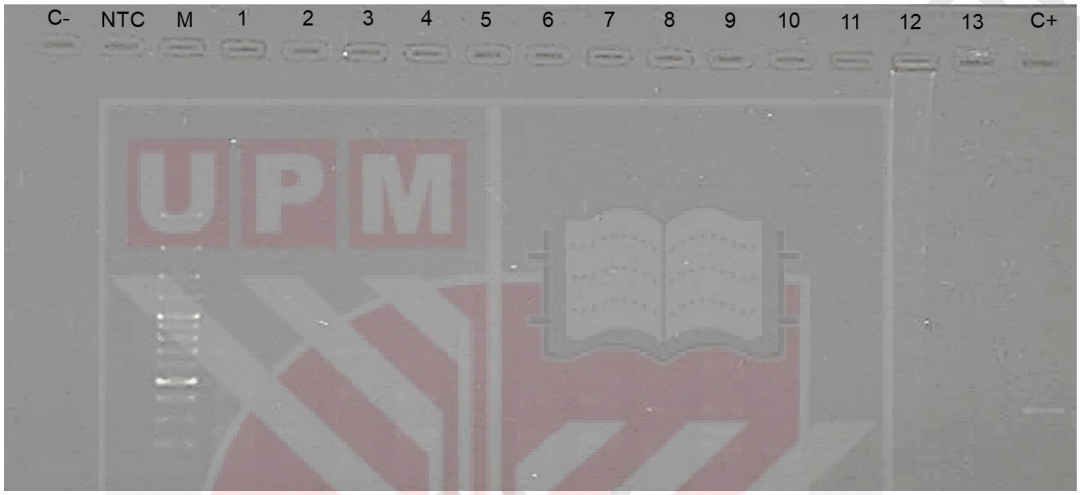


Figure 1.3 Uniplex conventional PCR for ASFV detection. C-: negative control; NTC: no template control; M: molecular weight ladder; 1-10 fresh tissue samples from domestic pigs; C+: positive control (ASFV DNA).

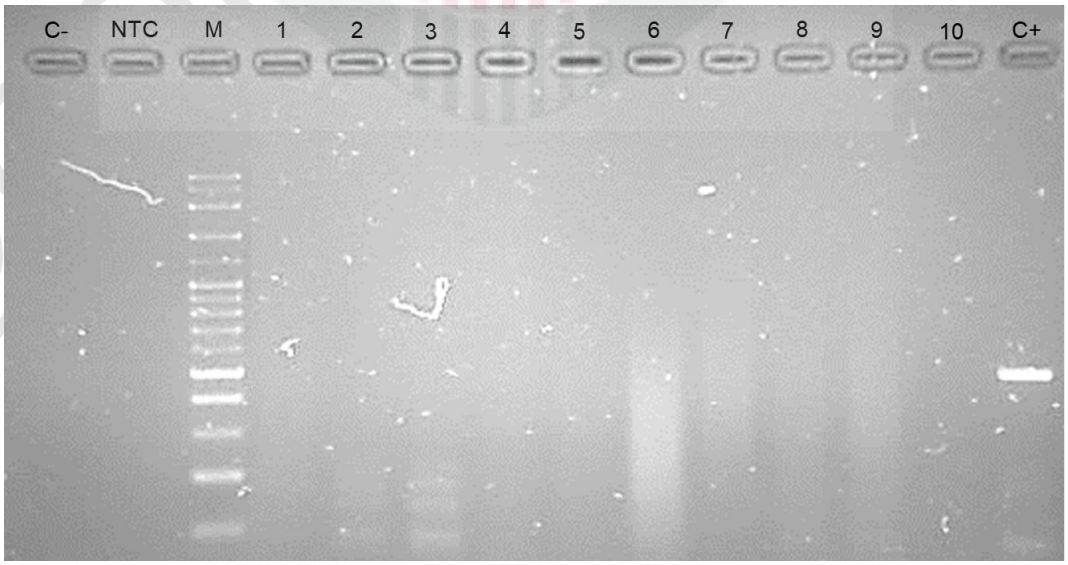
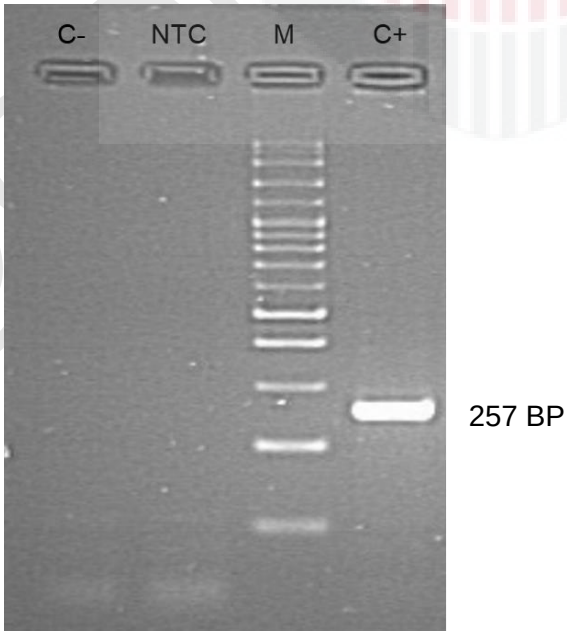


