MOLECULAR CHARACTERISATION OF PORCINE CIRCOVIRUSES IN MALAYSIA AND DEVELOPMENT OF A PCV TYPE 3 DIAGNOSTIC TOOL

By

TAN CHEW YEE

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor Philosophy

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DEDICATION

To Dr. Ooi, you ignited my passion for swine medicine from my very first year, thank you for sharing your expertise, knowledge, and experiences with me. Your guidance have been invaluable throughout this entire process. In you, I found both a magnanimous mentor and a cherished confidante.

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unwavering faith in me and endless encouragement kept me going through tough times. My career path is now clearer with my Ph.D. completion, and I wish the same for you – a fresh start into a positive and happier life chapter!

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This thesis is a testament to all the precious love, encouragement and support that I am blessed with in my life. From the bottom of my heart, thank you!

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the Degree of Doctor Philosophy

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Porcine circoviruses (PCVs) are globally distributed viruses that significantly impact pig populations, with four recognized species: PCV1, PCV2, PCV3, and PCV4. All PCVs share a non-enveloped, icosahedral structure and a single-stranded, circular DNA genome of approximately 2000 nucleotides encoding two main proteins: the replication-associated (Rep) protein and the capsid (Cap) protein. The Cap protein, a major target for host immune responses, is critical for virus detection and pathogenesis studies. PCV1, identified in 1974 as a non-pathogenic contaminant in pig kidney cell cultures, is apathogenic. In contrast, PCV2 emerged in the 1990s as the causative agent of post-weaning multisystemic wasting syndrome (PMWS), a major disease complex that severely affects pig health. The high mutation rate of PCV2, estimated at 10^{-3} – 10^{-4} substitutions per site per year, enables rapid evolution and genotype shifts, such as the transition from PCV2b to PCV2d, making it the most extensively studied and epidemiologically significant species. PCV3 and PCV4, identified more recently, are linked to multisystemic diseases, including

respiratory, gastrointestinal, and cardiovascular symptoms, with pathogenicity confirmed through infectious clone studies.

In Malaysia, PCV2 remains highly prevalent, with an 83.78% detection rate in pig farms and 94.74% in clinically healthy finisher pigs sampled at abattoirs. This study also confirms the presence of PCV3 and PCV4 in Malaysia, marking the first detection of these species in the country's commercial pig population, with detection rates of 17.02% and 6.67%, respectively. Wild boar samples revealed 100% positivity for PCV2, but PCV3 and PCV4 were not detected, suggesting distinct transmission patterns between domestic and wild pig populations. Phylogenetic analyses revealed a significant genotype shift in PCV2, with most Malaysian strains now classified as PCV2d. Moreover, the findings suggest the introduction of PCV3 and PCV4 into Malaysia via international trade, as Malaysian strains share close phylogenetic relationships with those from neighbouring countries such as Thailand and distant regions like the United States. Interestingly, Malaysian PCV3 strains were phylogenetically related to bat-associated and starling circoviruses, while PCV4 strains were closely linked to bat and mink circoviruses, indicating potential interspecies transmission or common evolutionary origins.

This study provides a comprehensive update on the epidemiology of PCVs in Malaysia, emphasizing the endemicity of PCV2 and the emergence of PCV3 and PCV4. The development of advanced diagnostic techniques, including a chromogenic in situ hybridization (ISH) method for PCV3, offers a significant improvement in local diagnostic capabilities. The ISH technique facilitates the identification of PCV3 antigens within infected tissues and aligns well with PCR

test results, supporting its utility as a cost-effective diagnostic tool. This advancement complements existing molecular tools like qPCR and aids in addressing knowledge gaps in PCV3 and PCV4 detection and pathogenesis. The findings underscore the need for continued surveillance, improved diagnostics, and biosecurity measures to control PCV transmission and evolution. Applying management strategies developed for PCV2 to PCV3 and PCV4 could mitigate the potential risks posed by these emerging viruses. Further research into the pathogenicity and immune responses associated with PCV3 and PCV4 is crucial for safeguarding the Malaysian swine industry against evolving threats.

Keywords: Porcine circovirus 2 (PCV2), porcine circovirus 3 (PCV3), porcine circovirus 4 (PCV4), in situ hybridization (ISH), genotype shift

SDG: GOAL 12: Responsible Consumption and Production

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Doktor Falsafah

SIRKOVIRUS BABI DI MALAYSIA: CIRI-CIRI MOLEKUL DAN PERKEMBANGAN TEKNIK DIAGNOSTIK

Oleh

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Pengerusi : Ooi Peck Toung, PhD
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Sirkovirus babi (PCV) adalah virus yang tersebar secara global dan memberi kesan signifikan kepada populasi babi, dengan empat spesies yang dikenal pasti: PCV1, PCV2, PCV3, dan PCV4. Semua PCV mempunyai struktur ikosahedral tanpa sampul dengan genom DNA berbentuk bulat, beruntai tunggal, dan sepanjang kira-kira 2000 nukleotida. Virus PCV mengekod dua protein utama: protein replikasi (Rep) dan protein kapsid (Cap). Protein Cap, yang menjadi sasaran utama tindak balas imun perumah, adalah elemen penting dalam pengesanan virus dan kajian patogenesis. PCV1 dikenal pasti pada tahun 1974 sebagai pencemar tidak patogenik dalam kultur sel ginjal babi. Sebaliknya, PCV2 muncul pada tahun 1990-an sebagai agen penyebab sindrom kelesuan multisistem pasca-cerai susu (post- weaning multisystemic wasting syndrome, PMWS). Kadar mutasi PCV2 yang tinggi, dianggarkan pada 10^{-3} – 10^{-4} perubahan/tapak/tahun, membolehkan evolusi pantas dan perubahan genotip, seperti peralihan daripada PCV2b kepada PCV2d, menjadikannya spesies yang paling banyak dikaji dan signifikan dari segi

epidemiologi. PCV3 dan PCV4, yang dikenal pasti kebelakangan ini, dikaitkan dengan penyakit multisistemik, termasuk simptom pernafasan, gastrointestinal, dan kardiovaskular, dengan patogenisiti disahkan melalui kajian klon berjangkit.

Di Malaysia, PCV2 kekal epidemik dengan kadar pengesanan 83.78% di ladang babi dan 94.74% dalam kalangan babi penggemuk sihat yang disampel di rumah sembelih. Kajian ini juga mengesahkan kehadiran PCV3 dan PCV4 di Malaysia buat julung kali, dengan kadar pengesanan 17.02% dan 6.67% masing-masing. Sampel babi hutan menunjukkan 100% positif untuk PCV2, tetapi PCV3 dan PCV4 tidak dikesan, menunjukkan corak penularan yang berbeza antara populasi babi domestik dan liar. Analisis filogenetik mendedahkan perubahan genotip yang ketara dalam PCV2, dengan kebanyakan strain Malaysia kini dikelaskan sebagai PCV2d. Selain itu, penemuan ini mencadangkan bahawa pengenalan PCV3 dan PCV4 ke Malaysia berlaku melalui perdagangan antarabangsa, memandangkan strain Malaysia menunjukkan hubungan filogenetik yang rapat dengan strain dari negara jiran seperti Thailand dan wilayah yang lebih jauh seperti Amerika Syarikat. Menariknya, strain PCV3 Malaysia berkait rapat secara filogenetik dengan sirkovirus kelawar dan burung jalak, manakala strain PCV4 Malaysia berkait rapat dengan sirkovirus kelawar dan cerpelai, menunjukkan potensi penularan antara spesies atau asal-usul evolusi yang sama.

Kesimpulannya, kajian ini menyediakan maklumat terkini yang menyeluruh mengenai epidemiologi PCV di Malaysia, dengan penekanan kepada prevalens yang tinggi dan endemik yang berterusan bagi PCV2, serta

kemunculan PCV3 dan PCV4. Pembangunan teknik diagnostik, termasuk kaedah hibridisasi in situ kromogenik (ISH) untuk PCV3, dapat membangunkan keupayaan diagnostik tempatan. Teknik ISH ini memudahkan pengesanan antigen PCV3 dalam tisu yang dijangkiti dengan hasil pengesanan yang selaras dengan hasil ujian PCR, lantas menjadikannya alat diagnostik yang berkesan dan efektif dari segi kos. Kemajuan diagnostik ini melengkapkan kaedah diagnostik molekul sedia ada seperti qPCR dan membantu menangani jurang pengetahuan dalam pengesanan dan patogenesis PCV3 dan PCV4. Demi pengawalan penularan dan evolusi PCV, pemantauan berterusan, kaedah diagnostik yang lebih baik, serta langkah biosekuriti perlu dititikberatkan. Strategi pengurusan yang dibangunkan untuk PCV2 boleh diaplikasikan untuk menangani risiko wabak penyakit PCV3 dan PCV4. Penyelidikan lanjutan mengenai patogenisiti dan tindak balas imun PCV3 dan PCV4 wajar diberi tumpuan untuk melindungi industri ternakan babi Malaysia daripada ancaman yang semakin berkembang.

Kata Kunci: Sirkovirus babi 2 (PCV2), sirkovirus babi 3 (PCV3), sirkovirus babi 4 (PCV4), in situ hybridization (ISH), peralihan genotip

SDG: MATLAMAT 12: Penggunaan dan Pengeluaran yang Bertanggungjawab

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LIST OF ABBREVIATIONS

APP	*Actinobacillus pleuropneumoniae*
AASV	American Association of Swine Veterinarians
aa	Amino acid
BFDV	Beak and feather disease virus
Cap	Capsid
CSF	Classical swine fever
DIG	Digoxigenin
dN	Non-synonymous substitution rates per codon
dS	Synonymous substitution rates per codon
FMD	Foot and mouth disease
FEL	Fixed-effects likelihood
FFPE	Formalin-fixed, paraffin-embedded
FUBAR	Fast, unconstrained Bayesian approximation
GTR	General Time Reversible
HKY	Hasegawa–Kishono–Yano
Hx	Shannon entropy
IACUC	Institutional Animal Care and Use Committee
ISH	In-situ hybridisation
ICTV	International Committee on Taxonomy of Viruses
MCMC	Markov chain Monte Carlo
MEME	Mixed effects model of evolution
ML	Maximum likelihood
MT	Metric tonnes
NJ	Neighbour-joining
Nt	Nucleotide
OR	Odds ratio
ORF1	Open reading frames 1
ORF2	Open reading frames 2
ORF3	Open reading frames 3
PASC	Pairwise sequence comparison
PCR-RFLP	PCR-restriction fragment length polymorphism
PCV	Porcine circovirus

PCV2	Porcine circovirus 2
PCV3	Porcine circovirus 3
PCV4	Porcine circovirus 4
PCVAD	Porcine circovirus associated diseases
PDNS	Porcine dermatitis and nephropathy syndrome
PEDV	Porcine epidemic diarrhea virus
PMWS	Post-weaning multisystemic wasting syndrome
pp	Posterior probability
PPV	Porcine parvovirus
PRDC	Porcine respiratory disease complex
PRRS	Porcine reproductive and respiratory syndrome
PRV	Pseudorabies virus
RR	Relative risk
Rep	Replicase
SEM	Standard error of mean
SLAC	Single-likelihood ancestor counting
SOC	Super optimal broth with catabolite repression
ssDNA	Single-stranded DNA
ssy	Substitutions per site per year
TTSuV	Torque teno sus virus
TTSuV1	Torque teno sus virus 1

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Porcine circoviruses (PCVs) are members of the genus Circovirus of the family Circoviridae. To date, four species of PCVs have been identified and sequentially named porcine circovirus 1 (PCV1) to porcine circovirus 4 (PCV4) (Tischer *et al.*, 1982; Allan *et al.*, 1998; Palinski *et al.*, 2017; Zhang *et al.*, 2020b). PCVs are widespread in the pig population worldwide and have been known to cause a variety of diseases in pigs. Porcine circovirus 2 (PCV2) is the primary etiological agent associated with a group of complex multi-factorial diseases classified as porcine circovirus associated diseases (PCVAD). PCVAD can cause a wide range of clinical signs in pigs, including respiratory disease, reproductive problems, and porcine dermatitis and nephropathy syndrome (PDNS). Multisystemic manifestation of Porcine circovirus 3 (PCV3) infection has been reported to encompass from respiratory tract, gastrointestinal, nervous to cardiovascular systems (Phan *et al.*, 2016; Chen *et al.*, 2017; Ku *et al.*, 2017; Zhai *et al.*, 2017; Arruda *et al.*, 2019; Qi *et al.*, 2019; Hou *et al.*, 2020). An infectious PCV3 molecular DNA clone study has further substantiated the pathogenicity of PCV3 (Jiang *et al.*, 2019a). On the other hand, PCV4 has been linked to clinical signs of respiratory disease, enteritis and PDNS (Zhang *et al.*, 2020b). Similarly, infection clone study has confirmed the pathogenicity of PCV4 which manifested through multi-organ pathological changes, with cytokine and chemokine upregulation (Niu *et al.*, 2022).

Clinical and subclinical PCVAD infection in herds could result in stunted growth rates, spiked mortality rates, impaired reproductive performance, and poorer meat quality. Consequently, pig producers would be faced with substantial economic implications to cover increased biosecurity, herd health and production cost. The economic impact of PCVAD on the global swine industry was estimated to be in the millions of dollars each year (Alarcon *et al.*, 2013). Over the decades, various control measures have been implemented to mitigate PCVAD situation, especially vaccination programs, supported by stronger biosecurity and sound management practices (Opriessnig *et al.*, 2020). These collective efforts have reduced the severity of PCVAD but the evolving nature of PCVs will continue to be a challenge. The emergence of PCV3 and PCV4 with their evident virulence would be expected to redefine the scope of PCVAD and exacerbate the impact on swine industry.

In Malaysia, the epidemiological status of PCV2 has not been updated for more than a decade (Jaganathan *et al.*, 2011). There are also no studies done on the newer PCV strains that have surfaced in the recent years, i.e PCV3 and PCV4. Hence, the aim of this thesis is to investigate the recent molecular epidemiology of PCVs in Malaysia. The results of this study will provide important information about the epidemiology of PCVs in Malaysia and will help to inform the development of control strategies for the disease. Further, the findings of this study will be of interest to pig producers, veterinarians, and government officials in Malaysia to further fortify PCVAD control strategies. The results will also be of interest to researchers working on PCVs in other countries.

1.2 Justifications of Study

Porcine circoviruses in Malaysia warrant investigation for several crucial reasons. The primary motivation for this study is the persistent impact of PCV2, a major problem affecting the swine industry locally and globally. Despite the widespread use of vaccinations, PCV2-associated post-weaning multisystemic wasting syndrome (PMWS) persists in Malaysian farms, suggesting possible gaps in vaccine efficacy or implementation. Given the substantial economic losses attributed to PCV2 outbreaks, which can reach up to $100 million annually (Alarcon *et al.*, 2013), a comprehensive epidemiological reassessment of PCV2 in Malaysia is urgently needed following a decade-long gap in updates.

Secondly, this study broadens the scope of previous research by incorporating samples from both clinically healthy pigs and wild boars. Unlike prior studies in Malaysia that focused on pigs exhibiting PMWS symptoms, this expanded sampling provides a more comprehensive view of PCV2 prevalence and distribution. Sampling healthy finishers allows for a clearer picture of subclinical infections, while wild boar populations, which often interact with domestic pigs, are included to assess their role as viral reservoirs. Additionally, phylogenetic comparisons between wild and domestic PCV strains will provide insights into viral evolution and adaptability in natural reservoirs.

The high substitution rates of PCV2 strains, on par with RNA viruses, facilitate frequent genotype shifts and influence virulence, transmissibility, and immune escape potential. Genotypes PCV2a to PCV2f have been reported (Franzo &

Segalés, 2018). Globally, shifts in PCV2 genotypes, such as from PCV2a to PCV2b and later to PCV2d, have impacted disease spread and vaccine efficacy. Thus, regular updates on the genetic characteristics of Malaysian PCV2 strains are crucial for adapting disease management strategies to evolving viral strains, as changes in genotype could alter the virus's pathogenicity or impact vaccine effectiveness. Genotyping Malaysian strains will provide insights into local strain evolution and assess if Malaysia follows global trends, enabling more precise management of PCV2-related diseases and informing vaccine updates if necessary.

The discovery of novel PCV species, PCV3 and PCV4, which have been shown to be pathogenic, further underscores the need for this study (Palinski *et al.*, 2017; Zhang *et al.*, 2020b). Reports of PCV3 cases have risen worldwide, including in neighbouring Thailand, indicating its potential spread and prompting a need for understanding its prevalence in Malaysia before implementing intervention strategies. PCV4 has only been documented in a limited number of countries, with cases recently emerging in Southeast Asia. This study seeks to provide foundational data on the prevalence and molecular characterisation of PCV3 and PCV4 in Malaysia. Conducting a pilot study on PCV3 and PCV4 prevalence is essential before investigating their roles in PCVAD and potential impacts on Malaysia's swine industry. Additionally, this study will develop chromogenic in-situ hybridization (ISH) for PCV3 detection, enhancing local diagnostic capacity. ISH will allow for detailed tracking of infection patterns using a method that is accessible and feasible within Malaysia, facilitating improved surveillance and response strategies.

International trade of breeding stock, boar semen and pork products is a significant factor justifying this study. Malaysia's imports of these products, and strategic role as a regional trade and travel hub, expose local swine populations to potential new PCV strains. Conducting phylogenetic analyses of Malaysian strains in relation to international strains will clarify relatedness and assess the need for improved screening and quarantine protocols to protect local pigs. Furthermore, for newly identified viruses like PCV3 and PCV4, robust phylogenetic and classification studies depend on a broad range of strain data. This study aims to contribute Malaysian PCV3 and PCV4 genetic data to GenBank research databases, supporting researchers worldwide in furthering PCV phylogenetic analyses and aiding global tracking efforts.

In summary, this study aims to enhance the understanding of the prevalence and genetic diversity of PCVs in Malaysia while improving diagnostic capabilities within local research facilities. By addressing critical gaps in PCV epidemiology, the study has implications for disease management, biosecurity and research collaboration. The findings are expected to inform more effective disease management strategies and guide the development of proactive biosecurity policies, ultimately strengthening the Malaysian swine industry.

1.3 Research Objectives

The objectives for this research are listed as below:

i. To determine molecular prevalence of PCV2 and PCV3 in domestic pigs of Malaysian commercial pig farms across different age groups & health status;
ii. To determine molecular prevalence of PCV2 and PCV3 in Malaysian wild boar population;
iii. To determine the molecular presence and prevalence of PCV4 in Malaysia
iv. To molecularly characterize Malaysian isolates of PCV2, PCV3 and PCV4;
v. To develop a chromogenic in-situ hybridization (ISH) technique for the detection of PCV3.

1.4 Research Hypotheses

i. PCV2 and PCV3 is detected in domestic pig population of Malaysian commercial pig farms across different age groups & health status, at molecular prevalence of 65 – 75% and 15 – 25% respectively.
ii. PCV2 and PCV3 is detected in wild boar population of Malaysian commercial pig farms across different age groups & health status, at molecular prevalence of 30 – 40% and 5 – 15% respectively.
iii. PCV4 is detected in domestic pig and wild boar population of Malaysian at molecular prevalence of lower than 10%.
iv. Molecular characteristics of Malaysian isolates of PCV2, PCV3 and PCV4 share similarities with isolates from countries with geographical and trading relationship with Malaysia;

v. Chromogenic ISH technique could be developed for detection PCV3 antigens in PCV3 PCR positive samples.

1.5 Outline of Manuscripts

Six publications are included in this manuscript as listed below in accordance to the compilation sequence:

1.5.1 Article 1: Porcine Circoviruses in Malaysia

i. Porcine circoviruses
ii. Swine industry in Malaysia
iii. Porcine circovirus 2 (PCV2) in Malaysia
iv. Porcine circovirus 3 (PCV3) in Malaysia
v. Porcine circovirus 4 (PCV4) in Malaysia
vi. Interaction between PCVs and other porcine pathogens
vii. Conclusion

1.5.2 Article 2: What Do We Know About Porcine Circovirus 3 (PCV3) Diagnosis So Far?

i. Introduction
ii. Worldwide detection of PCV3
iii. Different samples and detection rate of PCV3
iv. PCV3 in wild boar and other non-porcine species
v. Clinical signs and lesions of PCV3
vi. Detection method of PCV3

vii. Isolation of PCV3

viii. Porcine circovirus 4 (PCV4)

ix. Conclusion

1.5.3 Article 3: Genotype Shift of Malaysian Porcine Circovirus 2 (PCV2) from PCV2b to PCV2d within a Decade

i. Introduction

ii. Materials and Methods

iii. Results

iv. Discussion

   a. Molecular detection of PCV2

   b. Pairwise sequence comparison (PASC)

   c. Phylogenetic analysis & genotyping

   d. Nucleotide sequence polymorphism analysis

   e. Selection pressure

v. Conclusions

1.5.4 Article 4: First Molecular Detection and Complete Sequence Analysis of Porcine Circovirus Type 3 (PCV3) in Peninsular Malaysia

i. Introduction

ii. Materials and methods

iii. Results

   a. Molecular prevalence of PCV3

   b. Nucleotide sequence and complete genome analysis

iv. Discussion

v. Conclusion

1.5.5 Article 5: Chromogenic In-Situ Hybridisation Technique For The Detection of Porcine Circovirus 3 (PCV3)

i. Introduction

ii. Materials and methods

iii. Results and discussion

iv. Conclusion

1.5.6 Article 6: First Molecular Detection of Porcine Circovirus Type 4 (PCV4) in Malaysia

i. Introduction

ii. Materials and methods

iii. Results and discussion

iv. Conclusion

CHAPTER 2

LITERATURE REVIEW

2.1 Porcine Circoviruses in Malaysia (Article 1)

Over one decade since the first report, Malaysian porcine circovirus 2 (PCV2) molecular detection rates remain high at 83.78% and 83.54% for farm and sampled domestic pig population levels respectively. Clinically healthy finishers also showed a high detection rate of 94.74%. Most notably, a major genotype shift from genotype PCV2b to PCV2d was observed. PCV2 antigen was also detected in Malaysian wild boar population, sharing significant nucleotide identity similarities with PCV2 strains of domestic pigs. Porcine circovirus 3 (PCV3), which was discovered not too long ago in 2017, has been confirmed to be present among Malaysian commercial pig population at a molecular prevalence of 41.67% at farm level and 17.02% at sampled domestic pig population level respectively. Most recently, porcine circovirus (PCV4) had been detected in Malaysia, albeit at a much lower molecular prevalence of 4.08%. Both PCV4 positive samples originated from clinically healthy finishers. Phylogenetic analyses suggested the possibility of PCV3 and PCV4 being introduced by international trade activities. For practical and economic reasons, in the local Malaysian pig industry, field diagnostics of PCV2 cases generally rely on clinical and post mortem findings, together with herd antibody titer and qPCR viremia titer. Currently, commercial PCV3 diagnostics service and vaccines are not available locally. With updated local PCV2 epidemiology, control and management effort could be adapted into more effective strategies. Common management strategy of PCV2 challenge

in farms could be applied for the time being to control potential PCV3 problems in the farms. As for PCV4, a larger number of field samples from cases of different clinical manifestations needs to be included to obtain a more accurate epidemiological picture.