Figure 1.4 Uniplex conventional PCR for ASFV detection. C-: negative control; M: molecular weight ladder; 1-3 fresh tissue samples from wild boars; 4-6 fresh tissue samples from domestic pigs; 7-11 archived tissue samples from domestic pigs; C+: positive control (ASFV DNA).

Figure 1.5 Multiplex RT-PCR for CSFV and ASFV detection. C-: negative control; NTC: no template control; M: molecular weight; C+: positive control (ASFV DNA and CSFV RNA), the PCR fails to detect CSFV.



5.0 Discussion

5.1 Importance to differentiate between classical swine fever and African swine fever

5.1.1 Clinical signs and pathology

Animals infected with CSF and ASF exhibit similar clinical signs and post-mortem lesions. Post-mortem investigations commonly reveal respiratory lesions, including pneumonia, pleuritis, chronic bronchitis, pulmonary edema, conjunctivitis, and haemorrhages in multiple organs such as the renal pelvis, kidney, urinary bladder, lymph nodes, and spleen enlargement. Dry faecal contents in the colon and chronic gastric ulceration are also frequently observed (Armin et al., 2003).

There are various forms of ASF, including peracute, acute, subacute, and chronic. Highly virulent ASFV strains in Eastern Europe and Asia lead to acute and lethal diseases. Animals with acute ASF display loss of appetite, inactivity, and respiratory signs such as severe pulmonary edema. Pigs infected with highly virulent strains often die in shock approximately one week after the onset of fever, with foam typically observed around the mouth and nose. Affected pigs may also exhibit erythema and cyanosis on the skin of the ears, tail, distal extremities, chest, abdomen, and perianal area. Abortion may occur in pregnant sows due to fever. Those with acute ASF present with hyperemic splenomegaly, characterised by a spleen up to six times larger than normal. Lymph nodes, especially the gastrohepatic and renal nodes, display haemorrhages in the medulla, giving affected lymph nodes a marbled appearance. Kidneys typically show petechial haemorrhages in the cortex and renal pelvis. Other lesions in pigs with acute forms of ASF include petechial haemorrhages

in the mucosa of the urinary bladder, epicardium, endocardium, and pleura (Sánchez-Vizcaíno et al., 2015).

For CSF, there are prenatal, acute, and chronic courses of the disease, dependent on factors such as virus virulence, infection dose, health status, and age of the affected animal. Similar to ASF, animals with acute CSF exhibit fever, respiratory and gastrointestinal signs, lethargy, and inappetence. The acute lethal form may manifest with severe hemorrhagic or neurological signs, leading to high mortality in piglets. Adult pigs tend to withstand the disease better, developing lifetime immunity. The chronic form, caused by viruses with lower virulence, usually results in nonspecific symptoms such as runting, secondary infections of the respiratory and gastrointestinal tracts, skin lesions, and reduced fertility in sows. Transplacental infection can lead to abortion, stillbirth, mummification, or the development of persistently infected and virus-shedding piglets. In terms of pathology, acute forms of CSF may exhibit edematous and hemorrhagic lymph nodes, spleen, kidneys, and other organs. Spleen infarctions and necrotic regions in the tonsils are sometimes found in animals succumbing to the chronic form of CSF (Schulz et al., 2017).

In short, it is very hard to distinguish CSF and ASF based on clinical signs and postmortem findings. The status of CSF in Malaysia is still endemic, and in the meantime, it has been hit hard by ASF since 2021. There is a need for a highly sensitive and specific test to differentiate between these two diseases.

5.1.2 Policies in Malaysia

There are differences between the policies during the CSF outbreak and the ASF outbreak in Malaysia. When a farm has an outbreak of CSF, the farmers must report the case to state veterinary officers. All the infected pigs must be culled. The criteria

to confirm a positive CSF case is based on clinical and pathological findings, PCR test, and FAT test. Several policies are taken including, culling without vaccination, culling with vaccination, and vaccination without culling. Other steps that will be taken include movement control of the animals within 1 km, repeat testing every 6 months until the farm is negative for the virus, rapid tracking of the past movement of products or animals from the farms, active and passive surveillance, quarantine of infected animals, vaccination of at least 80 – 85% of the total pig population, and disinfection of the premises (Veterinary Malaysia Protocol, 2010). For ASF, the only policy we have is stamping out policy. This involves massive eradication and culling of all the pigs of the affected farms, quarantine, and closure of the affected farms, resulting in a heavy loss of industries and food supply chains. Furthermore, starting in 2018, Malaysia has restricted the import of pork or pork-related products from affected countries including China, Belgium, Vietnam, Thailand, Hong Kong, and so on. This further impacts our pork supply chains together with a significant drop in our self-sufficiency rate. Therefore, there is a need to distinguish between these two diseases, due to the different consequences, and to protect our local pork industries.

5.2 Comparison between uniplex PCR and multiplex PCR

The cost and time needed to scan a sample for both CSFV and ASFV by using uniplex PCR is more than multiplex PCR. For a uniplex PCR to detect both CSFV and ASFV, a conventional PCR to detect ASFV (DNA virus) and RT-PCR to detect CSFV (RNA virus) are needed. The total cost for uniplex PCR is approximately twice the multiplex PCR as double the amount of reagents and materials for DNA/RNA extraction and PCR are needed. More time is needed as well for uniplex PCR, as 32 minutes 15 seconds is needed for one conventional PCR while 131 minutes is needed for one RT-PCR. Besides, gel electrophoresis needs to be performed two times. Less cost

and time-consuming make multiplex PCR a practical, simple, economical, and reliable tool for rapid detection and accurate diagnosis for CSFV and ASFV (Liu et al.,2021). Multiplex PCR is also a highly desirable tool to perform herd-level surveillance (Song et al., 2023).

Table 1.0 Cost for scanning both CSFV and ASFV in 100 samples by uniplex PCR and multiplex PCR. Table includes the cost for DNA/RNA extraction kit/100 reactions, My Taq One-Step RT-PCR kit/ 100 reactions, Forward primers ASF, Reverse primers ASF, Forward primers CSF, and Reverse primers CSF.

Item	Uniplex PCR	Multiplex PCR
DNA/RNA extraction kit/100 reactions	RM 2029.60 x 2 = RM 4059.20	RM 2029.60
My Taq One-Step RT-PCR kit/ 100 reactions	RM 1920 x 2 = RM 3840	RM 1920
Forward primers ASF	RM 36.10	RM 38.00
Reverse primers ASF	RM 36.10	RM 36.10
Forward primers CSF	RM 39.90	RM 39.90
Reverse primers CSF	RM 39.90	RM 41.80
Total	RM 8051.20	RM 4105.40

Table 2.0 Time for scanning both classical swine fever virus and African swine fever virus in 100 samples by uniplex PCR and multiplex PCR. The table includes the time needed for conventional PCR, RT-PCR, and multiplex PCR.

Procedure	Uniplex PCR	Multiplex PCR
Conventional PCR	32 m 15s	
RT-PCR	131 m	
Multiplex PCR	-	121 m
Gel Electrophoresis	35 m x 2 = 70 m	35 m

Total	233 m 15 s	159 m

5.3 Optimization of mRT-PCR

Optimization of mRT-PCR can be done by varying single parameters while other parameters remain unchanged. Parameters to be changed include primer concentration, annealing temperature, and number of cycles. The parameters that produce the most amplified products when viewed under a UV transilluminator should be chosen. After optimization, the sensitivity and specificity of the test should be checked. The specificity of the test can be done by using DNA or cDNA of multiple common swine pathogens such as ASFV, CSFV, APP, PCV (Type 1, Type 2, and Type 3), PRRSV, PEDV, TGEV, PRoV, PDCoV, BVDV-1, BVDV-2, PPV, and PRV. High-specificity mRT-PCR can only simultaneously detect CSFV and ASFV with no amplicons from other pathogens. A sensitivity test should be done by performing a serial tenfold dilution of positive control for p-CSFV and p-ASFV to identify the detection limit for this PCR. The established mRT-PCR should have the same sensitivity as uniplex PCR. Last but not least, is the repeatability of the test. Uniform target bands should be obtained under five repeated reactions with the same serial dilutions to demonstrate the high reproducibility of the mRT-PCR (Liu et al., 2021).