2.1.1 Porcine Circoviruses

Porcine circovirus type 1 (PCV1) was first discovered in 1974 as an apathogenic cell culture contaminant (Tischer *et al.*, 1982). Two decades later, the first clinical outbreak of postweaning multisystemic wasting syndrome (PMWS) was reported and porcine circovirus 2 (PCV2) has become ubiquitous in the swine population since then (Harding, 1996). The American Association of Swine Veterinarians (AASV) coined the term porcine circovirus associated diseases (PCVAD) to collectively describe PCV2-related multi-factorial diseases (AASV, 2022). Currently, knowledge gaps in PCV2 research include differences in host response to PCV2 infection and possible immunological differences among PCV2 genotypes (Opriessnig *et al.*, 2020). Another two decades from the report of PCV2, porcine circovirus type 3 (PCV3) was uncovered in a field investigation of sow reproductive issue presented with porcine dermatitis and nephropathy syndrome (PDNS)-like syndrome, and in cases of myocarditis and multi-organ inflammation (Phan *et al.*, 2016; Palinski *et al.*, 2017). Most recently, porcine circovirus type 4 (PCV4) has been identified in both clinically ill and healthy pig population (Zhang *et al.*, 2020b; Nguyen *et al.*, 2022; Sirisereewan *et al.*, 2023; Tan *et al.*, 2023b). Association with clinical signs of respiratory disease, enteritis and PDNS has been suggested (Zhang *et al.*, 2020b). To date, PCV4 has yet to be reported out of

Asia (Franzo *et al.*, 2020b; Wang *et al.*, 2022). Pathogenicity of both PCV3 and PCV4 has been confirmed through infectious clone studies reporting multi-organ pathological changes, with cytokine and chemokine upregulation (Jiang *et al.*, 2019a; Niu *et al.*, 2022)

2.1.2 Swine Industry in Malaysia

Pig farming in Malaysia started off as backyard subsistence farming of the early Chinese settlement community. Over time, pig rearing evolved into a commercialized pig farming industry managed as public or private limited companies. The main driving force in the industry is the pork consuming population of Malaysia, making up roughly 40% of the 32 million total country population. In 2020, at a standing pig population of 1.8 million heads in over 470 farms, the pig sector ranked second in the livestock commodity segment. Ex-farm production of pork output in 2021 was valued to be RM4,066.62 million, representing a 17.7% proportion of the livestock industry. However, with smaller-scaled farms closing down to economic viability factor and the recent African swine fever (ASF) wave, as of January 2022, the pig industry has scaled down to 1.3 million SPP with estimated 430 farms in operation. The actual number may be lower as ASF and increasing production challenges continue to hit the swine farming community. Self-sufficiency of pork supply is expected to be lower than the 91.62% estimated in 2020. Currently, main pig farming states in Malaysia include Perak, Penang, Selangor, Johor, Sabah and Sarawak. Close to 50% of the pig farms are operating as small to medium-scale commercial farms with 100 – 2000 SPP (FLFAM, n.d.; Hassuzana *et al.*, 2004; Singh and Fong, 2014; DVS, 2022). Most farms adopt an open house

farrow-to-finish system with similar husbandry management, though large farms with SPP above 2000 tend to practice stricter biosecurity. Three-way commercial cross of Duroc, Landrace and Yorkshire remains the primary breed. General vaccination protocol includes but not limited to vaccinations against porcine circovirus 2 (PCV), *Mycoplasma hyopneumoniae* (Mhyo), porcine reproductive and respiratory syndrome (PRRS), classical wine ever (CSF), foot and mouth disease (FMD) and *Actinobacillus pleuropneumoniae* (APP).

2.1.3 Porcine Circovirus 2 (PCV2) in Malaysia

The Malaysian Veterinary Research Institute reported the first PCV2 case in 2004 (Ooi *et al.*, 2007). The pigs with respiratory problems were found to have interstitial pneumonia and lymphangitis. PCR-restriction fragment length polymorphism (PCR-RFLP) technique was used to confirm presence of PCV2 antigen in the pooled organ samples. An 83.33% (10 / 12 pigs; 5 / 6 farms) PCV2 positive detection rate was reported in a PMWS farm investigation in northern Malaysia (Jaganathan *et al.*, 2011). The pigs, aged between eight to 12 weeks of age, showed typical PMWS clinical signs of emaciation, retarded growth, pallor and visible enlargement of superficial inguinal lymph nodes. Microscopic findings of the PCV2 positive samples include lymphoid cells depletion, histiocytic infiltration of lymphoid tissue and interstitial pneumonia with mononuclear cell infiltration. This farm study had fulfilled the three-pronged case definition of PMWS: clinical signs, histologic lesions and PCV2 antigen within characteristic lesions. A wider PCV2 study covering 42 farms from Penang, Perak, Selangor, Melaka, Johor and Sarawak states reported

88.10% (37/42 farms) positive for PCV2 PCR detection (Seetha *et al.*, 2011). Sample population was still focused on pigs displaying typical PMWS clinical signs.

A recent PCV2 study reported a similar level of prevalence: 83.78% (31/37 farms) at farm level and 83.54% (66/79 pigs) in sampled domestic pig populations (Tan *et al.*, 2022). The sample population of this study expanded into clinically healthy finishers and wild boars. A relatively high molecular detection rate of 94.74% (18/19 pigs) was reported from abattoir samples originating from clinically healthy finishers. Previously, presence of PCV2 strains in Malaysia was only reported in clinically ill domestic pig population. PCV2 was also detected in clinically ill pigs from the same farms that supplied the said abattoirs. In addition, detection across all production age groups from foetus, piglet, weaner, grower to sow was reported in this study, reiterating that PCV2 infection can occur across all pig production age groups. Potential PCV2 transmission amongst swine herds through apparently healthy animals; and infectious and non-infectious factors to trigger PCV2 replication up to an extent of overwhelming the immune system would require further investigation.

Notably, PCV2 antigen was detected in all (28/28) of the tested Malaysian wild boar organ samples. Clades of Malaysian wild boar sequence interspersed among clades of domestic pig sequences, sharing at least 80.7% of nucleotide identity similarities. This finding could suggest a possible epidemiological link between wild boar and domestic pig populations of Malaysia. Pig farms in Malaysia are remotely located in the vicinity of forests and oil palm plantations. Moreover, the geographical location of Peninsular Malaysia is intersected by

wild boar migratory routes. Direct contact between the domestic pigs and wild boars, or indirect contact through fomites such as leftover feed are potential routes of disease transmission.

Previously, phylogenetic analysis of Malaysian PCV2 isolates classified all isolates (13/13 sequences) as PCV2b strain (Seetha *et al.*, 2011). More than one decade later, a genotype shift from genotype PCV2b to PCV2d (76/79 sequences) was observed (Tan *et al.*, 2022). This observation coincides with the global PCV2 epidemiological dynamics, where the emergence and spread of genotype PCV2d is set in motion to replace PCV2b (Grau-Roma *et al.*, 2008). Phylogenetic clusters of Malaysian PCV2d strains shared clades with PCV2d strains from numerous countries, including Canada, Denmark, France, Sweden, South Korea and U.S., countries from which Malaysia imported breeders since as early as back in 2000. Phylogenetic relationship was also shared among Malaysian strains with Thailand, Vietnam and China strains. Hence, it may be speculated that the phylogenetic relatedness might be a result of trade activities involving live breeder, semen stock and porcine products (Tan *et al.*, 2022). The Malaysian PCV2 study reported an estimated substitution rate of 1.102×10^{-3} substitutions/site/year (ssy), in agreement with previous global reports of PCV2 displaying very high substitution rates close to RNA viruses. Furthermore, co-existence of different PCV2 strains circulating in a same farm, and even within one individual pig was observed. This could serve as potential for viral recombination. Phylogenetic analysis also revealed strain clustering patterns that occur over time (Tan *et al.*, 2022). Hence, it is

speculated that PCV2 strains in Malaysia could continue to mutate under high substitution rates and selective pressures.

In Malaysian commercial pig farms, the most commonly observed porcine circovirus associated disease (PCVAD) is PMWS, with peak clinical prevalence two to three weeks post-weaning and often complicated with porcine reproductive and respiratory syndrome (PRRS) viral infection. Management program of PCV2 challenge in farms includes routine PCV2 vaccination, herd PCV2 serology monitoring and isolation of sick animals exhibiting PMWS clinical signs. PCV2 vaccination is widely practiced, with different farms adopting different strategies: sow vaccination, piglet vaccination, or sow and piglet vaccination. The strategy and timing of vaccination is often adjusted based on herd PCV2 serology profiling and anticipated PCV2 field challenge. Currently available commercial PCV2 vaccines, including Circovac® (Ceva), Ingelvac CircoFLEX® (Boehringer Ingelheim), Fostera® PCV (Zoetis) and Porcilis® (MSD). Positive outcomes of implementing PCV2 vaccination in local farms have been documented (Lim *et al.*, 2007; Kam *et al.*, 2013; Shen *et al.*, 2014; Wen *et al.*, 2015; Ng *et al.*, 2022). For practical and economic reasons, field diagnostic of PCV2 cases often rely on clinical findings of stunted growth, pallor, respiratory difficulties, increased mortality; post mortem findings of generalized lymphadenopathy and interstitial pneumonia in affected weaners. In recent years, serology antibody titer indication of suspected PCV2 challenge and qPCR detection of PCV2 viremia titer are increasingly employed for more accurate diagnosis.

2.1.4 Porcine Circovirus 3 (PCV3) in Malaysia

PCV3 has been confirmed to be present among Malaysian commercial pig populations, at a molecular prevalence of 41.67% (10/24 farms) at farm level and 17.02% (24/141 pigs) in sampled domestic pig population (Tan *et al*, 2020). PCV3 antigen has been detected in all nine organ types included in the study: lung, spleen, tonsil, kidney, heart, liver, brain, inguinal and mesenteric lymph nodes. PCV3 was most frequently detected in inguinal lymph node and lung samples, suggesting potential tissue tropism in relation with PCV3 pathogenesis. On the other hand, similar to PCV2, PCV3 was also detected across all production stages from foetus, piglet, weaner, finisher to sow. However, while PCV2 was detected in all the sampled clinically healthy finishers, none of them were positive for PCV3. As opposed to the global report of 23% – 42.66% PCV3 molecular prevalence in wild boar population, PCV3 has yet to be detected among Malaysian wild boars (Ouyang *et al*, 2019b; Tan *et al*, 2020). This finding may suggest that wild boar spillover infection is not implicated in introduction of PCV3 into Malaysian commercial swine population. Malaysian PCV3 strains shared a closer phylogenetic relationship with PCV3 strains from Spain, the U. S. and Mexico. Since 2001, Malaysia imports live breeders from the U.S., from 30 to 200 heads annually (DVS, 2022). The U.S. is also the main provider of live swine imports for Mexico, with a 72% share of the Mexican import market (Lara & Kuypers, 2019). Hence, it may be speculated that the phylogenetic relatedness among Malaysian, Spanish, U.S. and Mexico PCV3 strains may be linked to live breeder and semen stock movement. Phylogenetic relatedness with Italy, Thailand, Brazil, Japan and Germany, may be related to trade activities involving porcine

products in the past 10 years (DVS, 2022]. As with PCV2 described in the previous section, Malaysian PCV3 strains also showed a similar pattern of widening phylogenetic distance gap over time, suggestive of rapid mutation.

Six years into its discovery, commercial PCV3 antibodies are still not widely available possibly due to limited virus isolation, thus restricting their subsequent application in immunohistochemistry (IHC) and immunofluorescence assay (IFA) technique (Mora-Díaz *et al.*, 2020; Oh & Chae, 2020; Kroeger *et al.*, 2022). Similarly in Malaysia, there are no commercially available PCV3 diagnostics services and vaccines in the Malaysian pig industry. Locally, a chromogenic in situ hybridisation (ISH) technique using DIG-labeled probes targeting PCV3 ORF1 to detect PCV3 antigens in lung and lymphoid tissues had been developed (Tan *et al.*, 2023a). At early stages of PCV3 epidemiological investigation, ISH can complement PCR in detection studies for the localization of antigens in infected tissue sections. For the time being, ISH could be the economically feasible and practical option for local smaller-scale research or diagnostics laboratories.

2.1.5 Porcine Circovirus 4 (PCV4) in Malaysia

PCV4 was confirmed to be present in Malaysia at a detection rate of 4.08% (2/49 pigs), much lower compared to the most recent Malaysian PCV2 and PCV3 molecular prevalence (Tan *et al.*, 2022; Tan *et al.*, 2023b). To date, geographical distribution of PCV4 is still confined to Asian countries (Franzo *et al.*, 2020b; Wang *et al.*, 2022). Following China, South Korea and Thailand, Malaysia is the fourth country to have detected the presence of PCV4.

Malaysia does share trading relationships with the three aforementioned countries involving pork products importation of over 11,000 metric tonnes (MT) of pork products from year 2011 to 2018 (DVS, 2022). Hence, similar to the PCV3 situation, the relationship among Malaysia, China and South Korea PCV4 strains could also be associated with trade activities. Nonetheless, involvement of breeders and semen importation could not be ruled out from transboundary transmission of PCV4 into Malaysia. Similar to PCV2 and PCV3, PCV4 had been detected from pigs with a myriad of disease presentation ranging from respiratory, enteric and PDNS signs; and, in clinically healthy pigs including testicles of piglets (Zhang *et al.*, 2020b; Ha *et al.*, 2021; Tian *et al.*, 2021; Nguyen *et al.*, 2022). Both Malaysian PCV4 strains were detected from clinically healthy finishers. For a more accurate epidemiological picture, more field cases of different clinical manifestations need to be sampled. Overall, the current knowledge gap of global PCV4 genetic information and scarce number of Malaysian sequences impede further definitive interpretation of epidemiological and phylogenetic observations.

2.1.6 Interaction Between PCVs and Other Porcine Pathogens

Co-infection rate of PCV2 and PCV3 among the samples tested in the Malaysian study was 28.77% (21/73 samples). PCV3 positive animals could be 25% more likely to be positive for PCV2 (Tan *et al.*, 2020). Notably, one sample was positive for all PCV2, PCV3 and PCV4 (Tan *et al.*, 2023b). Co-infection of PCV2 with other common porcine pathogens have been extensively reported, including but not limited to PRRSV, pseudorabies virus

(PRV), porcine epidemic diarrhea virus (PEDV), torque teno sus virus (TTSuV), porcine parvovirus (PPV) and *Mhyo* (Ellis *et al.*, 2004; Grau-Roma, Fraile & Segalés *et al.*, 2011; Opriessnig & Halbur 2012*;* Ouyang *et al.*, 2019a). A similar co-infection case scenario was observed with PCV3 and PCV4. Most published PCV3 observations were made under field conditions and with frequent coinfections detected (Klaumann *et al.*, 2018a). In the earlier PCV3 discovery report, porcine astrovirus 4, porcine rotavirus A, porcine cytomegalovirus and porcine hemagglutinating encephalomyelitis virus were co-detected (Phan *et al.*, 2016).

However, it should be noted that in the other initial report, no viruses other than PCV3 were detected from sow samples with PDNS-like clinical signs (Palinski *et al.*, 2017). As more studies surfaced, high coinfection rates of PCV3 with similar aforementioned porcine pathogens were reported, as high as 61.54% and 61.54% with PCV2 and PRRSV respectively (Ouyang *et al.*, 2019a; Sukmak *et al.*, 2019). As for PCV4, co-infections with PCV2, PCV3, or both have been described in pigs with clinical signs of PMWS, PDNS and reproductive failure. Co-infection with PRV, PEDV and PRRSV in pigs with neurological symptoms, encephalitis, diarrhea and enteritis were also reported (Zhang *et al.*, 2020b; Sun *et al.*, 2021; Tian *et al.*, 2021; Hou 2022).

Several studies have demonstrated PCV2 to be the primary and necessary aetiological agent in PCVAD pathogenesis (Ellis *et al.*, 1999; Bolin *et al.*, 2001; Ladekjær-Mikkelsen *et al.*, 2002). There is no distinct co-pathogen that can be solely attributed to increasing the severity of PCVAD, instead it is speculated that different co-infecting aetiological agents utilize similar mechanisms which

result in enhancement of PCVAD (Opriessnig *et al.*, 2012). *Mhyo* potentiates the severity of PCV2-associated lung and lymphoid lesions by increasing the amount and prolonging the presence of PCV2-antigen (Opriessnig *et al.*, 2012). New cellular DNA synthesis has been reported to be the only in vivo prerequisite for PCV2 replication, suggesting that PCV2 requires support for activation of host cell replication (Kennedy *et al.*, 2002; Opriessnig *et al.*, 2012). Given that monocytes and macrophages are common cellular tropism for PCV2 and PRRS, co-infection with PRRS may potentiate PCV2 replication (Allan *et al.*, 2000). Host cytokine response could be altered to favour PCV2 upregulation (Meerts *et al.*, 2005). Increase IFN-γ in pulmonary alveolar macrophages and bronchial lavage fluid in response to PRRSV and *Mhyo* infection could then contributed to PCV2 upregulation (Thanawongnuwech & Thacker, 2003; Opriessnig *et al.*, 2012). Since PCV2 replication involves actively replicating cells, co-infecting agents such as PPV that induce cell apoptosis and subsequent regeneration of damaged tissue may indirectly enhance the replication of PCV2 (Meehan *et al.*, 1998; Ellis *et al.*, 2004). Other known host-pathogen interaction mechanism including inducing immune suppression, affecting macrophage function and interfering with pathogen clearance may contribute similarly to PCV2 and PCVAD pathogenesis (Chiou *et al.*, 2000; Jung *et al.*, 2009; Renukaradhya *et al.*, 2010; Sinha *et al.*, 2010). Current understanding of primary PCV3 clinical outcome points towards multi-organ inflammation. Three major pathogenesis mechanisms culminating to pathogenesis of multiorgan injuries by PCV3 infection have been proposed, i.e., virus-induced apoptosis, type III and type IV hypersensitivity (Sirisereewan, Thanawongnuwech & Kedkovid, 2022). Both PCV2 and PCV3

predominantly infect macrophages in lymphoid tissues (Palinski *et al.*, 2017; Jiang *et al.*, 2019a). To date, there is still no data regarding whether PCV3 could be upregulated, whether pigs are immunosuppressed or immunomodulated, or co-infection involving PCV3 is independent of the immune system affection (Klaumann *et al.*, 2018a). On the other hand, positive rate and viral DNA loads of PCV2 and PCV4 were found to be higher in spleen and lymph nodes. Considering this similar immune tissue tropism between PCV4 and PCV2, PCV4 may act as a co-factor for PCV2 (Xu *et al.*, 2022).

2.1.7 Conclusion on Porcine Circoviruses in Malaysia

To recap, Malaysian PCV2 molecular detection rates remain high at 83.78% and 83.54% for farm and sample population level respectively. Clinically healthy finishers also showed a high detection rate of 94.74%. Considering these high detection rates at farm and abattoir levels, it could be beneficial to investigate both subclinical and clinical PCVAD more thoroughly at the farms. Confirmation of PCV2 detection in Malaysian wild boar population warrants consideration of wild boars as potential PCV2 reservoir, especially when the Malaysian wild boar PCV2 sequences were found to share substantial genetic similarities with Malaysian domestic pig sequences. Most importantly, in face of an apparent genotype shift from genotype PCV2b to PCV2d, control and management effort must be adapted to the latest Malaysian PCV2 and PCVAD situation.

Presence of PCV3 and PCV4 has been confirmed in Malaysia, with molecular detection rates of 17.02% and 4.08% respectively. Phylogenetic analyses

suggested the possibility of these novel virus introduction through international trade activities. With the exception of vaccination, common management strategy of PCV2 challenge in farms could be applied to nip potential PCV3 and PCV4 problems at the bud. At national level, biosecurity screening procedures during live breeders and pork products importation may need to be revised to be more inclusive of emerging diseases. Continuous epidemiological studies are required to investigate the prevalence of novel porcine circoviruses in commercial pigs both healthy and ill, and also in wild boar population.

The future trend of pig farming in Malaysia is planned in the direction of Modern Pig Farming (MPF) and Pig Farming Areas (PFAs) schemes. Traditional farms are expected to transition in line with MPF, achieving a close house farming system with zero discharge or low-polluting effluent, sufficient buffer zone from residential areas and good animal husbandry practices. One step further, in PFAs, modern pig farms would consolidate with centrally managed facilities including biosecurity, breeding, waste management and abattoir units. Preventing, diagnosing and managing endemic disease challenges, such as PCVAD, should be one of key focus aspects in working towards MPF and PFA.

2.2 What Do We Know About Porcine Circovirus 3 (PCV3) Diagnosis So Far? (Article 2)

PCV3 was first discovered in 2016, almost concomitantly by two groups of researchers in the U.S. The novel case was reported in a group of sows with chronic reproductive problems with clinical presentation alike PDNS, where metagenomic sequencing revealed a genetically divergent porcine circovirus

designated PCV3. The discovery of PCV3 in a PDNS case, which used to be considered as part of PCVAD attributed to PCV2, has garnered attention and effort in further research of the novel virus. Just when an infectious molecular DNA clone of PCV3 has been developed and successfully used in an in vivo pathogenicity study, yet another novel PCV strain surfaced, designated PCV4. So far, PCV3 has been reported in the domestic swine population globally at low to moderate prevalence, from almost all sample types including organ tissues, faecal, semen and colostrum samples. PCV3 has been associated with a myriad of clinical presentations, from PDNS to PRDC. This review paper summarizes the studies on PCV3 to date, with focus on diagnosis.

2.2.1 Introduction

Circoviridae family consists of small (1.7 – 2.1 kb), circular, covalently closed, non-enveloped, single-stranded DNA (ssDNA) viruses (Breitbart *et al.*, 2017; Rosario *et al.*, 2017). They are among the smallest autonomously replicating viruses known (Biagini *et al.*, 2012; Rosario *et al.*, 2017). PCVs are members of the genus *Circovirus* of the family *Circoviridae*. Up till 2019, four species of PCVs have been identified. PCV1 was discovered in 1982 (Tischer *et al.*, 1982), followed by report of PCV2 in 1998 (Allan *et al.*, 1998). Two decades later, PCV3 was detected (Palinski *et al.*, 2017) and most recently in 2019, PCV4 became the newest PCV member (Zhang *et al.*, 2020b). International Committee on Taxonomy of Viruses (ICTV) defines a distinct *Circovirus* species based on sequence similarity: a novel circovirus must share <75% nucleotide (nt) identity over its entire genome and <70% amino acid (aa) identity of its Cap protein with other species in the genus (Rosario *et al.*, 2017).

PCV1 and PCV2 shares <80% similarity of overall nt identity, 86% and 65% similarity of ORF1 and ORF2 aa identity respectively (Meehan *et al.*, 1998; Morozov *et al.*, 1998; Gibbs *et al.*, 1999). PCV3 shares even lower similarity of only <50% of overall nt identity, 48% and 26 – 36% of ORF1 and ORF2 aa identity respectively with PCV2 (Phan *et al.*, 2016; Palinski *et al.*, 2017; Rosario *et al.*, 2017). This further supports the establishment of PCV3 as a novel porcine circovirus distinct from PCV1 and PCV2 (Biagini *et al.*, 2012; Palinski *et al.*, 2017). Porcine circovirus-associated diseases (PCVAD) of importance include PMWS, PRDC and PDNS. The pilot case of PCV3 was detected with concurrent Torque teno sus virus 1 (TTSuV1) infection of unknown pathogenic significance, with subsequent co-detection cases surfacing with high co-detection rates of 73.5% – 83.8% (110/132) (Palinski *et al.*, 2017; Ye *et al.*, 2018; Zheng *et al.*, 2018a). More importantly, co-infection rates of PCV3 and PCV2 were reported to range from 15.8% (35/222) to 70% (28/40) (Ku *et al.*, 2017; Ha *et al.*, 2018; Klaumann *et al.*, 2018b; Wen *et al.*, 2018; Zhao *et al.*, 2018; Dal Santo *et al.*, 2020; Guo *et al.*, 2020). Co-infection of PCV3 with other reproductive related pathogens including PRRSV, Ungulate protoparvovirus 1 (Porcine parvovirus, PPV) and *Leptospira* spp. has been reported (Ha *et al.*, 2018; Klaumann *et al.*, 2018b; Dal Santo *et al.*, 2020). However, it is postulated that occurrence of co-infections may be simply a reflection of widespread nature of major porcine pathogens such as PRRSV and PCV2, without an actual potential synergism or association with PCV3 (Franzo *et al.*, 2018b; Klaumann *et al.*, 2018b). Very low co-infection rates of PCV2 and PCV3, at 3% (16/624) to 7.5% (3/40) could also suggest independent circulation patterns of PCV2 and PCV3 (Saporiti *et al.*, 2020b; Chang *et al.*, 2021). On the other hand,

contrasting evidence where co-infection of PCV3 and PRRSV was found in 20.7% (29/140) of weaners to finishers showing severe respiratory clinical signs, in stark contrast of 0% co-infection cases in pigs with mild or no clinical signs has been reported (Zhai *et al.*, 2017). The possibility of synergism between PCV3 with other aetiological agents in affecting disease severity warrants further investigation (Zhai *et al.*, 2017).

2.2.2 Worldwide Detection of PCV3

PCV3 was first discovered in 2016 with metagenomic sequencing in domestic swine population in the U.S., in cases associated with reproductive failure and PDNS; cardiac and multi-organ inflammation respectively (Phan *et al.*, 2016; Palinski *et al.*, 2017). Thereafter, PCV3 has been detected, either retrospectively or presently, in Brazil (Tochetto *et al.*, 2018), China (Fan *et al.*, 2017), Italy (Faccini *et al.*, 2017), Poland (Stadejek *et al.*, 2017), South Korea (Kwon *et al.*, 2017), U.K. (Collin *et al.*, 2017), Denmark (Franzo *et al.*, 2018a), Japan (Hayashi *et al.*, 2018), Russia (Yuzhakov *et al.*, 2018), Spain (Klaumann *et al.*, 2018b), Sweden (Ye *et al.*, 2018), Thailand (Kedkovid *et al.*, 2018a), Vietnam (Nguyen, *et al.*, 2018), Colombia (Vargas-Bermudez *et al.*, 2019), German (Prinz *et al.*, 2019), Hungary (Deim *et al.*, 2019), Argentina (Serena *et al.*, 2020), India (Bera *et al.*, 2020), Malaysia (Tan *et al.*, 2020), Serbia (Savic *et al.*, 2020), Slovenia (Plut *et al.*, 2020) and Taiwan (Zeng, 2018).

Worldwide distributed strains from various countries interspersed in phylogenetic trees (Franzo *et al.*, 2018a; Ha *et al.*, 2018). Despite a broad global distribution involving massive circulation in numerous countries,

qualitative evaluation of major clades in many studies reached a consensus that PCV3 does not show specific geographical spatial clustering pattern (Klaumann *et al.*, 2018b; Franzo *et al.*, 2019a; Franzo *et al.*, 2020a). The rising detection reports of PCV3 could either be a result of increased pathogenic role of PCV3, or merely an outcome of wider viral circulation in a larger and more connected animal population (Franzo *et al.*, 2019a). In relation to the latter possibility, a long-lasting viral circulation was suggested for PCV3, rather than a recent emergence with subsequent progressive introduction in different countries (Franzo *et al.*, 2019a). A worldwide PCV3 circulation leading to multiple introduction events in different European countries followed by independent local evolution was proposed based on the broad mixing of strains from different countries and continents (Franzo *et al.*, 2018a). These assumptions are similar to that of being described for PCV2 (Franzo *et al.*, 2015b). On the other hand, haplotype networks analysis identified haplotypes of the U.S., South Korea, China and Brazil as corresponding to groups of viral isolates sharing identical ORF2 sequences. This observation also supports the suggestion that PCV3 is spreading globally, through the four dispersal routes. Trade flows among U.S., South Korea, China and Brazil that are potentially related to the dispersal routes of PCV3 could be identified, especially export flows from the U.S. to these countries from 2006 – 2016 (Saraiva *et al.*, 2018). Active PCV3 sequencing efforts and over representation of Chinese PCV3 strains, in contrast to the relatively low number of countries with available sequences of PCV3 isolates, may be a confounding factor in these findings (Saraiva *et al.*, 2018; Franzo *et al.*, 2019a).

2.2.3 Different Samples and Detection Rate of PCV3

PCV3 has been detected across all age groups of domestic pigs. In foetal cases, it is difficult to ascertain the exact time of infection, given the foetal time of exposure is secondary to dam viremia and the infection source could either be placenta or adjacent foetuses. It was suggested that in utero foetal death due to PCV3 was between 45 and 70 days of gestation, based on mummified foetus crown-rump length of 7 – 17cm (Arruda *et al.*, 2019). PCV3 has been reported in weaners of age 3 – 10 weeks old, with highest prevalence in the 6 weeks old group (Arruda *et al.*, 2019). A trend of positive PCV3 detection frequency increasing from piglets to weaners and then declining in older animals was observed (Franzo *et al.*, 2018b). An epidemiological study in Japan reported suckling piglets of 3 – 4 weeks old to have the highest PCV3 prevalence of 13.3% (2/15), followed by weaners of 5 – 8 weeks old at 9.0% (1/11) and growers-finishers above 9 weeks old at 9.4% (3/32) (Hayashi *et al.*, 2018). Similarly in Korea, growing (42.6%) and finisher (41.1%) pig groups had relatively lower prevalence than the weaned group (49.3%) (Kwon *et al.*, 2017). In Poland, it was reported that PCV3 was most frequently detected in serum pools of weaners (26.1%, 12/46) and finishers (28.0%, 33/118). However, a longitudinal study sampling pigs from weaning until the end of the fattening period concluded that PCV3 infection did not appear to be associated with any specific age group as PCV3 was detected across all ages, and that long-term infection spanned from 4 to 23 weeks (Klaumann *et al.*, 2019b). From a prevalence study involving serum samples of sows from first to fourth parity, it was found that PCV3 virus titre peaked at second parity then continuously declined until fourth parity, suggesting possible sow-to-piglet

transmission (Kedkovid *et al.*, 2018b). Shedding of PCV3 by sows has been reported with a prevalence of 44.74% (17/38) in colostrum samples, and 47.37% (18/38) in serum samples. Primiparous sow groups showed high prevalence of PCV3 shedding in colostrum, with up to 71% positive animals, compared to 33 – 43% in multiparous sows (Kedkovid *et al.*, 2018b). Another study reached a similar conclusion, that primiparous sows had a significantly higher number of PCV3-infected foetuses with higher PCV3 viral loads compared to multiparous sows (Saporiti *et al.*, 2020c).

PCV3 has been detected in both clinically healthy and ill pigs. Overall, detection rates in clinically ill pigs were moderate to high, as tabulated in Table 1 and Table 2. On the other hand, detection rates in clinically healthy pigs vary greatly from 0% (0/18; 0/42) (Qi *et al.*, 2018; Tan *et al.*, 2020), 6.7% (4/60) (Saporiti *et al.*, 2020a) to 90% (9/10) (Ye *et al.*, 2018). There are studies which reported homogeneous PCV3 frequency across different production phases and/or clinical health, with no statistically significant differences found among tested groups (Klaumann *et al.*, 2018b; Saporiti *et al.*, 2020a). Nevertheless, frequent detection of PCV3 in healthy animals could contribute to a wide, seemingly undetected and uncontrolled circulation of PCV3 (Franzo *et al.*, 2019a). A summary of sample types and detection rates of PCV3 was compiled into Table 1 and Table 2. The literatures cited in the Table 1 and Table 2 has been organized first by region, and then chronologically within each region from older to more recent sources. Table 1 focuses on studies involving organ samples, while Table 2 summarizes studies using non-organ samples such as serum and oral fluids. This structured arrangement allows for

a clearer understanding of regional and temporal trends in PCV research across different sample types.

Table 1: Detection Rates of PCV3 in Different Tissue Sample Types

Country		Sampling Population	Tissue type	PCV3 Molecular Detection Rate	Reference
Asia	China	Farms with congenital-tremor affected ≤ 3-day old piglets	Lung, spleen, liver, kidney, heart, brain	Higher copy numbers in heart, brain	Chen *et al.* (2017)
		Pigs with reproductive failure	Lung, lymph node, brain	85.71% (12/14)	Fan *et al.* (2017)
		Farms with reproductive issues (stillborn, sow mortality and acute loss of neonatal piglets)	Stillborn, tissues, semen, serum	34.7% (77/222) Brain 73.5% (25/34); including stillbirth brain 57.1% (8/14) Lung 57.1% (4/7); including stillbirth lung 66.7% (2/3) Lymph node 36.6% (15/41); tonsil 66.7% (4/6); semen 8.5% (4/47); serum 28.7% (25/87)	Ku *et al.* (2017)
		2 – 3 months old growers with severe respiratory signs	Lung	72.2% (13/18)	Zhai *et al.* (2017)
		Suspected PDNS cases	Lung, lymph nodes	42.9% (6/14)	Zhan *et al.* (2017)
		Natural stillborn foetuses; sow and live-born piglets without clinical signs	Stillborn foetus: lung, spleen, kidney, liver, heart, umbilical cord	59.46% (132/222)	
		Clinical samples from 2015 – 2017	Lung, lymph nodes, tonsil, kidney, stillbirth	26.7% (76/285)	

Table 1: Continued

Country		Sampling Population	Tissue type	PCV3 Molecular Detection Rate	Reference
		4 – 16 weeks old clinically ill pigs	Lung	33.3% (35/105)	Ha *et al.* (2018)
		Diagnostic samples	Lung, lymph nodes, spleen, liver, kidney, tonsil, brain, heart, stillbirth	14.25% (104/730) Lung 29.8% (25/84); lymph nodes 21.3% (16/75); stillbirth 14.07% (28/199); kidney 13.8% (9/65); brain 12.5% (7/56); tonsil 12.1% (7/58); spleen 7.3% (6/82); heart 6.5% (5/77); liver 2.9% (1/34)	Li *et al.* (2018c)
		Pigs with respiratory diseases or digestive diseases (2015 – 2017)	Lung (respiratory diseases); intestinal tissues (digestive diseases)	Respiratory disease: 26.6% (25/94) Digestive disease: 10.4% (50/480)	Qi *et al.* (2018)
		Diagnostic samples (1990 – 1999)	Lymph nodes, spleen, kidney	6.5% (13/200)	Sun *et al.* (2018)
		Healthy finishing pigs from slaughterhouses; diseased pigs from pig farms	Lung	Ill: 37.96% (41/108) Healthy: 19.14% (9/47)	
		Chinese commercial farms	Lung, lymph node, spleen, liver, kidney, brain, small intestine	31.82% (35/110)	

Table 1: Continued

Country		Sampling Population	Tissue type	PCV3 Molecular Detection Rate	Reference
		Piglets	Lung, lymph nodes, spleen, liver, kidney, heart, brain	16.6% (44/265)	Zhang *et al.* (2018)
		Weaners with PCV2 suggestive signs (2008 – 2017)	Lung, mesenteric and inguinal lymph nodes, spleen, liver, kidney, heart	14.7% (40/272)	Zhao *et al.* (2018)
		Farms with prolonged reproductive problems (abortion rate 3.9 – 9.3%, sow mortality 1.6 – 4.7%)	Aborted foetuses from <113 days of gestation: Pooled lung, heart, brain or entire carcass	60.6% (20/33)	Zou *et al.* (2018)
		Diseased pigs	Lung, lymph nodes, intestine	67.1% (190/283) 2014: 83.3% (40/48); 2015: 72.3% (68/94); 2016: 75.9% (44/58); 2017: 45.8% (38/83) Lung 87.5% (7/8); lymph nodes 81.0% (17/21); small intestine 69.0% (149/216); faeces 44.7% (17/38)	Geng *et al.* (2019)
		Farms (tonsil); slaughterhouses (lymph nodes)	Lymph nodes, tonsil	5.77% (315/5459) Lymph nodes 8.0% (114/1419); tonsil 5.0% (201/4040)	

Table 1: Continued

Country		Sampling Population	Tissue type	PCV3 Molecular Detection Rate	Reference
		Clinical samples from pigs with fever, diarrhoea and coughing	Lung, lymph nodes, spleens, pleural effusions, serum	22.39% (88/393) Lung 14.52% (9/62); lymph nodes 36.71% (29/79); spleen 23.66% (22/93); pleural effusion 22.81% (13/57); serum 14.71% (15/102)	Mengfan *et al.* (2019)
		Diseased and healthy pigs	Lung, lymph nodes, serum	13.3% (63/472)	Xia *et al.* (2019)
		Diagnostic samples from clinically ill pigs (2016 – 2019)	Lung, lymph nodes, spleen, liver, kidney, tonsil, brain, heart, intestines, muscle, joint fluid, aborted foetus, faecal samples	22.68% (105/463) 2016: 10.6% (7/66); 2017: 12.64% (22/174); 2018: 33.33% (36/108); 2019: 34.78% (40/115)	Chang *et al.* (2021)

Table 1: Continued

Country		Sampling Population	Tissue type	PCV3 Molecular Detection Rate	Reference
	Japan	Routine diagnostic samples; Foetus – adults (subclinical, sudden death, abortion, respiratory, enteric or neurological signs)	Lung: 6.4% (3/47); spleen: 4.3% (1/23); liver: 3.1% (1/32); kidney: 28.6% (4/14); intestine: 11.8% (2/17); brain: 8.3% (1/12); tonsil: 0% (0/11); pulmonary lymph node: 8.0% (2/25); mesenteric ln: 25.0% (1/4); mandibular ln: 33.3% (1/3); inguinal ln: 0% (0/2); foetal organs: 1.8% (1/55); placenta: 0% (0/2)	9.6% (7/73) Foetus 6.7% (1/15); suckling piglet 13.3% (2/15); weaner 93 9.0% (1/11); grower-finishers 9.4% (3/32)	Hayashi *et al.* (2018)
	Korea	PCV2 diagnostic samples	Lung, lymph nodes, serum	8.6% (59/690) Lung, lymph nodes, serum 9.8% (57/582); aborted foetus 1.9% (2/108)	Kim *et al.* (2018a)
		Fattening pig farm with 10% increase in sow abortion rate and a 20% increase in suckling pig mortality	Aborted foetus (gestational age 60 to 100 days): lung, heart	42.86% (6/14) Lung 28.6% (4/14); heart 21.4% (3/14)	Kim *et al.* (2018b)
			Suckling piglet (weak, 3 – 7 days old): lung, heart, lymph node, kidney, spleen, liver	25% (2/8) Kidney: 37.5% (3/8); spleen: 25% (2/8); lung, heart, liver, lymph node: similarly 12.5% (1/8)	

Table 1: Continued

Country		Sampling Population	Tissue type	PCV3 Molecular Detection Rate	Reference
	Taiwan	Aborted foetus	Mixed tissues	4% (17/423)	Zeng (2018)
		Sick pigs	Mixed tissues	22.68% (105/463)	Chang *et al.* (2021)
	Thailand	Farm with endemic PRRS and PRDC in 18 weeks grower	Lung, lymph nodes, spleen, liver, kidney, tonsil, heart, salivary gland	Lung & lymph nodes: 62.5% (5/8)	Kedkovid *et al.* (2018a)
		Diagnostic samples	Serum and tissue samples	36.70% (29/79)	Sukmak *et al.* (2019)
	Malaysia	Pigs suspected of PRDC or PCVAD	Weaner and grower of 4 – 12 weeks old; archived lung (2016 – 2017); lung from abattoirs and retail shops (clinically healthy finisher); wild boar lung	17.02% (24/141) Tissue samples from clinically ill pigs: Lung 73.33% (11/15); inguinal lymph node 90.00% (9/10); mesenteric lymph node 36.36% (4/11); spleen 57.14% (8/14); tonsil 60.00% (6/10); kidney 50.00% (5/10); heart 20.00% (1/5); liver 16.67% (1/6); brain 33.33% (1/3) Lungs from clinically healthy finishers: 0% (0/18) Wild boar lung: 0% (0/28)	

Table 1: Continued

Country		Sampling Population	Tissue type	PCV3 Molecular Detection Rate	Reference
	Vietnam	Pigs with varied clinical signs of wasting, diarrhea, respiratory signs	Pooled lung, lymph nodes, spleen, kidney, heart	6/135 (4.44%)	Nguyen, Chung & Huynh (2018)
South America	Brazil	Healthy sows and weaners, slow growing pigs, foetus	Lung, lymph nodes, spleen, liver, heart, cerebrum, vaginal swab, umbilical cord, intestine, serum	China	
		Farms with >2.5% foetal mummification rates	Mummified foetus: Lung, liver, kidney, heart, brain	97% (270/276)	
		Healthy finishing pigs from slaughterhouses (2017 – 2018); diagnostic samples (1967 – 2017)	Lung, lymph nodes, spleen, liver, heart, kidney, brain	14.6% (26/178) Healthy finishers 40% (14/35); diagnostic samples 8.4% (12/143)	
	Colombia	PDNS case	Lung, mesenteric ganglia	25% (1/4) *mesenteric ganglia	
Europe	Denmark, Italy	Diagnostic samples for respiratory, reproductive or PCV2 evaluation	Lymph node, placenta (sow); lung	Denmark: Lung 10% (2/20), lymph nodes 94.74% (36/38) Italy: Lung 34.48% (10/29); organ pool 68.97% (20/29)	