6.0 Conclusion

All of the fresh tissue samples from domestic pigs and wild boars are negative for ASFV and CSFV. Three out of five archived samples from domestic pigs are positive with CSFV. Multiplex PCR saves time and cost compared to uniplex PCR to detect both ASFV and CSFV from one sample.

7.0 Recommendations

The positive samples are recommended to be sent for sequencing to identify the genotypes of current circulating strains of both CSFV and ASFV in Malaysia, followed by epidemiological studies of the diseases. An increase in sample sizes is

recommended for statistical analysis. Wild boar samples should be obtained for further studies of the roles of wild boars in the transmission of the disease in Malaysia. Multiple PCR should be established in our laboratory in the future to meet the demand of our current situation in Malaysia.

8.0 References

Adekunle, B. R., Angus, N. O., Mercy, T. A., Ezinne, J. N., Moses, O. N., Olayinka, O. A., Suliat, A. S., & Nancy, A. M. (2023). Design of multi epitope vaccine candidate from a major capsid protein of the African swine fever virus, *Veterinary Vaccine, 2*(1).

Armin, R. W., Elbers, J. H., Vos, A. B., Ad C. A., Exsel, V., & Arjan, S. (2003). Assessment of the use of gross lesions at post-mortem to detect outbreaks of classical swine fever, *Veterinary Microbiology, 96*(4), 345-356.

Ata, E. B., Li, Z. J., Shi, C. W., Yang, G. L., Yang, W. T., & Wang, C. F. (2022). African swine fever virus: A raised global upsurge and a continuous threaten to pig husbandry. *Microbial pathogenesis, 167,* 105561.

Brown, V.R., & Bevins, S. N. (2018). A Review of African Swine Fever and the Potential for Introduction into the United States and the Possibility of Subsequent Establishment in Feral Swine and Native Ticks. *Front Vet Sci, 5,* 11.

Brown, V.R., & Bevins, S. N. (2018). A Review of Classical Swine Fever Virus and Routes of Introduction into the United States and the Potential for Virus Establishment. *Front Vet Sci, 5,* 31.

Coronado, L., Bohórquez, J. A., Muñoz-González, S., Perez, L. J., Rosell, R., Fonseca, O., Delgado, L., Perera, C. L., Frías, M. T., & Ganges, L. (2019). Investigation of chronic and persistent classical swine fever infections under field conditions and their impact on vaccine efficacy. *BMC Vet Res, 5*(1), 247.

Coronado, L., Perera, C. L., Rios, L., Frías, M. T., & Pérez, L. J. (2021). A Critical Review

about Different Vaccines against Classical Swine Fever Virus and Their Repercussions in Endemic Regions. *Vaccines*, *9*(2), 154.

Edwards, S., Fukusho, A., Lefèvre, P. C., Lipowski, A., Pejsak, Z., Roehe, P., & Westergaard, J. (2000). Classical swine fever: the global situation. *Veterinary microbiology*, *73*(2-3), 103–119.

Fatima, M., Luo, Y., Zhang, L., Wang, P. Y., Song, H., Fu, Y., Li, Y., Sun, Y., Li, S., Bao, Y. J., & Qiu, H. J. (2021). Genotyping and Molecular Characterization of Classical Swine Fever Virus Isolated in China during 2016-2018. *Viruses*, *13*(4), 664.

Francis, M., & Kevin, J. D. (2023). Epidemiological drivers and control strategies for African swine fever transmission cycles at a wildlife-livestock interface, *Ecological Modelling, 481*, 110344,

Gallardo, C., Fernández-Pinero, J., Pelayo, V., Gazaev, I., Markowska-Daniel, I., Pridotkas, G., Nieto, R., Fernández-Pacheco, P., Bokhan, S., Nevolko, O., Drozhzhe, Z., Pérez, C., Soler, A., Kolvasov, D., & Arias, M. (2014). Genetic variation among African swine fever genotype II viruses, eastern and central Europe. *Emerg Infect Dis. 20*(9), 1544-1547.