Table 1: Continued

Country		Sampling Population	Tissue type	PCV3 Molecular Detection Rate	Reference
	Italy	Diagnostic samples	Aborted foetuses and stillborn piglets: Pooled lung, heart, thymus / foetus	12.5% (2/16)	Faccini *et al.* (2017)
		Domestic pigs, hunted wild boars, culled feral free ranging pigs	Lung, spleen, liver, aborted foetus, intestine, brain, placenta, EDTA blood	Domestic pigs 17.64% (6/34); feral free ranging pigs 77.39% (89/115); wild boar 61.54% (24/39)	Dei Giudici *et al.* (2020)
	Hungary	Farm with reproductive failure	Aborted, stillborn or weakborn piglets	89.10% (49/55) Thymus: 89.1% (49/55); lymph node: 60% (33/55); placenta: 50.9% (28/55); spleen: 21.8% (12//55; kidney: 7.2% (4/55); liver: 5.4.% (3/55)	Deim *et al.* (2019)
	Poland	Farms with reproductive failure	Foetuses or stillborn piglets	36.0% (9/25)	Woźniak, Miłek & Stadejek, (2020)
	Serbia	Pigs with varied signs from PRDC, PDNS to congenital tremor	Pooled lung, lymph nodes, spleen, kidney, tonsil (frozen / FFPE), brain homogenate	PCR: 84.8% (28/33) qPCR of PCR negatives: 100% (5/5)	Savic *et al.* (2020)

Table 1: Continued

Country		Sampling Population	Tissue type	PCV3 Molecular Detection Rate	Reference
	Spain	Wild boar	Lung, submandibular lymph node, spleen, liver, kidney, tonsil, brain and faecal sample	91.43% (32/35)	Klaumann *et al.* (2019a)
		Farms with good reproductive parameters	Sows with paired serum; Mummies and stillborn: lung, brain	33.7% (86/255) Foetuses from primiparous sows: 80.2% (73/91) Foetuses from multiparous sows: 7.9% (13/164)	Saporiti *et al.* (2020c)
		Diagnostic samples for reproductive failure	Foetus/stillborn piglet: heart, lung, spleen, kidney, thymus, liver, intestine, cerebrum, cerebellum	33.9% (18/53)	Saporiti *et al.* (2021)
	Sweden	Archived samples (1993 – 2007)	Lymph nodes	20.41% (10/49)	Ye *et al.* (2018)
	United Kingdom	Archived samples (2002 – 2017)	Mixed tissues	20% (48/240) Positive: Lymph node, tonsil, spleen, faeces Negative: Lung, liver, colon 5% (4/80) Positive: Lymph node (3/70), lung (1/3) Negative: Liver, spleen	

Table 1: Continued

Country		Sampling Population	Tissue type	PCV3 Molecular Detection Rate	Reference
		Reproductive failure cases (increased mummification and stillbirth rate)	Pooled foetal heart and lung	61.11% (22/36)	Arruda *et al.* (2019)
		Wild boar	Spleen	Region 1: 50% (10/20) Region 2: 23% (16/69)	Prinz *et al.* (2019)
Eurasia	Russia	Samples for PCV2 and PRRS diagnostics	Spleen, kidney, heart tissues, serum, pleural effusion and peritoneal cavity fluid	77 samples: Sow serum of sows 10%; weaner kidneys 40%; fattener spleen 50%; thoracic and abdominal cavity of stillborn piglets 100%; stillborn piglets spleen 75%	Yuzhakov *et al.* (2018)

Table 2: Detection Rates of PCV3 in Non-Tissue Sample Types

Sample Type		**Country**	**Sampling Population**	**PCV3 Detection Rate (Clinical Signs and Age Group)**	Reference
Serum	Asia	China	Commercially imported live boars (2011 – 2017)	8.96% (54/601) United States 11.01% (37/336); United Kingdom 6.35% (8/126); France 6.47% (9/139)	Feng *et al.* (2019)
			PCV2 diagnostic samples	28.7% (25/87)	Ku *et al.* (2017)
			Clinical samples from pigs with fever, diarrhoea and coughing	14.71% (15/102)	Mengfan *et al.* (2019)
			Pigs suspected with PCV2, PRRS, PEDV, PRV or PPV infection (2013 – 2017)	30% (18/60)	Xu *et al.* (2018)
			Weaners with respiratory signs	Severe respiratory signs: 63.75% (51/80); mild respiratory signs: 13.14% (23/175); asymptomatic: 4/216 (1.85%)	Zhai *et al.* (2017)
			Sows with prolonged reproductive problems	Sow with reproductive failure: 45.9% (39/85) Healthy sow: 21.9% (23/105)	Zou *et al.* (2018)
		Taiwan	Nursery pigs, sows	Nursery pigs 7.1% (85/1195); sows 3.2% (3/94)	Zeng (2018)
		Thailand	5 – 18 weeks old growers, parity 1 – 4 sows	PRDC-affected pigs: 60% (15/25); clinically healthy pigs: 28% (7/25); sows: 60% (12/20)	

Table 2: Continued

Sample Type		Country	Sampling Population	PCV3 Detection Rate (Clinical Signs and Age Group)	Reference
	North America	United States	Respiratory disease diagnostic samples	12.5% (34/271)	Palinski *et al.* (2017)
	South America	Brazil	Post-farrowing sows (2015 – 2016)	Sows with stillbirths: 67.4% (31/46) Sows without stillbirths: 60.5% (26/43)	Tochetto *et al.* (2020)
		Columbia	Serum pools from 3, 7, 10, 13, 16, 20 weeks old pig	16.67% (1/6 pool)	Vargas-Bermudez *et al.*, (2019)
	Europe	Denmark Italy Spain	Diagnostic samples for respiratory, reproductive or PCV2 evaluation	Denmark 30% (6/20); Italy 18.18% (6/33); Spain 14.89% (14/94)	Franzo *et al.* (2018a)
		German	20 – 24 weeks finishers with respiratory signs	Herd level: 75% (40/53 farms; 79/212 serum pools)	Fux *et al.* (2018)
		Italy	Juvenile to adult wild boar population (Italy)	33.15% (62/187)	Franzo *et al.* (2018c)
		Poland	Archived diagnostics samples of sows and 3 – 20 weeks old pigs (2014 – 2017)	25.0% (46/184) Suckling piglet 5.0% (1/20); weaner 26.1% (12/46); finisher 28.0% (33/118)	
			Routine diagnostics samples of 3 – 21 weeks old pigs and sows	PCV2 vaccinated farm: 29.2% (31/106) PCV2 non-vaccinated farms: 20.0% (12/60)	

Table 2: Continued

Sample Type		Country	Sampling Population	PCV3 Detection Rate (Clinical Signs and Age Group)	Reference
		Serbia	Routine diagnostics samples of grower-finishers and sows	PCR: 15.6% (5/32) qPCR of PCR negatives: 88.8% (24/27)	Savic *et al.* (2020)
		Slovenia	Survey diagnostic samples of weaners to sows	31/237 (13.1%)	Plut *et al.* (2020)
		Spain	Archived samples of 1 – 4 months old pigs with enteric or respiratory histopathological lesions (1997–2018)	Enteric group: 5.5% (7/126) Respiratory group: 6.2% (8/129) Healthy: 6.6% (4/60)	Saporiti *et al.* (2020a)
			10 – 25 week clinically healthy grower-finisher	8% (52/624)	Saporiti *et al.* (2020b)
			Sows from farrowing herd with good reproductive parameters	Primiparous sow: 33.3% (19/57); multiparous sow: 0% (0/64)	Saporiti *et al.* (2020c)
			Longitudinal sampling from suckling piglets to finishers (2 – 25 weeks)	Farm A 82.35% (28/24); farm B 72.72% (32/44); Farm C 78.57% (22/28); Farm D 71.74% (34/46)	
			Resident wild boars	42.66% (221/518)	
			Diagnostic samples (1996 – 2017)	75/654 (11.46%)	

Table 2: Continued

Sample Type		Country	Sampling Population	PCV3 Detection Rate (Clinical Signs and Age Group)	Reference
Oral fluid	Asia	China	Clinically healthy pigs	12.3% (39/318) Sow stall: 15.06% (25/166); pen: 9.21% (14/152)	Guo *et al.* (2019)
		Korean	Pen-based (weaner, grower, finisher, sick pen)	44.2% (159/360) Hospital pen 51.5%; grower 42.6%; weaner 49.3%; grower-finisher 41.1%	Kwon *et al.* (2017)
	Europe	Poland	Archived samples from previously PCV3 positive farms	37.3% (122/327)	
		Slovenia	Survey diagnostic samples of weaners to sows	25/34 (73.5%)	Plut *et al.* (2020)
Faecal sample	Asia	China	Weaned pigs with / without respiratory signs	Diarrheal: 17.14% (6/35); non-diarrheal: 2.86% (1/35)	
			Clinically healthy pigs	0% (0/42)	
			Diseased pigs	44.7% (17/38)	

Table 2: Continued

Sample Type		Country	Sampling Population	PCV3 Detection Rate (Clinical Signs and Age Group)	Reference
			Faeces and/or intestinal contents from diarrheal / non-diarrheal sucklings / weaners	16.31% (23/141) Diarrheal suckling piglets: 27.28% (15/55); diarrheal weaners: 14.29% (5/35); non-diarrheal suckling pigs: 6.67% (2/30); non-diarrheal weaners: 4.76% (1/21)	Zhang *et al.* (2020a)
	Europe	Poland	1451 pairs of serum and faeces samples	Faeces: 15.0% (217/1451) *Only 30.5% (43/141 PCV3-positive serum samples) of viremic pigs shed PCV3 with faeces *Only 19.8% (43/217 PCV3-positive faeces samples of pigs shedding PCV3 with faeces showed viremia Serum: 9.7% (141/1451)	Woźniak, Miłek & Stadejek (2020)
		Slovenia	Survey diagnostic samples of weaners to sows	8/34 (23.5%)	
		United Kingdom	2002 – 2017 archived samples	Presence of PCV3 positive samples	
Colostrum	Asia	Thailand	Matched colostrum and serum samples from 38 sows of parity 1 – 6	Colostrum: 44.74% (17/38); serum: 47.37% (18/38); colostrum & serum: 26.32% (10/38)	

Table 2: Continued

Sample Type		**Country**	**Sampling Population**	**PCV3 Detection Rate (Clinical Signs and Age Group)**	Reference
Nasal swab	Europe	Italy	Diagnostic samples for respiratory, reproductive or PCV2 evaluation	12.5% (1/8)	Franzo *et al.* (2018a)
Pleural effusion	Asia	China	Clinical samples from pigs with fever, diarrhoea and coughing	22.81% (13/57)	Mengfan *et al.* (2019)

To summarize findings that directly inform the design and scope of this study, these tables highlights several critical points regarding the detection of PCV3 across diverse tissues, reflecting its multisystemic nature. This underscores the need to include a variety of tissue samples to gather comprehensive prevalence data, as done in this study. High detection rates in organs such as the brain and lung point to a possible tropism for respiratory and central nervous tissues, while consistent presence in lymphoid tissues like lymph nodes and tonsils suggests PCV3's involvement in immune response pathways. These reports also support the development of chromogenic in-situ hybridization (ISH) to enable more detailed tissue-specific investigations, providing insights into pathogenesis and transmission of PCV3. Additionally, studies employing serum, nasal swabs, and oral fluids have broadened PCV3 detection methods. The higher sensitivity of PCR and qPCR in serum, especially among asymptomatic pigs, facilitates non-invasive diagnostic practices in field conditions. Research in countries like Poland and the United States has shown PCR's effectiveness in identifying asymptomatic infections, uncovering hidden reservoirs within herds. These findings justify including non-organ samples in this study to assess silent PCV3 circulation in swine populations.

Regional variation in detection methodologies, from Asia to Europe and the Americas, likely affects PCV prevalence data. Asian studies, especially in China, report high detection rates across both tissue and non-tissue samples, reflecting comprehensive diagnostic coverage. In contrast, European research often relies on archived tissue samples, focusing on retrospective analyses.

This diversity in sample types and methodologies informs the selection of sample types and diagnostic techniques in this study, ensuring compatibility with global data for meaningful comparative analyses. In this study, most samples consist of freshly collected tissues, yet archived lung samples from as far back as 2016 were also tested, yielding positive PCV detections. Given PCV3 detection has been reported across multiple continents, along with Malaysia's role as a regional hub for trade and imports, phylogenetic analysis of Malaysian PCVs is essential to gauge cross-border transmission risks. Phylogenetic comparisons will help assess the relatedness between Malaysian and international strains, evaluating the exposure risk of local pig populations to potential new strains. Understanding the genetic relationships between Malaysian PCVs and those in imported breeding stock, boar semen, and pork products may reveal the need for improved screening and quarantine measures, helping prevent the spread of emerging strains like PCV4 across Southeast Asia. Furthermore, European studies emphasize wild boars as potential PCV reservoirs, with some wild populations exhibiting significant prevalence. Including wild boar samples in this study addresses potential cross-species transmission, crucial for managing PCV spread in areas where wild and domestic pig populations intersect. Comparative phylogenetic analysis of domestic and wild strains will elucidate viral evolution patterns and adaptability in natural reservoirs, supporting more comprehensive management strategies.

In conclusion, these findings collectively guide the study's design, underscoring the importance of a multi-sample, multi-method approach that

includes both domestic and wild populations. Phylogenetic and molecular characterisations are vital for understanding local strains, monitoring genotype shifts and enhancing biosecurity protocols. Additionally, developing diagnostic tools like ISH will support in-depth investigations of PCV3 infection patterns in Malaysia, aligning with global research efforts and strengthening regional disease management capabilities.

2.2.4 PCV3 in Wild Boar and Other Non-Porcine Species

PCV3 has been reported in wild boar population with a molecular prevalence range of 33.15% – 50.00% (Franzo *et al.*, 2018c; Franzo *et al.*, 2019b; Klaumann *et al.*, 2019a; Prinz *et al.*, 2019). Rep gene sequence of PCV3 strains collected from wild boars were found to be identical to that of domestic pigs (Franzo *et al.*, 2019b). The high viral prevalence reported in wild boars which even exceeded the average prevalence in domestic pig populations, on top of the genetic similarity between PCV3 strains isolated from domestic pigs and wild boars, may suggest the potential role of wild boars as PCV3 reservoirs (Franzo *et al.*, 2019b). PCV1 and PCV2, while mainly detected in porcine, have been reported in bovine, canine and rodent hosts (Kappe *et al.*, 2010; Zhai *et al.*, 2016; Song *et al.*, 2019). The presence of PCV3 in non-porcine species too has been reported. When PCV3 strains isolated from different collection hosts were analysed phylogenetically, the strains interspersed in different clades (Franzo *et al.*, 2020a). PCV3 has been detected in bovine serum samples at a molecular detection rate of 34.7% (74/213), with strains sharing 97.5 – 99.8% nt identities with reference PCV3 strains (Wang *et al.*, 2019). In dogs, PCV3 strains have been isolated from hosts with various clinical signs

including respiratory and enteric signs, at a prevalence of 9.1% – 23.6%. At 96.28 – 100% nt identities similarities, the canine PCV3 strains readily fit into PCV3 clade (Zhang *et al.*, 2018; Sun *et al.*, 2019). In wildlife, PCV3 has been detected in 1/9 (12.5%) chamois (*Rupicapra rupicapra*) and 2/50 (4%) roe deer (*Capreolus capreolus*) sampled from mountain areas in Italy (Franzo *et al.*, 2019b). A Spanish paper reported PCV3 detection in 1/108 (0.9%) red deer (*Cervus elaphus*), 1/104 (0.96%) mouflon (*Ovis aries*) and 1/104 (0.96%) fallow deer (*Dama dama*) (Czyżewska-Dors *et al.*, 2020). Further, PCV3 has been detected in invertebrates. Two (4.25%, 2/47) *Ixodus ricinus* tick pools collected from roe deer tested PCV3 positive despite not originating from positive roe deer. The positive status of non-engorged ticks, negative status of the roe deer host, and genetic difference of the PCV3 strain isolated from ticks and roe deer, suggest a transstadial PCV3 transmission rather than accidental detection due to blood meal or contamination (Franzo *et al.*, 2019b). Closer to farms, high molecular detection rates of 27.1% – 37.5% have been reported in different mosquito species including *Aedes vexans, Anopheles sinensis, Culex tritaeniorhynchus* and *Culex pipiens pallens*, captured in swine farms in China (Ha *et al.*, 2020). The farms showed similar PCV3 prevalence of 21.25% (17/80). An 100% nt identity similarity between the PCV3 strains isolated from the mosquitos and pigs of the same farm was discovered.

2.2.5 Clinical Signs and Lesions of PCV3

In the first field report of PCV3, PDNS-like disease was a major observation (Palinski *et al.*, 2017). PCV3 was first discovered when metagenomic sequencing was performed on tissue homogenate pool of three mummified

foetus aborted from sows with skin lesions consistent with PDNS, and on tissues from sows that died with PDNS-like clinical signs, including anorexia, multifocal papules, macules and superficial dermatitis (Palinski *et al.*, 2017). The aborted litters consisted of mummified foetus of various gestational ages, similar to description of PCV2-associated abortion (Pensaert *et al.*, 2004; Palinski *et al.*, 2017). High PCV3 viral titres were detected in the aborted foetal tissue pools, suggesting a correlation between PCV3 and reproductive failure on the basis of both nucleic acid amount and tissue distribution (Palinski *et al.*, 2017). Association of PCV3 with respiratory disease is also widely reported. A retrospective study in China reported highest molecular detection in lung samples, at 27.6% (37/134) (Fu *et al.*, 2018). A molecular detection rate of 57.1% (4/7) in lung samples was reported in China, including 66.7% (2/3) detected in the lung of stillbirth samples (Ku *et al.*, 2017). A higher rate of 73.33% (11/15) was reported in lung samples in a Malaysian PCV3 study (Tan *et al.*, 2020). An epidemiological study carried out in China reported a higher PCV3 prevalence in weaned pigs with respiratory disease: 63.75% (51/80) when the respiratory disease was severe and 13.14% (23/175) in mild respiratory disease, compared to a mere 1.85% (4/216) in asymptomatic weaners. PCV3 positive samples collected from pigs with respiratory disease had higher frequency of lower Ct values (Zhai *et al.*, 2017). Further, a study in Thailand which recorded a 100% (5/5) PCV3 molecular detection rate in the lung and lymph node samples among PCV3 positive pigs also found the corresponding PCV3 titres to be highest, compared to other organs including spleen, kidney and heart (Kedkovid *et al.*, 2018a).

Apart from the respiratory tract, multisystemic manifestation of PCV3 infection encompasses gastrointestinal, nervous and cardiovascular system (Phan *et al.*, 2016; Chen *et al.*, 2017; Ku *et al.*, 2017; Zhai *et al.*, 2017; Arruda *et al.*, 2019; Qi *et al.*, 2019; Hou *et al.*, 2020). An infectious PCV3 molecular DNA clone has been synthesized based on a Chinese PCV3 strain (GenBank accession no.: MF318451). The rescued PCV3 from transfection of PCV3 DNA was intranasally inoculated into 4 weeks and 8 weeks old specific-pathogen free (SPF) piglets, with resulting peak PCV3 viremia at 21 days post-inoculation (dpi) and seroconversion of anti-PCV3 Cap antibodies detected from 21 dpi onwards. Clinical signs were seen from as early as 8 dpi until the end of the experiment at 28 dpi, progressing from anorexia, coughing, sneezing and diarrhoea to increased respiratory rates, lethargy, skin lesions (rubefaction on the skin and the ears, multifocal papules, macules, superficial dermatitis), shivering, and/or hyperspasmia within 15 dpi; and four deaths of 4 weeks old piglets were recorded from 16 to 25 dpi (Jiang *et al.*, 2019a). Grossly, lungs were of diffuse mottled tan-red appearance with lesions including lobular pneumonia, bronchopneumonia, multifocal haemorrhage and multifocal tan-to-purple consolidation. Hyperplastic lymph nodes were enlarged and firm, tan to haemorrhaging. Hepatitis (hyperaemic and necrotic with grey-white nodules), nephritis (swollen, oedematous with multiple pinpoint haemorrhages) and splenitis (splenomegaly with necrotic foci at marginal zone) were also seen. Microscopic lesions include but not limited to lymphoplasmacytic and histiocytic bronchointerstitial pneumonia, glomerulonephritis, tubulo-interstitial nephritis, congestive hepatitis, epicarditis, myocarditis and mucosal enteritis. Lymphoid related lesions

include hyperplasia, cortical lymphocytic reduction, lymphocytic necrosis, lymphoid depletion and eosinophil infiltration of lymph nodes; hypoplastic splenic white pulp with haemorrhagic and necrotic lymphoid follicles. Another infectious PCV3 DNA clone study revealed changes in alveolar and cardiac tissues of intraperitoneally infected mice, notably interstitial pneumonia (Jiang *et al.*, 2020). These results demonstrate the pathogenicity of PCV3 as a single aetiological agent, and that PCV3 infection could be characterized by moderate to severe clinical PDNS-like diseases in piglets.

2.2.6 Detection Method of PCV3

2.2.6.1 In situ Hybridisation (ISH)

In situ hybridisation (ISH) permits the direct correlation of molecular findings with the cytologic and histologic features of samples (Nuovo, 1994). ISH on PCV3 was first performed on lung and heart tissues of infected pigs by using RNAscope (Phan *et al.*, 2016). Gross examination of stillborn foetuses was impaired due to utero dehydration or mummification, with many cases showing unremarkable findings in histologic examination of the foetal heart, lung, spleen, liver, kidney and placenta. However, detection rates as high as 100% (8/8 assays) by the ISH method have been reported. The most abundant PCV3 positive labelling was present in a section of heart with lymphocytic myocarditis. Replication of PCV3 was observed in cardiac arterial leiomyocytes, cardiac myocytes, renal arterial and arteriole leiomyocytes, renal tubular epithelium, neural cells and gut-associated lymphoid tissue (Phan *et al.*, 2016; Arruda *et al.*, 2019). Presence of PCV3 was also reported in

trophoblasts, colonic leiomyocytes and adipocytes (Arruda *et al.*, 2019). PCV3 mRNA was detected infrequently in macrophages in the alveolar wall and in histiocytes of lymph node lymphoid follicles in respiratory cases (Kim *et al.*, 2018b). When PCR and ISH detection rates of PCV3 in lung, inguinal and mesenteric lymph node tissue samples were compared, the detection status was in agreement except for one PCR positive sample turning out to be ISH negative (Tan, 2020). The false negative result was postulated to be caused by insufficient mesenteric lymph node tissue section. In the same study, ISH staining of PCV3 nucleic acid was observed in the cytoplasm of infected cells, matching the description of PCV3 being virus-like particles (VLP) in intracytoplasmic inclusion bodies (Oh & Chae, 2020; Tan, 2020). More recently, a PCV2/PCV3 differential RNAscope to facilitate specific detection of low copy PCV3 transcripts and to provide strong evidence of productive replication has also been described (Mora-Díaz *et al.*, 2020).

2.2.6.2 Immunohistochemistry (IHC)

Monoclonal antibodies against PCV3 Cap protein with a titre of 1/25,600, designated mAb 1H1, have been developed and shown to react strongly with recombinant PCV3 Cap protein without cross-reaction to PCV2 Cap protein. MAb 1H1 has been applied successfully for immunohistochemistry (IHC) detection of PCV3 Cap protein in PCV3 positive tonsil and lymph node tissue samples (Li *et al.*, 2018c). In lung lesions, occasional lymphocytes and scattered macrophages showed moderate intracytoplasmic IHC staining against PCV3 (Palinski *et al.*, 2017). PCV3 mRNA was visualized infrequently in macrophages in the alveolar wall (Kim *et al.*, 2018b). In PCV3 infectious

clone inoculation experiment, strong IHC signals were observed in bronchial epithelial cells, interstitial vascular contents, alveolar exudate, dust cells and septal cells. Given that the inoculation study solely involved PCV3 molecular clones, the findings of abundant and strong IHC staining ought to be specific to PCV3 antigen (Jiang *et al.*, 2019a). In lymph nodes with diffuse granulomatous lymphadenitis and moderate cortical lymphoid depletion, intense and diffuse intracytoplasmic IHC staining against PCV3 was observed in the follicular and perifollicular lymphocytic population (Palinski *et al.*, 2017). PCV3 mRNA was detected in histiocytes of the lymphoid follicles in the lymph nodes. (Kim *et al.*, 2018b). In tracheobronchial, mesenteric and inguinal lymph nodes, IHC stainings were observed in cortical and medullary reticular cells, macrophages and eosinophils (Jiang *et al.*, 2019a). In PDNS-like skin lesions, marked intracytoplasmic IHC staining against PCV3 was observed in dermal lymphocytic infiltration. PCV3 nucleic acid has been demonstrated in splenic arterial leiomyocytes and necrotic cells, renal artery, tubular epithelial cells, intestinal epithelial cells and inflammatory cells in lamina propria, hepatic lobular stroma and sinus antral wall, myocardial fibres, tissues and vascular contents; as well as foetal lung and cerebral samples with no apparent histopathologic lesions (Palinski *et al.*, 2017; Jiang *et al.*, 2019a; Hou *et al.*, 2020; Saporiti *et al.*, 2021).

2.2.6.3 PCR and qPCR

Following the discovery of PCV3 through metagenomic sequencing, conventional PCR and qPCR protocols were widely used to detect PCV3 antigens in field cases. PCV2 primers were omitted due to extensive research

already available on this virus. Some of the primers utilized in these protocols are listed in Table 4, which includes primers targeting either the cap or rep gene of PCV3, with varying amplicon sizes. The primer sequences have been standardized in the respective studies. Organizing these primers in a single table provides a comprehensive overview of the different primer sets chosen to detect each virus, offering insights into their design specifications, such as amplicon size and gene target. This detailed compilation facilitates easy access for researchers to assess the methodological consistency across studies and understand primer selection rationale in different research contexts. For instance, the rep gene sequences generated using the primers from Ye *et al.* (2018) or Plut *et al.* (2020) could be useful for phylogenetic studies focused on investigating the evolutionary history of PCV3. Additionally, it serves as a valuable reference for future studies, facilitating comparisons of detection efficacy and ensuring reproducibility when working with similar primer-based detection methods in PCV research.

At this stage, only a few genotypic variants of PCV3 and PCV4 have been identified—namely, PCV3a, PCV3b, PCV4a, and PCV4b—distinguished primarily through minor mutations identified by phylogenetic analysis. These variants do not exhibit significant genetic divergence, so most primers are designed to detect them universally, regardless of strain. Therefore, primer selection in this context focuses less on variant specificity and more on study objectives. Primer sets vary in amplicon length (from 78 bp to 649 bp) to suit different molecular methods, with longer amplicons typically suited for PCR

and phylogenetic analysis, while shorter amplicons are often employed in qPCR for diagnostic purposes.

In the first detection of PCV3, the assembly of a 2,000 nt circular genome from foetal tissue homogenate was achieved by rolling-circle amplification (RCA) and subsequent PCR and Sanger sequencing of resulting amplicons (Palinski *et al.*, 2017). Another RCA protocol with 8 exoresistant PCV3-specific primers was designed to increase the DNA load of PCV3, given its limited amount in a single positive sample (Ye *et al.*, 2018). Additionally, a direct PCR method which eliminates the need for DNA extraction step has been developed (Franzo *et al.*, 2018d). Sample processing time is significantly reduced as the samples only need to be pre-treated by adding a dilution buffer and DNA release additive, with <10 min incubation time. This detection method has successfully identified 34.17% (41/120) PCV3 positive samples from various sample types including lungs, organ pools, sera, oral fluid, nasal swabs and environmental samples; at high results agreement with qPCR replicates (35%, 42/120) and equally high sensitivity of 10 viral genome/µL limit of detection (LOD).

A SYBR green-based qPCR assay specific to PCV3 Rep protein has been established with LOD of 1.73×10^2 copies/µL and a single melt peak at 82.5°C (Chen *et al.*, 2018). SYBR green-based qPCR protocol specific to PCV3 Cap protein has also been developed, with LOD of 10^1copies/µL and a single melt peak at 83.19°C (Zou *et al.*, 2018). TaqMan-based qPCR specific for PCV3 Cap protein was first designed by Palinski *et al.* (2017), followed by Wang *et al.* (2017a), similarly with LOD of 10^2 copies/µL. Primers amplifying a 107 bp

section of porcine 18S ribosomal gene were suggested as an endogenous control for qPCR assay (Assao *et al.*, 2020). Detection of PCV3 nucleic acid from formalin-fixed, paraffin-embedded tissues was possible (Palinski *et al.*, 2017). Optimized TaqMan protocols with LOD of 10 – 15 copies/µL have been described, further evidencing that positive detection rate of qPCR protocols are much higher compared conventional PCR (Chen *et al.*, 2018; Zou *et al.*, 2018; Feng *et al.*, 2019; Yuan *et al.*, 2020). Notably, a qPCR protocol that incorporates an exogenous internal control (IC), with high sensitivity of LOD 10 viral genome/µL, reports an almost perfect results agreement with its PCR replicate as described above (Franzo *et al.*, 2018d). The said protocol was the first successful implementation of a commercially available enhanced green fluorescent protein as a reliable exogenous IC for PCV3 qPCR validation. The lowest LOD reported to date is 1 copy/µL, ten times greater sensitivity compared to current qPCR protocols, achieved by droplet digital PCR (ddPCR). The said ddPCR targets PCV3 Cap protein and shares the same primers and probe with its comparison qPCR assay (Liu *et al.*, 2019a). To facilitate epidemiological data collection for evaluation of impact of the four known PCVs, various multiplex qPCR assays capable of simultaneous differential detection of PCVs have been developed. A PCV2/PCV3 duplex qPCR with 2.9 copies and 22.5 copies LOD for PCV2 and PCV3 respectively reported a 27.6% (94/340) co-infection rate (Li *et al.*, 2018d). Another PCV3/PCV4 duplex qPCR with 51.7 copies and 67.7 copies LOD for PCV3 and PCV4 respectively was designed, detecting a co-infection rate of 17.19 % (11/64) (Hou *et al.*, 2021). Further, a comprehensive quadruplex qPCR assay detecting all four known PCVs with LOD of 2.8 copies across all strains was

established. The assay detected 39.17% (47/120) of PCV positive samples, of which 4 co-infections of PCV1/PCV4 (1/47), PCV2/PCV3 (2/47) and PCV2/PCV4 (1/47) were identified (Chen *et al.*, 2020). Detecting PCVs co-infection cases with these multiplex molecular detection assays is a critical early investigative step when new PCVs surface.

2.2.6.4 Enzyme-linked Immunosorbent Assay (ELISA)

The first PCV3 prevalence study was conducted using a recombinant PCV3 capsid ELISA to detect anti-PCV3 Cap antibodies in porcine serum samples, where 56.6% (47/83) – 63% (17/27) of detection rates were reported with absorbances of 0.88 to 1.37. In the farm where PCV3 was first discovered and isolated, an 100% (10/10) detection rate with an average absorbance of 1.27 was reported from serum samples collected from 10 sows, 3 months after the initial PDNS outbreak (Palinski *et al.*, 2017). Another in-house PCV3 Cap recombinant protein-based indirect ELISA was developed and used in a caesarean-derived, colostrum-deprived (CD/CD) pigs' experimental study (Mora-Díaz *et al.*, 2020). Indirect ELISA assay was also developed using a single optimized PCV3 nuclear signal sequence-deleted Cap protein, where all positive serum samples were verified to be positive at a maximum dilution of 1:3200, indicating high sensitivity of the ELISA test (Deng *et al.*, 2018). Further, PCV3 VLPs have been used as coating antigen of an indirect ELISA, detecting 52.82% (197/373) serum samples as PCV3 positive. A partially optimized full-length PCV3 Cap protein was synthesized based on the codon bias of *Escherichia coli* and inserted into pET30a expression vector; and formation of PCV3 VLPs was verified with transmission electron microscopy

(Wang *et al.*, 2020). Baculovirus Expression Vector System (BEVS) too has been deployed to express PCV3 capsid protein to be used as coating antigen for indirect ELISA. The assay identified 33.68% (64/190) of sow serum samples as PCV3 positive, showing similar results as its qPCR replicate assay (Zhang *et al.*, 2019a). PCV3 qPCR DNA level was reported to be negatively correlated with indirect ELISA antibody titre when serum sample replicates were tested for comparison (Geng *et al.*, 2019).

2.2.6.5 Others

Real-time recombinase polymerase amplification (rt-RPA) assay is an option for field diagnostics where resources are limited and time is crucial at point-of-need. A rt-RPA assay specific for PCV3 Cap has been designed for such potential rapid diagnostic, requiring only 5 – 14 min as compared to the 35 – 52 min required by qPCR. The assay reported a positive rate of 27.4% (51/186) (Wang *et al.*, 2017b). In addition, loop-mediated isothermal amplification (LAMP) assays using hydroxynaphthol blue have been developed for rapid and visual detection of PCV3 in 40 – 60 min (Park *et al.*, 2018; Zheng *et al.*, 2018b). A colorimetric isothermal multiple-self-matching-initiated amplification (IMSA) using cresol red for PCV3 Cap antigen detection has been described. Upon isothermal incubation at 63°C for 60 min, positive PCV3 samples would change the colour of the reaction mixture from red to yellow. A perfect 100% agreement with qPCR and higher sensitivity than PCR has been reported (Gou *et al.*, 2020). Further, polymerase spiral reaction (PSR), also an isothermal amplification assay based on PCV3 Cap gene, has

been described. The PSR assay, run at 62 °C for 50 min, detected PCV3 at LOD 1.13 × 102 copies/µL with phenol red and cresol red (Ji *et al.*, 2019).

Progress made in detection of PCV3 antigen and antibodies to date is summarized in Table 3.

Table 3: Progress Made in Detection of PCV3 Antigen and Antibodies

Reference	Progress in PCV3 Antigen and Antibodies Detection
Phan *et al.* (2016)	• RNA expression of PCV3 was detected by ISH in cardiac tissue of pigs with unexplained cardiac and multi-organ inflammation. • PCV3 mRNA was detected intralesionally and multifocally in leiomyocytes of the tunica media of an inflamed cardiac arteriole, scattered cardiomyocytes and inflammatory cells (presumably macrophages) infiltrating the adjacent myocardium; all showing diffuse sarcoplasmic/cytoplasmic reactivity.
Palinski *et al.* (2017)	• PCV3 was first discovered in 2016 with metagenomic sequencing in the U.S., in sows that died acutely with PDNS-like clinical signs. • Complete genome sequences of PCV3 were determined by Sanger sequencing of four overlapping amplicons. • PCV3 antigen was demonstrated in skin, kidney, lung and lymph node samples with typical PDNS lesions via IHC analysis. • Direct ELISA detection of anti-PCV3 capsid antibodies revealed 56.6% (47/83 unrelated diagnostic samples) – 63% (17/27 replacement gilts) PCV3 seroprevalence. • MAbs against PCV3 cap were produced by hybridoma technology with purified N-terminally truncated capsid protein (35 – 214 aa); specificity of the MAbs was confirmed by IFA.
Wang *et al.* (2017a)	• First TaqMan-based qPCR assay for PCV3 detection was described, with positive detection rate was reported at 12.5% (14/112).
Kim *et al.* (2017)	• The first multiplex qPCR assay was developed for differential detection of PCV2 and PCV3. • Co-infection rate of PCV3 with PCV2 was reported at 28.3% (13/46).

Table 3: Continued

Reference	Progress in PCV3 Antigen and Antibodies Detection
Wang *et al.* (2017b)	• A rt-RPA protocol with specific RPA primers and exo probes targeting ORF2 gene of PCV3 was published. • Rapid detection was achieved at 38ºC in 20 min, reporting a positive detection rate of 27.4% (51/186) at 96.2% diagnostic agreement with qPCR replicate.
Zhang *et al.* (2018)	• First molecular detection of PCV3 in non-porcine host is reported. • PCV3 DNA detected via nested PCR in 9.09% (4/44) of dogs negative for PCV2 and canine circovirus
Deng *et al.* (2018)	• First indirect enzyme-linked immunosorbent (ELISA) assay was developed. • Surveillance revealed samples positive for anti-PCV3 Cap antibodies increased from 22.35% (76/370) to 51.88% (152/293) between 2015 and 2017.
Park *et al.* (2018)	• A LAMP assay using hydroxynaphthol blue was first described for rapid and visual detection of PCV3 capsid gene, incorporating six primers hybridizing to eight distinct sequences. • The amplification protocol only requires 40 min at 62°C with positive results indicated by color change in the reaction tubes. • PCV3 detection rate by the LAMP assay was 42.3% (33/78), matching the results of qPCR replicate and higher than conventional PCR replicate (26.9%, 21/78).
Fux *et al.* (2018)	• Classification of PCV3 genotypes a and b, subtype a1, a2, b1 and b2 was suggested. • Phylogenetic analysis of 54 PCV3 strains revealed two clearly separated groups of PCV3 strains with distinctive nucleotide and amino acid marker positions in ORF1 and ORF2 gene.
Franzo *et al.* (2018d)	• Direct PCR method that does not require DNA extraction step was first described. • Sample processing only involves addition of dilution buffer and DNA release additive, with <10 min incubation time. • The authors also developed a qPCR protocol with exogenous internal control, which showed comparable detection sensitivity with said direct PCR method.

Table 3: Continued

Reference	Progress in PCV3 Antigen and Antibodies Detection
Li *et al.* (2018e)	• Production of MAb against PCV3 cap also by hybridoma technique but based on full-length recombinant PCV3 cap protein was described. • Specificity of the Mab isotype IgG2a/k produced by clone 1H1 was confirmed by Western-blot analysis, with successful subsequent downstream application in IHC PCV3 cap detection reported.
Ji *et al.* (2019)	• First polymerase spiral reaction (PSR) based on the PCV3 Cap gene was described. • The assay was run at 62 °C for 50 min and had successfully detected PCV3 at LOD 1.13 × 102 copies/μL with phenol red and cresol red.
Liu *et al.* (2019a)	• Droplet digital PCR (ddPCR) assay targeting PCV3 cap gene was designed with the same primers and probe as qPCR. • LOD was reported to be 1 copy/μL, ten times greater sensitivity compared to current qPCR protocols.
Zhang *et al.* (2019)	• Baculovirus Expression Vector System (BEVS) was used to express PCV3 capsid protein for indirect ELISA coating antigen application. • Detection rate was comparable with qPCR.
Wang *et al.* (2020)	• An indirect ELISA with PCV3 VLPs as coating antigen was designed and used to detect 52.82% (197/373) PCV3 positive serum samples. • A partially optimized full-length PCV3 Cap protein was synthesized based on the codon bias of *Escherichia coli* and inserted into pET30a expression vector; and successful formation of PCV3 VLPs was verified with transmission electron microscopy.
Mora-Díaz *et al.* (2020)	• First successful isolation of PCV3 in PCV1 and PCV2 free pig PK-15 cells. • Three PCV3 isolates were isolated from three separate reproductive cases, from various organs of weak-born piglets, stillborn and mummified foetuses. • Inoculation of the isolated PCV3 strain results in a successful in vivo replication and characterisation of PCV3 in a CD/CD pig model; viremia detected as early as 7 dpi; and detected in all experimental animals by 28 dpi.
Assao *et al.* (2020)	• An endogenous control was introduced to existing PCV3 qPCR protocol, namely primers amplifying a 107 bp section of porcine 18S ribosomal gene

Table 3: Continued

Reference	Progress in PCV3 Antigen and Antibodies Detection
Gou *et al.* (2020)	• Colorimetric isothermal multiple-self-matching-initiated amplification (IMSA) using cresol red detecting PCV3 cap is first being described. • Upon isothermal incubation at 63°C for 60 min, samples positive for PCV3 are identified by red to yellow reaction mixture color change. • Equal sensitivity with qPCR and higher sensitivity than PCR has been reported.
Chung *et al.* (2021)	• Each coding gene of PCV3, namely capsid, replicase and ORF3 protein, was reassembled into 16 sequences by connecting the nucleotide sequences with different combinations. • From the diverse combination, a new classification of three genotypes (1, 2, 3) and several subtypes (2a and 2b, 3a to 3h) was discovered.

2.2.7 Isolation of PCV3

First successful PCV3 isolation was reported in South Korea, where PCV3 strain MK503331 was successfully isolated from inguinal lymph nodes of a clinically healthy 91-day old pig positive exclusively for PCV3 by using primary porcine kidney cells. PCV3-positive ISH signals were detected predominantly in the cytoplasm of infected cells, with no cytopathic effect. Electron microscopy observation revealed small aggregates of circular, ~17nm PCV-like particles similar to the description of Tischer *et al.* (1982) and non-membrane bound intracytoplasmic inclusion bodies containing VLPs arranged in paracrystallline arrays (Oh & Chae, 2020). Further, three PCV3 strains (GenBank accession no.: MK058528, MK568470 and MK568469) were isolated from three separate cases of reproductive issues in the U.S. The isolation involved lung, brain, heart, liver and kidney tissues from weak-born

piglets, stillborn and mummified foetuses in PCV1 and PCV2 free pig kidney epithelial cells (PK-15). Virus replication in cell culture was confirmed by qPCR, immunofluorescence assay (IFA) and strong intracytoplasmic ISH signals indicating active-replicating PCV3. Neither distinct cytopathic effect as expected of porcine circoviruses, nor nuclear and/or intracytoplasmic PCV3-specific immunofluorescence was observed. The same study also reported the first successful in vivo replication and characterisation of PCV3 infection in a CD/CD pig model (Mora-Díaz *et al.*, 2020).

Previously, viral isolation in swine testicular (ST) and porcine kidney cells PK-15 has failed (Faccini *et al.*, 2017; Palinski *et al.*, 2017; Bera *et al.*, 2019). PCV3 DNA replication may depend on cellular enzymes, given that its replication in vitro was best achieved by inoculation of semi confluent monolayers of PK-15 mitotically active cells as described for other PCVs (Tischer *et al.*, 1987; Mora-Díaz *et al.*, 2020). PCV3 replicates less efficiently than PCV1 and PCV2, and the difference in replication efficiency could be influenced by their respective *ori* and *rep* genes, and certain aa mutations in *cap* gene (Fenaux *et al.*, 2004; Beach *et al.*, 2010; Mora-Díaz *et al.*, 2020). Based on experiences with PCV2, it was also suggested to supplement the culture medium with interferon inhibitors and to select a homogeneous subpopulation of PK15 cell line to enhance PCV3 replication in vitro (Meerts *et al.*, 2005; Zhu *et al.*, 2007; Walia *et al.*, 2014; Ma *et al.*, 2017; Mora-Díaz *et al.*, 2020). It was also proposed that virus isolation of PCV3 in a smooth muscle cell culture may be rewarding, given that PCV3 replication was observed in the smooth muscle of arteries by ISH (Arruda *et al.*, 2019).

2.2.8 Porcine Circovirus 4 (PCV4)

PCV4 was first detected in a pig farm in Hunan province, China (Zhang *et al.*, 2020b). This novel PCV was first picked up by degenerate nested PCR utilizing primers targeting conserved *rep* genes of genera Circovirus and Cyclovirus (Li *et al.*, 2010). Molecular prevalence was then determined by TaqMan qPCR targeting the *cap* gene (Zhang *et al.*, 2020b; Sun *et al.*, 2021). A conventional PCR protocol for detecting PCV4, similarly targeting the *cap* gene, was described later (Tian *et al.*, 2021). Primers used in PCR and qPCR detection of PCV4 are listed in Table 4.

The first PCV4 strain (HNU-AHG1-2019, GenBank accession no: MK986820) has a total length of 1770 nt, with an 891 nt *rep* gene encoding 296 aa replicase protein, and a 687 nt Cap gene encoding 228 aa capsid protein. The conserved (T/n)A(G/t)TATTAC nonanucleotide stem-loop motif indicating the *ori* which is present in the genomes of all known PCVs, has been determined to be CAGTATTAC in PCV4. PCV4 shares the highest overall nt identity, Rep and Cap aa identity with mink circovirus (MiCV); and has been shown to be genetically and phylogenetically related to bat associated circovirus (BatACV) and mink MiCV. In comparison, % identity similarities are lower between PCV4 and the three known PCVs, with PCV3 being the least similar to PCV4 (Zhang *et al.*, 2020b; Sun *et al.*, 2021).