Gallardo, C., Mwaengo, D. M., Macharia, J. M., Arias, M., Taracha, E. A., Soler, A., Okoth, E., Martín, E., Kasiti, J., & Bishop, R. P. (2009). Enhanced discrimination of African swine fever virus isolates through nucleotide sequencing of the p54, p72, and pB602L (CVR) genes. *Virus genes*, *38*(1), 85–95.

Gallardo, C., Nieto, R., Soler, A., Pelayo, V., Fernández-Pinero, J., Markowska-Daniel, I.,

Pridotkas, G., Nurmoja, I., Granta, R., Simón, A., Pérez, C., Martín, E., Fernández-Pacheco, P., & Arias, M. (2015). Assessment of African Swine Fever Diagnostic Techniques as a Response to the Epidemic Outbreaks in Eastern European Union Countries: How To Improve Surveillance and Control Programs. *Journal of clinical microbiology*, *53*(8), 2555–2565.

Goatley, L. C., Reis, A. L., Portugal, R., Goldswain, H., Shimmon, G. L., Hargreaves, Z., Ho, C. S., Montoya, M., Sánchez-Cordón, P. J., Taylor, G., Dixon, L. K., & Netherton, C. L. (2020). A Pool of Eight Virally Vectored African Swine Fever Antigens Protect Pigs Against Fatal Disease. *Vaccines*, *8*(2), 234.

Gong, W., Li, J., Wang, Z., Sun, J., Mi, S., Lu, Z., Cao, J., Dou, Z., Sun, Y., Wang, P., Yuan, K., Zhang, L., Zhou, X., He, S., & Tu, C. (2019). Virulence evaluation of classical swine fever virus subgenotype 2.1 and 2.2 isolates circulating in China. *Veterinary microbiology*, *232*, 114–120.

Green, M. R., & Sambrook, J. (2019). Nested Polymerase Chain Reaction (PCR). *Cold Spring Harbor protocols*, *2019*(2), 10.

Hsu, W. L., Chen, C. L., Huang, S. W., Wu, C. C., Chen, I. H., Nadar, M., Su, Y. P., & Tsai, C. H. (2014). The untranslated regions of classical swine fever virus RNA trigger apoptosis. *PloS one*, *9*(2), e88863.

Hu, L., Lin, X. Y., Yang, Z. X., Yao, X. P., Li, G. L., Peng, S. Z., & Wang, Y. (2015). A multiplex PCR for simultaneous detection of classical swine fever virus, African swine fever virus, highly pathogenic porcine reproductive and respiratory syndrome virus, porcine reproductive and respiratory syndrome virus and pseudorabies in swines. *Polish journal of veterinary sciences*, *18*(4), 715–723.

Jabatan Perkhidmatan Veterinar. (2010). Deman Babi (Classical Swine Fever). *Protokal Veterinar Malaysia, 5*(2)

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.

Li, Y., Zhao, J. J., Li, N., Shi, Z., Cheng, D., Zhu, Q. H., Tu, C., Tong, G. Z., & Qiu, H. J. (2007). A multiplex nested RT-PCR for the detection and differentiation of wild-type viruses from C-strain vaccine of classical swine fever virus. *Journal of virological methods*, *143*(1), 16–22.

Liu, H., Shi, K., Sun, W., Zhao, J., Yin, Y., Si, H., Qu, S., & Lu, W. (2021). Development a multiplex RT-PCR assay for simultaneous detection of African swine fever virus, classical swine fever virus and atypical porcine pestivirus. *Journal of virological methods*, *287*, 114006.

Malik, Y. S., Bhat, S., Kumar, O. R. V., Yadav, A. K., Sircar, S., Ansari, M. I., Sarma, D. K., Rajkhowa, T. K., Ghosh, S., & Dhama, K. (2020). Classical Swine Fever Virus Biology, Clinicopathology, Diagnosis, Vaccines and a Meta-Analysis of Prevalence: A Review from the Indian Perspective. *Pathogens (Basel, Switzerland)*, *9*(6), 500.

McGoldrick, A., Lowings, J. P., Ibata, G., Sands, J. J., Belak, S., & Paton, D. J. (1998). A novel approach to the detection of classical swine fever virus by RT-PCR with a fluorogenic probe (TaqMan). *Journal of virological methods*, *72*(2), 125–135.