Table 4: Commonly Used Primers in PCV3 and PCV4 PCR and qPCR Detection

Antigen Detection	Reference		Primer Sequence	Amplicon target size
PCV3 Cap gene	Wang *et al.* (2017a)	qPCR	F: 5'–CGGACTTGTAACGAATCCAAACT–3' R: 5'–GGAGCATTTATGCCCCGGAAA–3' Probe: FAM–5'–CTTTSGTGCCGTAGAAGTCTGTCATTCCA–3'–Eclipse	78 bp
	Palinski *et al.* (2017)	qPCR	F: 5'–AGTGCTCCCCATTGAACG–3' R: 5'–ACACAGCCGTTACTTCAC–3' Probe: 5'–FAM–ACCCCATGG–Zen–CTCAACACATATGACC–Iowa Black–3'	112 bp
	Zhai *et al.* (2017)	qPCR	Modified probe from Palinski *et al.* (2017) 5'–FAM–ACCCCATGGCTCAACACATATGACC–BHQ1–3'	
	Feng *et al.* (2019)	qPCR	F: 5'–TGTGGCTAAGGTATTATATATTAC–3' R: 5'–TCTCCATGTCTTTCTTTACC–3' Probe: 5'–FAM–AGTAATGTTGTACCGGAGGAGTGG–BHQ1–3'	149 bp
	Zou *et al.* (2018)	SYBR qPCR	F: 5'–YAGTGCTCCCCATTGAACGG–3' R: 5'–GCTCCAAGACGACCCTTATGC–3'	79 bp
	Tochetto *et al.* (2020)	SYBR qPCR	F: 5'–AACGGTGGGGTCATATGTGTTG–3' R: 5'–AGACGACCCTTATGCGGAAA–3'	175 bp
	Wen *et al.* (2018)	PCR	F: 5'–TCCAAACTTCTTTCGTGCCGTAG–3' R: 5'–GGCTCCAAGACGACCCTTATGC–3'	264 bp
	Sun *et al.* (2018)	PCR	F: 5'–GTGCCGTAGAAGTCTGTCATT–3' R: 5'–TACACTCAGCCCTGTAATTTCT–3'	345bp
	Mengfan *et al.* (2019)	PCR	F: 5'– CCGTAGAAGTCTGTCATTCCAG–3' R: 5'– AAGCCCTGGCACGCCAACCAC–3'	434 bp
	Ku *et al.* (2016)	PCR	F: 5'–TTACTTAGAGAACGGACTTGTAACG–3' R: 5'–AAATGAGACACAGAGCTATATTCAG–3'	649 bp

Table 4: Continued

Antigen Detection	Reference		Primer Sequence	Amplicon target size
PCV3 Rep gene	Franzo et al, (2018d)	qPCR	F: 5'–TGACGGAGACGTCGGGAAAT–3' R: 5'–CGGTTTACCCAACCCCATCA–3' Probe: 5'–FAM–GGGCGGGGTTTGCGTGATTT–BHQ1–3'	113 bp
	Chen *et al.* (2018)	SYBR qPCR	F: 5'–TGAAGTTGCGGAGAAGAT–3' R: 5'–CCTGGAGGACCAATAAAA–3'	132 bp
PCV3 Rep gene	Xu *et al.* (2018)	SYBR qPCR	F: 5'–GCTACGAGTGTCCTGAAGAT–3' R: 5'–GCCTCCACACTCCACAATAG–3'	138 bp
	Franzo *et al.* (2018d)	Direct PCR	F: 5'–AAAGCCCGAAACACAGGTGGTGT–3' R: 5'–TTTTCCCGCATCCTGGAGGACCAAT–3'	510 bp
	Plut *et al.* (2020)	PCR	F: 5'– AAAGCCCGAAACACAGGTGGTGT–3' R: 5'–TTTTCCCGACATCCTGGAGGACCAAT–3'	483 bp
	Ye *et al.* (2018)	PCR	F: 5'–TTTACGATAAACAACTGGACCC–3' R: 5'–CATCTTCTCCGCAACTTCAG–3'	528 bp
PCV4	Tian *et al.* (2021)	PCR	F: 5'–GTTTTTCCCTTCCCCCACATAG–3' R: 5'–ACAGATGCCAATCAGATCTAGGTAC–3'	391 bp of Cap gene
	Zhang *et al.* (2020b)	qPCR	F: 5'–GCAGTAATGACGTAGTCCCGGAG–3' R: 5'–CAGCGACCTTAAAGCGGCTGTG–3' Probe: FAM–5'–CCGCCCTGAATGCCGGCAGCTCAATG–3'–BHQ1	77 bp of Cap gene

Molecular prevalence in Chinese provinces and in South Korea ranges widely from 3.28%, 5.1%, 12.8% to 25.40% (Zhang *et al.*, 2020b; Nguyen *et al.*, 2021; Sun *et al.*, 2021; Tian *et al.*, 2021). Co-infections with PCV2 (25 – 53.80%), PCV3 (23.1%), PRRS (50%), PRV (12.5%) and PEDV (18.75%) have been detected. Concurrent PCV2 and PCV3 (0.08%, 1 /13 positive cases); PCV2,

PCV3 and PCV4 (68.75%, 11/16 positive cases) infection was also detected (Zhang *et al.*, 2020b; Sun *et al.*, 2021; Tian *et al.*, 2021). It was suggested that PCV4 might have synergistic effect on PCV2 pathogenesis (Tian *et al.*, 2021). PCV4 has been detected in lung, lymph node, spleen, liver, kidney and small intestines tissues (Tian *et al.*, 2021). The wide tissue tropism of PCV4 is in accordance with the different clinical presentations of PCV4 positive animals, including lymphadenopathy, respiratory, enteric, neurological and PDNS-like signs (Zhang *et al.*, 2020b; Sun *et al.*, 2021; Tian *et al.*, 2021). Positive animals have been identified in all age groups from suckling piglet, weaner, grower, finisher to sow and boar groups; and even aborted foetuses (Zhang *et al.*, 2020b; Nguyen *et al.*, 2021). Notably, DNA of PCV4 is also detected in clinically healthy pigs: testicles of suckling piglets, serum samples, faecal and nasal swabs from suckling piglets to sows and boars (Zhang *et al.*, 2020b; Nguyen *et al.*, 2021). The clinical importance of PCV4 remain ambiguous at this moment as it was detected in both clinical disease and healthy animal. Isolation attempts of PCV4 in porcine kidney cell line (PK-15) and swine testicular (ST) were unsuccessful (Zhang *et al.*, 2020b).

2.2.9 Conclusion

Since the discovery of PCV3 in 2016 in a case of sow reproductive issues, more studies discovered PCV3 antigen and/or anti-PCV3 antibodies in archived samples dating back to 1993, and in a myriad of clinical cases from nervous disorder, respiratory to enteric diseases. Considering the role of PCV2 in PRDC and PDNS, studies of PCV3 often involve respiratory and reproductive samples, with PCR and qPCR being the most commonly used

methods currently. Published qPCR PCV3 detection protocols have been shown to be very sensitive, thus recommended for field detection. Despite having higher genetic variability, molecular detection of PCV3 Cap protein has been shown to be just as reliable as that of Rep protein. PCR remains useful for complete genome sequencing, and as an economical substitute of qPCR albeit with lower sensitivity. In farms where PCV3 has been identified without the presence of other co-infecting agents, development of commercial ELISA kit for seroprevalence survey could be considered, but at this point, management of PCV3-related syndromes is only by minimizing introduction and contact to PCV3.

With the discovery of yet another novel strain of PCV4, the knowledge gap in PCV3 ought to be closed fast. Further, the concern of whether PCV3 will pick up its speed in transmission and launch into an epidemic as PCV2 did before the advent of PCV2 vaccination must be addressed. Although we have the data of PCV3 prevalence globally, the possibility of underestimating the true prevalence due to low viral titres and subclinical infection still needs to be considered. Given that the clinical and economic impact of PCV3 infection is still being ascertained, co-infection(s) with common porcine pathogens must be considered when diagnosing field cases. With subclinical circulation of PCV3 remains unclear, molecular detection method(s) that can detect minimal viral amounts and minimize false-negative results such as qPCR with exogenous IC validation and ddPCR are of diagnostic value. Through several successful inoculation studies involving PCV3 infectious clone, pathogenicity of PCV3 as a single aetiological agent has been demonstrated, with PCV3

infection manifesting as multisystemic clinical signs and lesions. By now, it should be clear that PCV3 is indeed pathogenic and is capable of inducing disease even on its own. The potential severity of PCV3 clinical manifestation in field conditions warrants further research to facilitate the direction of PCV3 vaccine development.

CHAPTER 3

MATERIALS AND METHODS

Materials and methods for each chapter are described in detail in their respective publications as paginated below:

Chapter 4: Molecular Detection and Characterisation of Porcine Circovirus 2 (PCV2)

Chapter 5: Molecular Detection and Characterisation of Porcine Circovirus 3 (PCV3)

Chapter 6: Diagnostic Development for Porcine Circovirus 3 (PCV3)

This study received ethical approval from the Universiti Putra Malaysia (UPM) Institutional Animal Care and Use Committee (IACUC) under AUP Code UPM/IACUC/AUP-R030/2019. All procedures adhered strictly to the guidelines outlined in the UPM Code of Practice for the Care and Use of Animals for Scientific Purposes. The collection of samples was overseen by qualified veterinarians from the Faculty of Veterinary Medicine, Universiti Putra Malaysia.

CHAPTER 4

MOLECULAR DETECTION AND CHARACTERISATION OF PORCINE CIRCOVIRUS 2 (PCV2)

Article 3: Genotype Shift of Malaysian Porcine Circovirus 2 (PCV2) from PCV2b to PCV2d Within a Decade

4.1 Introduction

Porcine circovirus 2 (PCV2) is considered ubiquitous in the swine population. In the history of circoviruses of swine, porcine circovirus type 1 (PCV1) was first discovered in 1974 as an apathogenic cell culture contaminate (Tischer *et al.*, 1982). Two decades later, the first clinical outbreak of postweaning multisystemic wasting syndrome (PMWS) was reported from December 1994 to March 1996, with a characteristic presentation of unthriftiness, jaundice, respiratory distress, diarrhea and increased postweaning mortality rate (Clark, 1996; Harding, 1996). PCV2 antigen was detected in multiple organs from postweaning pigs with PMWS-associated clinical signs and pathological changes (Ellis *et al.*, 1998). The term porcine circovirus associated diseases (PCVAD) was coined by American Association of Swine Veterinarians (AASV) in March 2006 to classify the complex PCV2-related multi-factorial diseases under one umbrella term (AASV, 2006). Another two decades from the report of PCV2, a genetically divergent circovirus, designated porcine circovirus type 3 (PCV3), was uncovered through metagenomic sequencing when investigating a case of increased sow mortality rate and decreased conception rates with presenting dermal and renal lesions, suggestive of porcine dermatitis and nephropathy syndrome (PDNS) (Palinski *et al.*, 2017). Most

recently in 2019, porcine circovirus type 4 (PCV4) was reported to be the newest PCV member. The index herd was reported to show severe clinical signs of respiratory disease, enteritis and PDNS (Zhang *et al.*, 2020b). As with other members of the family Circoviridae and genus Circovirus, PCV2 is an icosahedral, non-enveloped virus which assembles into a single-stranded ambisense circular genome (Delwart & Li, 2012). With genome length ranging from 1766 bp to 1769 bp, PCV2 is larger than PCV1 (1758 – 1760 bp); smaller than PCV3 (1999 – 2001 bp) and PCV4 (1770 bp) (Meehan *et al.*, 1997; Fenaux *et al.*, 2000; Palinski *et al.*, 2017; Zhang *et al.*, 2020b). Despite their different lengths, three common major open reading frames (ORFs): ORF1, ORF2 and ORF3, have been recognized in the genomes of all porcine circoviruses. Respectively, ORF1, ORF2 and ORF3 are responsible for encoding proteins involved in viral genome replication (Rep and Rep'), capsid protein (Cap), and possibly in pathogenesis (Nawagitgul *et al.*, 2000; Mankertz *et al.*, 2004; Liu *et al.*, 2005; Liu *et al.*, 2007). Based on a more recent genome-wide, pairwise identity-based genome identity calculation method, pairwise identity cut-off for circovirus species fell between 78% to 80%; with PCV1 and PCV2 sharing 79% genome-wide pairwise identity. PCV1 and PCV2 shares 86% and 65% similarity of amino acid (aa) identity in their ORF1 and ORF2 respectively (Meehan *et al.*, 1998; Morozov *et al.*, 1998). Considering the fact that PCV1 and PCV2 have always been considered as two separate species as supported by their distinct biological differences in terms of pathogenicity, species demarcation for circoviruses is delineated at 80% (Rosario *et al.*, 2017*).* PCV3 and PCV4 show lower identity similarity. Compared to PCV2, PCV3 shows only < 50% of overall nucleotide (nt) identity, 48% and 26 – 36%

aa identity of ORF1 and ORF2 respectively (Phan *et al.*, 2016; Palinski *et al.*, 2017). Respective to the genomes of PCV1, PCV2 and PCV3, PCV4 showed 50.3%, 51.5% and 43.2% nt similarities (Zhang *et al.*, 2020b).

Cap gene was focused in this study given that it is known to show the highest genetic variation when compared to other regions of the entire PCV2 genome sequence (Nawagitgul *et al.*, 2000; Olvera, Cortey & Segales, 2007). By observing the differences over nucleotide sequences of *cap* genes, the population diversity and genetic variation of PCV2 strains could be assessed (Davies *et al.*, 2016). After 16 years of vaccination effort against PCV2, although the virus is still circulating in production systems, PCV2 positive PCVAD cases are reported at a declining rate (Afghah *et al.*, 2017). Being a single-stranded DNA virus with high nucleotide substitution rates comparable to RNA viruses, genome mutation of PCV2 is expected to be high (Firth *et al.*, 2009). Indeed, nucleotide polymorphism of PCV2 has been characterized in one decade post its first discovery (Hughes & Piontkivska, 2008). Since then, more novel genotypes and phylogenetic clusters of PCV2 continued to be discovered. To date, eight PCV2 genotypes, PCV2a through PCV2h, have been described (Franzo & Segalés, 2018). In Malaysia, presence of PCV2 and PCV3 in commercial swine herds has been confirmed (Jaganathan *et al.*, 2011; Tan *et al.*, 2020). The circulating PCV2 strains were determined to be 1767 bp in length and clustered as PCV2b genotype. This paper aims to update the molecular Characterisation of PCV2 strains circulating in both Malaysia commercial swine herds and in the wild boar population. Considering the continuing economic impact of PCV2 on the swine farming industry

worldwide, there is a need to update the genetic characteristics of current Malaysian PCV2 strains as part of the collective effort in optimizing PCV2 disease management and control.

4.2 Materials and Methods

4.2.1 Animals

A total of 111 lung and inguinal lymph nodes samples from the authors' previous PCV3 sample collection were included in this study. Samples origin and collection method were as previously described (Tan *et al.*, 2020). Briefly, 37 commercial intensive farms in Penang, Perak; Selangor; Melaka and Johor states representing northern; central and southern regions of Peninsular Malaysia respectively were involved. The farms were run on a semi-closed house, farrow-to-finish system, with farm size ranging from 200 to 1500 sows. Samples consisted of archived lung samples from 2016 to 2017; farm clinical samples, and abattoir lung samples collected from 2018 to 2021. Apart from that, 28 lung samples from the wild boar population in Peninsular Malaysia collected in 2019 were also included in this study.

4.2.2 Molecular Detection of PCV2

DNA extraction was performed using the DNeasy Blood & Tissue Kit extraction kit (Qiagen, Germany) in accordance with the manufacturer's instructions. Conventional PCR were performed using MyTaq™ Red Mix 2X (Bioline, United Kingdom). Published primers, FortF–5'–CTTTTTTATCACTTCGTAATG–3' and FortR–5'–

CGCACTTCTTTCGTTTTC–3', were used to amplify the capsid (cap) region of PCV2 (Fort *et al.*, 2007). Briefly, 12.5 µL of Taq DNA polymerase master mix and 1.0 µM each from the primer pair were used in a 25 µL total PCR reaction volume. Cycling conditions of the conventional PCR were as referred (Fort *et al.*, 2007). PCR products were stained using RedSafe™ nucleic acid staining solution (iNtRON Biotechnology, South Korea) and analysed by agarose electrophoresis. Expected product length was a 760 bp representing PCV2 cap region spanning from position nt 998 – 1757. Samples that showed a positive band at the between 700 – 800 bp region as marked by GelPilot 100 bp Plus Ladder (Qiagen, Germany) were further sequenced (Macrogen, South Korea). Since the samples were obtained from a PCV3 study, the relative risk (RR) and odds ratio (OR) of co-infection with PCV3 were computed as well, using IBM SPSS Statistics for Windows v23 software (SPSS, I. B. M., 2016).

4.2.3 Pairwise Sequence Comparison (PASC) Analysis

Seventy-nine samples positive for PCV2 *cap* gene by PCR detection were sequenced to confirm the identity of the PCV2 *cap* gene nucleotide sequences: 51 sequences from domestic pigs and 28 sequences from wild boars. Sequence assembly and multiple sequence alignment were generated with the MUSCLE algorithm using MEGA v7.0.26 software (Kumar, Stecher & Tamura, 2016). The resulting sequences were analyzed using NCBI Nucleotide-BLAST® for a final identity confirmation as PCV2 by comparing the nucleotide similarity with reference PCV2 sequences deposited in the GenBank (Altschul *et al.*, 1990). Another 162 PCV2 *cap* gene nucleotide sequences were downloaded from GenBank to be used as reference

sequences in the subsequent phylogenetic analysis. The sequences were ensured to be well-aligned, in the correct reading frame without degenerate nucleotides or indels, of different country origin, and representative of clusters PCV2a through PCV2h. Pairwise distance estimation based on a p-distance nucleotide substitution model with 1000 bootstrap replicates was calculated across the entire set of 241 PCV2 *cap* gene nucleotide sequences, using the same MEGA v7.0.26 software. Based on the pairwise distance estimation data matrix, pairwise sequence comparison (PASC) analysis was performed by constructing a p-distance/frequency histogram using Microsoft Excel 2019 to determine possible cut-off values for distinguishing different PCV2 genotypes.

4.2.4 Phylogenetic Analysis & Genotyping

A neighbor-joining (NJ) phylogenetic tree based on p-distance nucleotide substitution model with 1000 bootstrap replicates was constructed to analyze the 241 PCV2 *cap* gene nucleotide sequences dataset, similarly with MEGA v7.0.26 software described previously (Kumar, Stecher & Tamura, 2016). Resulting phylogenetic clustering was used to classify the PCV2 sequences into genotypes PCV2a to PCV2h. The phylogenetic-based genotype classification was tallied with genotype classification systems proposed in recent years (Franzo *et al.*, 2015a; Franzo & Segalés, 2018).

4.2.5 Nucleotide Sequence Polymorphism Analysis

Malaysian PCV2 *cap* gene nucleotide sequences obtained in this study were further analysed for selection pressure. Tajima's D, Fu and Li's D, and Fu and

Li's F statistical tests of neutrality were performed using DnaSP v6.12.03 software programme (Tajima, 1989; Fu & Li,1993; Rozas *et al.*, 2017). Statistical significance was set at $p \leq 0.05$ for all three tests. Positive and negative selective pressures acting on codon of the *cap* gene sequences were estimated based on calculated difference between non-synonymous (dN) and synonymous (dS) substitution rates per codon. Single-likelihood ancestor counting (SLAC), fixed-effects likelihood (FEL), fast, unconstrained Bayesian approximation (FUBAR) and mixed effects model of evolution (MEME) selection pressure inferring methods were run in the DataMonkey web server (http://www.datamonkey.org/) (Kosakovsky Pond & Frost, 2005; Murrell *et al.*, 2012; Murrell *et al.*, 2013; Weaver *et al.*, 2018). To infer dN and dS rates, FUBAR uses Bayesian approach; FEL and MEME utilize ML approach; while SLAC incorporates additional counting approaches. FEL, SLAC and FUBAR detect both positive and negative selection, but MEME focuses on detecting aa sites evolving under positive selection. For the FUBAR method, positively-selected sites were identified when $\alpha < \beta$ at posterior probability (pp) > 0.9 where α represents mean posterior synonymous substitution rate at a site and β represents mean posterior non-synonymous substitution rate at a site. Negatively-selected sites were identified when $\alpha > \beta$ at pp > 0.9. For the FEL method, positively-selected sites were identified when $\beta > \alpha$ at $p < 0.05$ where α represents synonymous substitution rate at a site and β represents non-synonymous substitution rate at a site. Negatively-selected sites were identified when $\beta < \alpha$ at $p < 0.05$. For the SLAC method, positively-selected sites were identified when $dN / dS > 1$ at $p < 0.05$ where dS represents inferred synonymous substitution rate at a site and dN represents inferred non-

synonymous substitution rate at a site. Negatively-selected sites were identified when dN / dS < 1 at p < 0.05. For the MEME method, positively-selected sites were identified when p+ > 0 at p < 0.05, where p+ represents mixture distribution weight allocated to β+ and β+ represents non-synonymous substitution rate at a site for positive selection. For each site where statistically significant selection pressure had been identified, statistical method consensus (n) across FUBAR, FEL, SLAC and MEME methods was recorded. Further, to evaluate aa residue diversity of PCV2 *cap* gene nucleotide sequences, Shannon entropy (Hx) values were calculated with BioEdit software v7.2.5 using the Shannon entropy formula: $-(\sum_{j=1}^{4} p_{ij} \log_2 p_{ij})$ where *i; j* is equal to 1, 2, 3 and 4, corresponding to A, C, G and T nucleotides and *pij* being the proportion of nucleotide *j* in site *i.* Entropy plots of aa sequences of the *cap* genes were constructed to plot the diversity of aa residues at a given position (Shannon, 1948; Hall, 1999). In the context of diversity of aa residues at a given position, Hx value of entropy will present as 0.0 when only a single residue is present. As the Hx value increases, it would be more likely to observe different aa residues diversity at the same codon position. A Hx value of 4.322 indicates all 20 residues are equally represented. Aa with Hx $\geq$ 2.0 are considered variable, while highly conserved aa would be expected to have Hx of $\leq$ 1.0 (Litwin & Jores, 1992). Range, mean and standard error of mean (SEM) were calculated using previously described IBM SPSS Statistics (SPSS, I. B. M., 2016).