Nah, J. J., Kwon, O. K., Choi, J. D., Jang, S. H., Lee, H. J., Ahn, D. G., Lee, K., Kang, B., Hae-

Eun, K., & Shin, Y. K. (2022). Development of an indirect ELISA against African swine fever virus using two recombinant antigens, partial p22 and p30. *Journal of virological methods*, *309*, 114611.

Nguyen, N. H., Nguyen, B. T. P., Do, D. T., Nguyen, T. Q., Nguyen, D. T. M., & Nguyen, M. N. (2021). Genetic diversity and molecular characterization of classical swine fever virus envelope protein genes E2 and Erns circulating in Vietnam from 2017 to 2019. *Infection, genetics and evolution : journal of molecular epidemiology and evolutionary genetics in infectious diseases*, *96*, 105140.

Paton, D. J., McGoldrick, A., Greiser-Wilke, I., Parchariyanon, S., Song, J. Y., Liou, P. P., Stadejek, T., Lowings, J. P., Björklund, H., & Belák, S. (2000). Genetic typing of classical swine fever virus, *Veterinary Microbiology, 73*(2–3), 137-157.

Postel, A., Schmeiser, S., Bernau, J., Pridotkas, A., Dirbakova, G., Mojzis, Z., Becher, M., & Paul. (2012). Improved strategy for phylogenetic analysis of classical swine fever virus based on full-length E2 encoding sequences. *Vet Res* 43, 50.

Revilla, Y., Pérez-Núñez, D., & Richt, J. A. (2018). African Swine Fever Virus Biology and Vaccine Approaches. *Advances in virus research*, *100*, 41–74.

Saatkamp, H. W., Berentsen, P. B., & Horst, H. S. (2000). Economic aspects of the control of classical swine fever outbreaks in the European Union. *Veterinary microbiology*, *73*(2-3), 221–237.

Sánchez-Vizcaíno, J. M., Mur, L., Gomez-Villamandos, J. C., & Carrasco, L. (2015). An update on the epidemiology and pathology of African swine fever. *Journal of comparative pathology*, *152*(1), 9–21.

Schulz, K., Staubach, C., & Blome, S. (2017). African and classical swine fever: similarities, differences and epidemiological consequences. *Veterinary research*, *48*(1), 84.

Song, X.P., Xia, Y.J., Xu, L., Zhao, J. J., Wang, Z., Zhao, Q.Z., Liu, Y. B., Zhang, Q.Y., & Wang, Q. (2023). A multiplex real-time PCR assay for simultaneous detection of classical swine fever virus, African swine fever virus, and atypical porcine pestivirus, *Journal of Integrative Agriculture, 22*(2), 559-567.

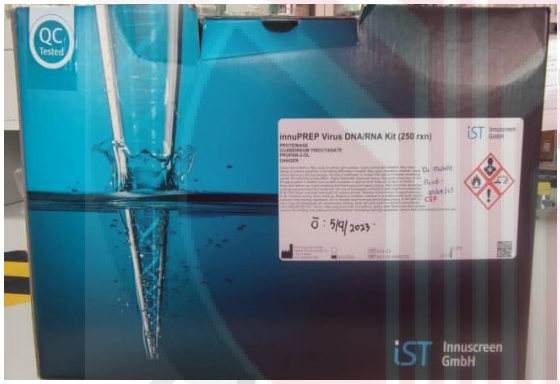
Wang, Q., Liu, H., Xu, L., Li, J., Wu, H., Yang, C., Chen, X., Deng, Y., Sun, Y., Tu, C., Chen, N., Gong, W., & Chen, G. (2021). Different clinical presentations of subgenotype 2.1 strain of classical swine fever infection in weaned piglets and adults, and long-term cross-protection conferred by a C-strain vaccine. *Veterinary microbiology*, *253*, 108915.

Wang, Z. Z., Qi, C. X., Ge, S. Q., Li, J. M., Hu, Y. X., Zhang, X. Y., Yan, L., Han, N., Wu, X. D., Wang, Z., & Qian, Y. J. (2022). Genetic variation and evolution of attenuated African swine fever virus strain isolated in the field: A review. *Virus research*, *319*, 198874.

APPENDIX

Appendix I My Taq One-Step RT-PCR Kit

Appendix II immuPREP Virus DNA/RNA Kit (200 rxn)



Appendix III Agarose Powder

Appendix IV Classical Swine Fever Live Attenuated Vaccine