4.2.6 Rates of Substitution

Estimations of the rate of substitution of PCV2 *cap* gene nucleotide sequences were calculated by running Bayesian Markov chain Monte Carlo (MCMC) algorithm using BEAST v1.10.4 software (Drummond *et al.*, 2012). Three independent MCMC runs were computed for the complete dataset of 241 PCV2 *cap* gene nucleotide sequences, under relaxed molecular clock model, Hasegawa–Kishono–Yano (HKY) substitution model, gamma site heterogeneity model and the remaining default parameters in the prior's panel. Bayesian Skygrid tree prior was selected to account for different population dynamics through time, set at 52 parameters and one time at the last transition point (Gill *et al.*, 2013; Hill & Baele, 2019). The MCMC run was set with 2×10^8 length of chain, with posterior probability distribution of the long chains to be sampled every 1000 steps. Convergence to the same posterior distribution was assessed using Tracer v1.7.2, first by visual appraisal of the runs' trace plot, followed by ensuring effective sampling size of greater than 200 after a 10 % burn-in (Suchard *et al.*, 2018). Estimations of rates of substitution were obtained from the three independent runs using LogCombiner software v1.10.4 (part of the BEAST v1.10.4 package), with 95 % CI calculated. MCMC runs were then repeated as described above, on data subsets of PCV2a, PCV2b and PCV2d *cap* gene nucleotide sequences independently.

4.3 Results

4.3.1 Molecular Detection of PCV2

Out of the 79 domestic pigs sampled from 37 commercial swine farms in Peninsular Malaysia, 66 pigs were positive for PCV2 based on PCR detection, amounting to a molecular detection rate of 83.54%. At farm level, 31 out of 37 farms (83.78%) were tested positive (Table 5). Notably, higher PCV2 molecular detection rates were observed in abattoir lung samples and wild boar samples: 18 out of 19 (94.74%) abattoir samples from clinically healthy finishers and all 28 (100%) wild boar lung samples were tested positive for PCV2 antigen.

Table 5 : PCV2 PCR Positive Rate Reported in This Study. Samples Consisted of Organ Samples Collected from Domestic Pig Farms, Abattoir and Wild Boar Population of Peninsular Malaysia

PCV2 PCR Detection Rate	Domestic Pig				Abattoir	Wild Boar
	Northern Malaysia	Central Malaysia	Southern Malaysia	Overall		
% PCR Positive (Animals)	93.10% (27/29)	82.76% (24/29)	71.43% (15/21)	83.54% (66/79)	94.74% (18/19)	100.00% (28/28)
% PCR Positive (Farms)	92.86% (13/14)	85.71% (12/14)	66.67% (6/9)	83.78% (31/37)	-	-

Among the farm samples, PCV2 was detected across all production age groups as tabulated in Table 6.

Table 6 : Distribution of PCV2 Positive Samples Across Production Age Groups. Molecular Detection Status of PCV2 Was Tabulated for Each Production Age Group from Foetus to Sow

Production Age Group	% PCR Positive (Number of Positive Animals / Tested Animal)
Foetus	100% (7/7)
Piglet	100% (2/2)
Weaner	80.36% (45/56)
Grower	83.33% (10/12)
Sow	100% (2/2)

Co-infection rate of PCV2 and PCV3 among the samples tested in this study was 28.77% (21 / 73). Relative risk of PCV2 and PCV3 co-infection was determined to be 1.25 (95% CI, p: 0.01); suggesting that PCV3 positive animals could be 25% more likely to be positive for PCV2 (Table 7). Odds ratio was found to be statistically insignificant (OR: 6.46, 95% CI, p: 0.08).

Table 7 : Distribution of PCV2 and PCV3 Co-Infection Molecular Detection Status. Relative Risk (RR) and Odds Ratio (OR) of PCV2 Co-Infection with PCV3 Were Computed Based on the Molecular Detection Status Tabulated Above

Detection Status of Tested Pig (Number of Individuals)	PCV2 Positive	PCV2 Negative
PCV3 Positive	21	1
PCV3 Negative	39	12

4.3.2 Pairwise Sequence Comparison (PASC)

Pairwise distance estimation based on a p-distance nucleotide substitution model calculated across the 241 PCV2 *cap* gene nucleotide sequence dataset computed a p-distance value matrix ranging from 0.000 to 0.193. The sequences shared at least 80.7% of nucleotide identity similarities. Excluding three strains that appeared to be outliers (PCV2/PG43I/UPM/MY007/OM524604, PCV2/WB1/UPM/MY052/OM524649 and PCV2/WB10/UPM/MY060/OM524657), the p-distance range narrowed to show that Malaysian PCV2 *cap* gene nucleotide sequences shared at least 91.16% of nucleotide identity similarities. When sequences from domestic pigs and wild boars were compared independently, sequences from domestic pigs were more closely related to each other at 94% nucleotide identity similarities; compared to 88.46% when wild boar sequences were analysed jointly.

Pairwise sequence comparison (PASC) analysis based on PCV2 cap gene sequences resulted in a multimodal curve with several distance thresholds ranging from 0.021 to 0.162 (Figure 1). All the threshold values observed in this analysis did not match previously proposed cut-off values of 0.035, 0.068 and 0.09 (Grau-Roma *et al.*, 2008; Franzo *et al.*, 2015a). Further, only seven threshold values could be identified in this PASC graph. Numerous frequency bar clusters were too low for clear demarcation, leaving two ambiguous threshold points between 0.113 – 0.162 and two points between 0.162 – 0.201. This PASC analysis could not distinctly classify all currently known eight genotypes of PCV2.

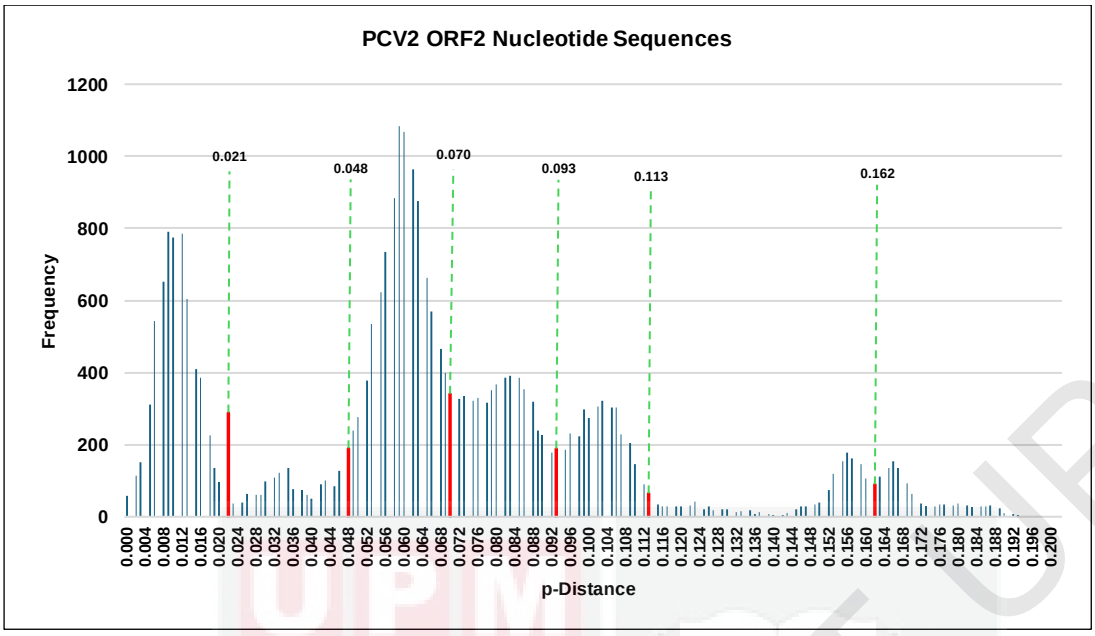

Figure 1: PASC analysis of PCV2 cap gene nucleotide sequences. Pairwise sequence comparison for PCV2 cap gene nucleotide sequences were calculated based on pairwise p-distances within a 0.01 p-distance interval. PASC distance thresholds were previously used to define PCV2a and PCV2b genotypes. Seven distance thresholds ranging from 0.021 to 0.162 were identified in this multimodal curve, denoted by red lines

4.3.3 Phylogenetic Analysis & Genotyping

Seventy-nine PCV2 *cap* gene nucleotide sequences were sequenced successfully in this study; 51 sequences from domestic pigs and 28 sequences from wild boars. The Malaysian PCV2 *cap* gene sequences reported in this study have been deposited at GenBank (http://www.ncbi.nlm.nih.gov) under accession numbers OM524598 – OM524676. NCBI Nucleotide-BLAST® analysis results confirmed the identity of the 79 nucleotide sequences as PCV2, sharing over 99% similarity with at least 100 reference PCV2 *cap* gene nucleotide sequences recorded in GenBank. All but one of the Malaysian *cap* gene nucleotide sequences are 701 bp in length, encoding a 233 aa Cap protein; the exception being sequence OM524649 which is 702 bp long.

A neighbor-joining (NJ) phylogenetic tree was constructed to analyze the 79 Malaysian PCV2 *cap* gene nucleotide sequences in relation to 162 GenBank reference sequences from different countries and different genotype clades. The condensed resulting phylogenetic trees were shown as Figure 2. Wild boar *cap* gene nucleotide sequences were indicated with “WB” suffix. All 241 *cap* gene nucleotide sequences were grouped in genotype clades PCV2a through PCV2h. The resulting clustering matches the PCV2 genotype classification obtained by phylogenetic analysis generated based on a collection of strains representative of the proposed PCV2 genotypes (Franzo & Segalés, 2018).

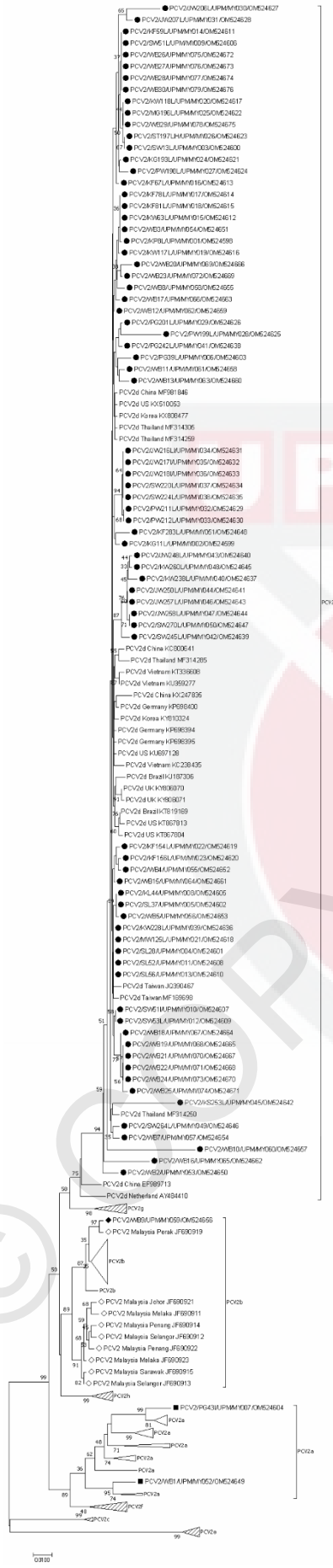

Figure 2: Condensed phylogenetic analysis of PCV2 cap gene nucleotide sequences. Seventy-nine Malaysian PCV2 cap gene nucleotide sequences (■ PCV2a, ◆ PCV2b, ● PCV2d) were compared with 162 other GenBank reference sequences from different countries and different genotype clades including previously published Malaysian sequences (denoted ◇). PCV2a and PCV2b clades were partially condensed, showing only Malaysian sequences. PCV2c, PCV2e, PCV2f, PCV2g and PCV2h clades were entirely condensed. GenBank accession numbers, origin country and genotype are as indicated. Malaysian PCV2 cap gene sequences were additionally labelled with sequence ID. The tree was constructed using the NJ method, p-distance nucleotide substitution model with 1000 bootstrap replicates. The scale bar indicates branch length measured in number of substitutions per site

The most apparent observation was that a 96.2% majority of the latest Malaysian sequences (76 / 79 sequences) were categorized as PCV2d. Among the domestic pig PCV2 strains, 98.04% (50 / 51) were classified as genotype PCV2d. As for the wild boar PCV2 strains, 92.86% (26 / 28) belonged to genotype PCV2d. Genotypes PCV2a and PCV2b were also identified in this study. The sole PCV2b sequence was identified from a wild boar sample (WB9/MY059/OM524656), sharing a clade with a Malaysian PCV2 strain dated back in 2007 (JF690919), originating from the northern region. Two PCV2a sequences were identified, one strain collected from a domestic pig (PG43I/MY007/OM524604) and the other strain originating from a wild boar (WB1/MY052/OM524649). These phylogenetic-based clustering were tallied with the pairwise distance analysis, which reaffirmed that Malaysian PCV2 *cap* gene nucleotide sequences reported in this study shared more similarities with PCV2d reference strains in GenBank deposit. The sole Malaysian PCV2b sequence (WB9/MY059/OM524656) and the only two Malaysian PCV2a sequences (PG43I/MY007/OM524604 and WB1/MY052/OM524649) also turned out to be have further pairwise distances from all other Malaysian PCV2 strains. PCV2 genotypes reported in Malaysia since 2007 were tabulated chronologically in Figure 3, including the previous Malaysia report (Jaganathan *et al.*, 2011).

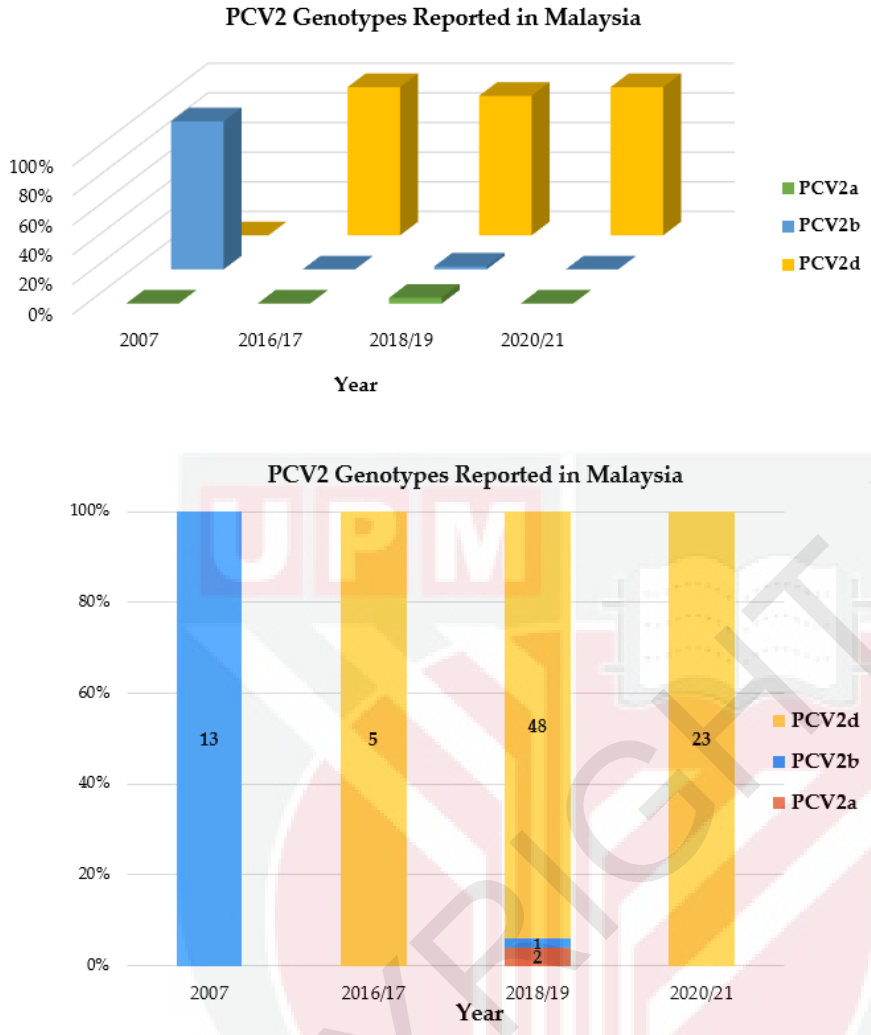

Figure 3: Chronology of PCV2 genotypes reported in Malaysia. PCV2 genotypes re-ported in Malaysia since 2007 were tabulated chronologically to illustrate the trend of PCV2d genotype predominance. Figures within the bars represent the total number of Malaysian PCV2 cap gene sequences

When the genotypes of 79 Malaysian PCV2 cap gene nucleotide sequences were tallied with the genotype specific marker nucleotide system proposed by Franzo *et al.* (2015a), Malaysian PCV2a and PCV2b strains fit completely into the system classification. For Malaysian PCV2d strains, 88.16% (67/ 76) strains completely match the marker positions. The nine strains with dissimilar nucleotides at the marker positions were listed in Table 8.

Table 8: Malaysian PCV2d cap gene nucleotide strains agreement with marker nucleotide positions. Malaysian PCV2a and PCV2b strains match completely into Franzo *et al.* (2015) system classification. Nine PCV2d strains that did not fit into the system were tabulated above together with overall agreement %

Marker Nucleotide Position Malaysian PCV2d Sequences	157 A	162 T	513 C	585 T	643 A
KL44/OM524605	A	T	A	T	A
SL37/OM524602	A	T	A	T	A
PG201L/OM524626	A	T	A	T	A
PG242L/OM524638	A	T	A	T	A
PG39L/OM524603	A	T	A	T	A
PW199L/OM524625	A	T	A	T	A
WB2/OM524650	T	A	C	T	A
WB5/OM524653	A	T	A	T	A
WB10/OM524657	T	T	C	T	A
% Sequences not matching marker nucleotides	2.63% (2/76)	1.32% (1/76)	9.21% (7/76)	0.00%	0.00%

Although wild boar sequences tend to form distinct clades, their clades and several individual strains could be found interspersed within clades of domestic pig sequences. Some clades between domestic pigs and wild boar sequences were supported by substantial bootstrap of 33% to 97%. Generally, the Malaysian PCV2 *cap* gene nucleotide sequences tend to form large clusters according to their year of origin. This pattern was especially evident in the clade of 2016 / 2017 sequences; and the large cluster containing the majority of 2020 / 2021 sequences. As expected, sequences collected from a same farm at one single time point would be grouped in one clade, such as sequences JW216LI/MY034/OM524631, JW217I/MY035/OM524632, JW218I/MY036/OM524633 from Farm S5; SW220L/MY037/OM524634,

SW224L/MY038/OM524635 from Farm C8/C9; SW51I/MY010/OM524607, SW53L/MY012/OM524609 from Farm C1; and JW257L/MY046/OM524643, JW258L/MY047/OM524644 from Farm S2. However, when sampling intervals spanned over months to years, sequences originating from one farm may no longer share a close phylogenetic relationship. For instance, sequences KG11L/MY002/OM524599, KW63L/MY015/OM524612, KF67L/MY016/OM524613, KF78L/MY017/OM524614, KF81L/MY018/OM524615 and KF283L/MY051/OM524648 collected from Farm N3 over 2018/2019 to 2020/2021, were dispersed from each other even though they were still within a bigger cluster. In February 2019, PCV2a genotype (PG43I/MY007/OM524604) was detected in Farm N4. Later in December 2019 and July 2020, strains from the same farm (PG201L/MY029/OM524626 and PW212L/MY033/OM524630) were identified as genotype PCV2d.

A unique phylogenetic pattern was noted for two neighboring farms: Farm N4 and N7. PCV2 strains of the two farms appeared to evolve phylogenetically in tandem. Sequence PW212L/MY033/OM524630 (Farm N4) and sequence PW211L/MY032/OM524629 (Farm N7) collected in year 2020/2021 were observed to be closely related; just as sequence PG201L/MY029/OM524626 (Farm N4) and sequence PW199L/MY028/OM524625 (Farm N7) collected back in year 2018/2019 were found with closer phylogenetic relationship. Furthermore, co-existence of two different PCV2 strains within one individual pig was noted when sequences PCV2/SW51L/UPM/MY009/OM524606 and PCV2/SW51I/UPM/MY010/OM524607 obtained from lung and inguinal lymph

node respectively of the same pig were found to be genetically and phylogenetically distinct.

4.3.4 Nucleotide Sequence Polymorphism Analysis

To determine positive and/or negative selections in the PCV2 *cap* gene nucleotide sequences, neutrality test values, dN – dS values and Hx values of the *cap* gene were evaluated. The sequences were analyzed separately according to their genotype: PCV2a, PCV2b or PCV2d. Results from the statistical tests of neutrality ran on PCV2 *cap* gene nucleotide sequences were summarized in Table 9. All three tests showed unanimous negative neutrality values, of which analysis values for PCV2b and PCV2d genotypes showed statistical significance.

Table 9: Results tabulation of PCV2 cap gene nucleotide sequences statistical tests of neutrality. PCV2 cap gene nucleotide sequences were analyzed separately according to their genotype to obtain their neutrality test values which determine positive and/or negative selection pressure

PCV2 Genotype	Tests of Neutrality					
	Tajima's D		Fu and Li's D		Fu and Li's F	
	Test statistic	Statistical significance, p-value	Test statistic	Statistical significance, p-value	Test statistic	Statistical significance, p-value
PCV2a	-1.2028	> 0.10	-2.2422	0.10 > P > 0.05	-2.2331	0.10 > P > 0.05
PCV2b	-2.1293	< 0.05	-3.3600	< 0.05	-3.4549	< 0.02
PCV2d	-2.5171	< 0.001	-5.8800	< 0.02	-5.2945	< 0.02

4.3.5 Selection Pressure

Further, positive and negative selective pressures acting on codons of PCV2 cap gene nucleotide sequences were determined based on calculated difference between non-synonymous (dN) and synonymous (dS) substitution rates per codon. Summary of single-likelihood ancestor counting (SLAC), fixed-effects likelihood (FEL), fast, unconstrained Bayesian approximation (FUBAR) and mixed effects model of evolution (MEME) selection pressure inference results were tabulated in Table 10. At the statistical significance thresholds applied in this study, a higher number of negative selections was observed across all PCV2 genotypes. Up to 13.73% (32 / 233) of codons were identified to be under negative selection, contrasting to only 1.72% (4 / 233) – 3.86% (9 / 233) of codons identified to be influenced by positive selection. Proportion of negatively selected codons reaching consensus across all selection pressure inferring methods was also higher in the negative selection group, at 28.13% (9 / 32) – 43.75% (14 / 32), compared to the 14.29% (1 / 7) in the positive selection group. Among the four positive selection pressure inferring methods used, descending detection rates were observed from MEME, FUBAR, FEL to SLAC method. Similar detection rate patterns were seen across FUBAR, FEL to SLAC methods for negative selection.

Table 10: Selection pressures acting on codons of PCV2 cap gene nucleotide sequences. Proportion of codons under statistically significant positive and negative selective pressure were identified. Selection pressure inferring methods applied were FUBAR, FEL and SLAC for both positive and negative selection; with an additional method MEME for positive pressure. Statistical significance was set at pp > 0.9 for FUBAR and p < 0.05 for FEL, SLAC and MEME

PCV2 Genotype	Positive Selection									Negative Selection						
	Total Positively Selected Codon	Selection Pressure Inference Methods				Statistical Consensus across Methods				Total Negatively Selected Codons	Selection Pressure Inference Methods			Statistical Consensus across Methods		
		FUBAR	FEL	SLAC	MEME	n = 1	n = 2	n = 3	n = 4		FUBAR	FEL	SLAC	n = 1	n = 2	n = 3
PCV2 a	3.86% (9/233)	3	1	0	7	8	0	1	0	13.73% (32/233)	29	31	14	4	14	14
PCV2 b	1.72% (4/233)	3	0	0	1	4	0	0	0	13.73% (32/233)	30	22	9	12	11	9
PCV2 d	3.00% (7/233)	3	1	1	6	5	1	0	1	13.30% (31/233)	27	26	9	9	13	9

Shannon entropy value (Hx) for each aa position of PCV2 cap gene codon was plotted in a line chart (Figure 4), with the analysis summarized in Table 11. With the exception of aa position 63 and 131 of genotype PCV2a where the Hx values were 1.078 and 1.192 respectively, all the recorded Hx values were well below 1.0. Comparatively among genotypes, PCV2d cap gene nucleotide sequences have the most aa sites with entropy values of > 0.0, at 31.76% (74 / 233), values ranging from 0.055 to 0.8977.

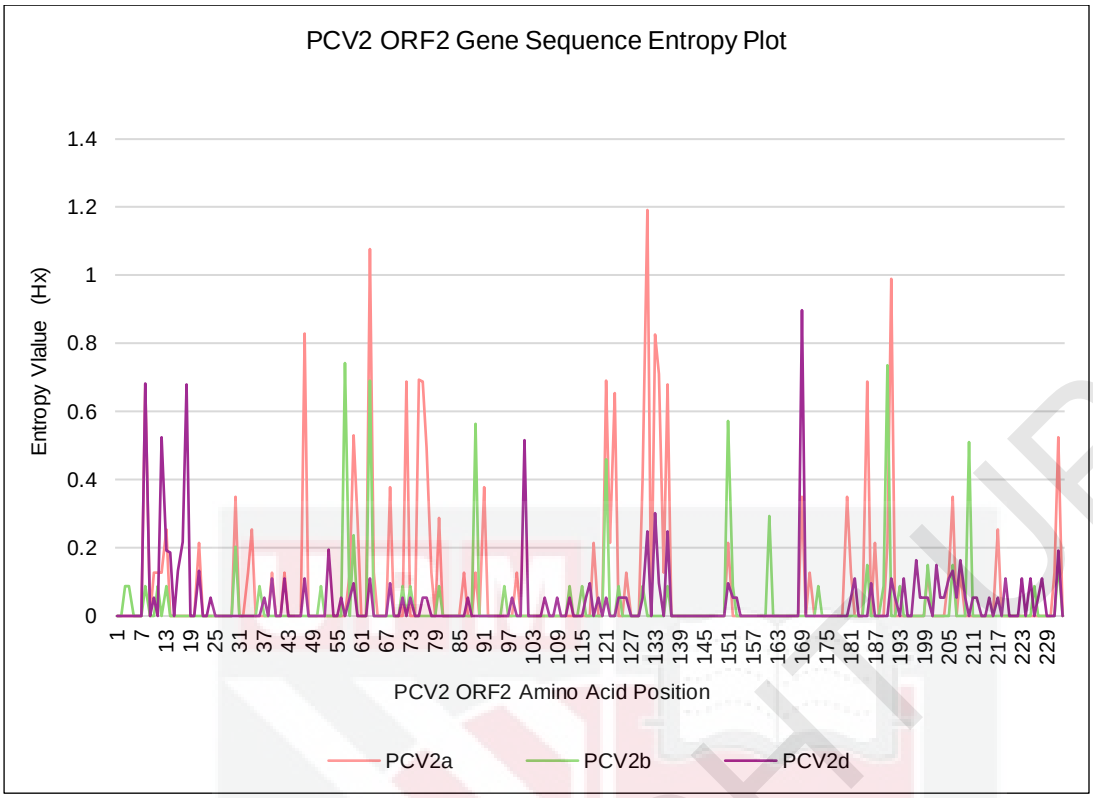

Figure 4: Entropy plot of PCV2 ORF2 gene sequences. Shannon entropy analysis were carried out separately for each genotype PCV2a, PCV2b and PCV2d; and Hx value for each aa position was plotted

Table 11: Shannon entropy values for PCV2 cap gene nucleotide sequences. Hx values computed for each aa position of the gene nucleotide sequences were summarized separately for each genotype PCV2a, PCV2b and PCV2d

PCV2 Genotype	ORF2 aa site with Hx values > 0.0	Lowest Hx Value	Highest Hx Value	Standard Deviation, σ
PCV2a (36)	23.61% (55 / 233)	0.127	1.192	0.208
PCV2b (58)	15.02% (35 / 233)	0.087	0.743	0.111
PCV2d (103)	31.76% (74 / 233)	0.055	0.898	0.108

4.3.6 Rates of Substitution

Estimations of the rate of substitution of PCV2 *cap* gene nucleotide sequence dataset generated from the Bayesian Markov chain Monte Carlo (MCMC) algorithm were detailed in Table 12. For all the replicate runs in the three independent MCMC analysis, all parameters and densities of interest were found to converge to the same posterior distribution of a 10% burn-in, with large effective sample size (ESS) values well exceeding 200.

Table 12: Estimations of rate of substitution for PCV2 cap gene nucleotide sequences. Estimations of the rates of substitution were generated from the Bayesian MCMC algorithm. For all replicate runs of the three independent MCMC analysis, the results were found to fit burn-in and ESS validity criteria

PCV2 Genotype	Mean Rate of Substitution (Substitution Per Site Per Year)	95% Highest Posterior Density (HPD)	Effective Sample Size (ESS)
PCV2a	4.746×10^{-4}	8.463×10^{-4}	246638
PCV2b	6.571×10^{-4}	1.024×10^{-3}	255628
PCV2d	2.111×10^{-3}	2.925×10^{-3}	68174
Combined analysis for all genotypes	1.102×10^{-3}	1.374×10^{-3}	82401

Overall, when all three genotypes PCV2a, PCV2b and PCV2d were analysed collectively, the substitution rate for PCV2 cap gene was estimated to be 1.374 x 10-3 substitutions per site per year (ssy). When fractionated according to genotypes, estimated substitution rate of PCV2d genotype was the highest at 2.111 x 10-3 ssy; compared to that of 4.746x 10-4 ssy and 6.571 x 10-4 ssy observed in PCV2a and PCV2b genotypes respectively.

4.4 Discussion

First case of porcine circovirus 2 (PCV2) in Malaysia was reported by the Malaysian Veterinary Research Institute. The said PCV2 DNA was identified using PCR-restriction fragment length polymorphism (PCR-RFLP) assay in a respiratory-related clinical case (Hassuzana *et al.*, 2004). Later, PCV2 was reported in the first described post-weaning multisystemic wasting syndrome (PMWS) clinical case in Malaysia, with a PCR molecular detection rate of 83.33% (10 / 12 organ samples) (Ooi *et al.*, 2007). The last published update of Malaysian PCV2 reported a PCR molecular detection rate of 88.10% (37 / 42 farms) (Jaganathan *et al.*, 2011). This present study reported a similar level of prevalence: 83.78% (31 / 37) at farm level and 83.54% (66 / 79) in sampled domestic pig populations. Southern region of Malaysia showed the lowest molecular detection rate, possibly due to farther farm-to-farm distances which contribute to stronger biosecurity control.

Previously, presence of PCV2 strains in Malaysia was only reported in clinically ill domestic pig populations. This study reported a relatively high molecular detection rate of 94.74% (18 / 19) in abattoir samples originating from clinically healthy finishers. PCV2 was also detected in clinically ill pigs from the same farms that supplied the said abattoirs. It is known that PCV2 can be present in both clinically healthy and ill pigs, in pigs with or without PMWS presentation. This observation suggested the need for additional infectious and non-infectious factors to trigger PCV2 replication up to an extent of overwhelming immune system (Calsamiglia *et al.*, 2002; Madec *et al.*, 2008; Grau-Roma, Fraile & Segalés, 2011; Segalés, 2012). Although the implication

of detecting PCV2 in healthy animals in this study still warrants further investigation, potential PCV2 transmission amongst swine herds through apparently healthy animals should not be ignored (Madson *et al.*, 2009). Detection across all production age groups from foetus, piglet, weaner, grower to sow at 80.36% (45/56) to 100% (7/7) was reported in this study, reiterating that PCV2 infection can occur across all pig production age groups (Sibila *et al.*, 2004; Grau-Roma *et al.*, 2009). PCV-associated disease (PCVAD) manifests as different clinical syndromes, and also as subclinical infection without conspicuous clinical sign have been shown to be associated with PCV2 (Sorden, 2000; Segalés, 2012).

Further, this is the first study to confirm the circulation of PCV2 in wild boar population roaming Peninsular Malaysia. PCV2 antigen was detected in all (28 / 28) of the tested wild boar organ samples. Serological and direct detection of PCV2 antigens in wild boars have been reported in various countries including Belgium, Brazil, Croatia, Czech Republic, Germany, Greece, Hungary, Italy, Korea, Poland, Romania, Serbia and Uruguay (Franzo *et al.*, 2015b; Sanchez, Nauwynck & Pensaert, 2001; Vicente *et al.*, 2004; Knell *et al.*, 2005; Cságola *et al.*, 2006; Lipej *et al.*, 2007; Sedlak *et al.*, 2008; Sofia *et al.*, 2008; Petrini *et al.*, 2009; Turcitu *et al.*, 2011; Fabisiak *et al.*, 2012; An *et al.*, 2014; Ramos *et al.*, 2018; Nisavic *et al.*, 2022). Seroprevalence of 20 – 48% have been reported in the European wild boar population, suggesting high circulation rate of PCV2 (Ruiz-Fons, Segalés & Gortázar, 2008). All 28 Malaysian wild boar PCV2 *cap* gene nucleotide sequences in this study shared at least 80.7% of nucleotide identity similarities with Malaysia domestic pig PCV2 *cap* gene

sequences. Reports from Greece, Hungary and Germany also found wild boar PCV2 isolates to be identical to those from domestic pigs, even when the sampling regions were distant (Knell *et al.*, 2005; Cságola *et al.*, 2006; Sofia *et al.*, 2008). In this study, clades of Malaysian wild boar sequence were observed to be interspersed among clades of domestic pig sequences, with several clades between domestic pigs and wild boar sequences supported by substantial bootstrap of 33% to 97%. This could suggest a possible epidemiological link between wild boar and domestic pig populations (Cságola *et al.*, 2006; Rudova *et al.*, 2022).

Pig farms in Malaysia are usually located in remote areas, in the vicinity of forests and oil palm plantations. Considering the increasing population and migratory behavior of wild boars, contact between domestic pigs and wild boars and resulting two-way pathogen transmission is highly possible (Brown, Bowen & Bosco-Lauth, 2018; Choi *et al.*, 2020). Direct contact between the domestic pigs and wild boars, or indirect contact through fomites such as leftover feed, are both possible. Moreover, through Thailand, Peninsular Malaysia is linked to neighboring countries including Vietnam and China, which are intersected by one of the many possible wild boar migratory routes (Choi *et al.*, 2020). It was noted that the wild boar sequences clustered with domestic pig sequences from every state except for Johor. The farms sampled in Johor have farther farm-to-farm distances and stronger biosecurity control including proper perimeter fencing which could minimize contact between wild boar and domestic pigs. However, the sample size from the region needs to be increased to be more conclusive.

Incidences of PMWS have also been reported in wild boars with clinical presentation and microscopic lesions similar to PMWS in domestic pigs (Vicente *et al.*, 2004; Sofia *et al.*, 2008; Petrini *et al.*, 2009). Recently, PCV2 was detected in samples of pregnant wild boar and their foetuses, suggesting that PCV2 may have similar tropism for foetal tissues and thus is also involved in PCV-RD just as in domestic sows (Pacini *et al.*, 2021). High seroprevalence of PCV2 and widespread geographical distribution pattern may suggest an endemic status of PCV2 in the wild boar population (Vicente *et al.*, 2004). Histopathologic lesions consistent with typical PMWS had been reported in wild boars (Schulze *et al.*, 2004; Vicente *et al.*, 2004; Toplak *et al.*, 2004). More recently, a case close to fulfilling PMWS's definition has been published: clinical signs of wasting and dyspnea were observed and PCV2 antigen were detected in lung, heart, intestine, and lymph nodes samples. Moreover, the wild boar in question was aged less than six to eight months old, nearer to the PMWS susceptible age group in cases of domestic pigs (Sorden, 2000; Sofia *et al.*, 2008).

Wild boars may potentially act as reservoir of PCV2, given that (1) there are a large number of wild boars and they may come in come in contact with confined domestic pigs; (2) PCV2 have been shown to infect wild boar and could cause PMWS; (3) PCV2 could transmit from wild boars to domestic pigs (Corner, 2006; Wobeser, 2007; Ruiz-Fons *et al.*, 2008; Franzo *et al.*, 2020d). It has been suggested that wild boars are potential reservoirs of viruses infectious to domestic pigs, and possibly vice versa from domestic pigs to wild boars. Reported virus transmission between these two groups include African

swine fever, classical swine fever virus, Aujeszky's disease virus and bovine viral diarrhea virus (Albina *et al.*, 2000; Laddomada, 2000; Vicente *et al.*, 2002; Zupancić *et al.*, 2002; Sauter-Louis *et al.*, 2021). Considering the significant impact of PCV2 disease in the domestic pig population, the direction of PCV2 transmission between domestic pigs and wild boars, as well as similarity of pathogenesis and clinical manifestation of PCV2 infection between the two groups should be further investigated. At the time being, avoiding close contact between wild boars and domestic pigs should be highlighted as an integral part of PCV2 disease control programme in farms. With the recent ASF outbreaks in several high domestic pig density areas, this biosecurity measure is especially critical in Malaysian domestic pig farming scenario now. Sightings of wild boars near pig farms are not uncommon; thus contact between wild boars and domestic pigs is certainly plausible and must be managed.

This study also reports co-detection of PCV2 and PCV3, where relative risk analysis found PCV3-positive pigs to be 25% more likely to be positive for PCV2. Porcine circovirus 3 (PCV3) and porcine circovirus 4 (PCV4) are two newly emerging virus in the swine industry, first reported in 2016 and 2019 (Palinski *et al.*, 2017; Zhang *et al.*, 2020b). Reports of co-infection of PCV2 with these two relatively new PCV species have surfaced, with the clinical implication of their co-existence in the swine host still being debated (Ouyang *et al.*, 2019a; Guo *et al.*, 2020; Visuthsak *et al.*, 2021; Xu *et al.*, 2022). PCV3-positive clinical samples were frequently found to be co-infected with PCV2 with co-infection rates of 27.6% to 69.74%, suggesting common occurrence of mixed PCV2 and PCV3 infection (Kim *et al.*, 2017; Li *et al.*, 2018d; Guo *et al.*,

2020). In view that both PCV2 and PCV3 predominantly infect macrophages of lymphoid tissues, co-infection of PCV2 and PCV3 could have clinical implication worth investigating further (Palinski *et al.*, 2017; Jiang *et al.*, 2019a).

The genomic variability of Malaysian PCV2 strains were compared by having their 701 – 702 bp long *cap* gene nucleotide sequences phylogenetically analysed as cap gene is the most variable region spanning the entire PCV2 genome sequence (Nawagitgul *et al.*, 2000; Olvera, Cortey & Segales, 2007). It has been established that PCV2 displays very high substitution rates, higher than expected for a DNA virus and instead akin to RNA viruses (Duffy, Shackelton & Holmes, 2008). For the PCV2 *cap* gene nucleotide sequences in this study, the estimated substitution rates for PCV2a, PCV2b and PCV2d genotypes were 4.746×10^{-4}, 6.571×10^{-4} and 2.111×10^{-3} substitutions/site/year (ssy) respectively. When all the sequences were analyzed together, the overall estimated rate was 1.102×10^{-3} ssy. High substitution rates as such, within the order of magnitude of $10^{-3} – 10^{-4}$, were in agreement with previous reports (Firth *et al.*, 2009; Perez *et al.*, 2011; Franzo *et al.*, 2015a; Franzo *et al.*, 2016a).

Furthermore, co-existence of two different PCV2 strains within one individual pig was observed, when PCV2 strains obtained from lung and inguinal lymph node respectively (PCV2/SW51L/UPM/MY009/OM524606 and PCV2/SW51I/UPM/MY010/OM524607) originating from the same pig were found to non-identical genetically, sharing 99.43% nucleotide identity similarity and belonging to rather distant phylogenetic clades. Circulation of different

PCV2 strains in a farm, or concurrent infection of different strains in one same pig, were suggested as potential for viral recombination, through recombination of two parental strains with various patterns and crossover regions (Hesse, Kerrigan & Rowland, 2008; Cai *et al.*, 2011; Zhai *et al.*, 2011; Chiou *et al.*, 2012). Strain clustering according to the year of origin was also a pattern observed in the phylogenetic analysis of this study. The evident clustering of the 2016 / 2017 clade and year 2020 / 2021 clade could suggest that mutation of PCV2 strains that occurs over time. As reported in the results section, when longitudinal sampling was carried out in one same farm, sequences showed further phylogenetic relationship when sampling interval spanned longer over months to years, again proffering speculation of PCV2 strain mutation with time.

High substitution rates can represent a substrate for selective pressures to act (Franzo *et al.*, 2016a). The unanimous negative values of all three neutrality tests signify an excess of low frequency polymorphisms relative to expectation, an effect resulting from either purifying selection or expansion of population size (Tajima, 1989; Fu & Li, 1993). Considering the general negative trend of dN – dS values as shown in Table 7, it was suggested that *cap* genes of PCV2 may be heavily influenced by negative selection pressure. In this study, *cap* gene codons indicated higher negative selection of 13.73% (32 / 233) compared to positive selection of 1.72 – 3.86% (4 – 9 / 233) at significance threshold used in this study, translating as a strong negative selection acting over *cap* gene of PCV2. In the context of Shannon entropy values (Hx), a large majority of the recorded Hx values were well below 1.0, supporting the

conjecture of PCV2 *cap* gene being under strong purifying selection processes (Nawagitgul *et al.*, 2000).

Pairwise sequence comparison (PASC) analysis based on PCV2 *cap* gene nucleotide sequences resulted in a multimodal curve with several distance thresholds, ranging from 0.021 to 0.162. This methodology could not distinctly classify all currently known eight genotypes of PCV2 (Franzo & Segalés, 2018). A neighbor-joining phylogenetic tree based on pairwise p-distance with 1000 bootstrap replicates divided the PCV2 strains into eight clusters, matching the previously published genotype classification (Franzo & Segalés, 2018). Most importantly, the most apparent observation in this study was a genotype shift from genotype PCV2b to PCV2d. In this study, 96.2% majority of the latest Malaysian sequences (76 / 79 sequences) were identified to be genotype PCV2d; a stark contrast to the previous Malaysian study conducted ten years ago which reported only PCV2b strains (Jaganathan *et al.*, 2011). However, the limitation of sample size in the aforementioned study needs to be taken into account. Nevertheless, the clear-cut predominance of genotype PCV2d revealed in this study warrants our attention. This observation of genotype shift matches the well-described PCV2 epidemiological dynamics, characteristically beginning with an initial rise of genotype PCV2a in the 90s which was rapidly superseded by the wave of PCV2b about ten years later (Cheung *et al.*, 2007; Carman *et al.*, 2008; Dupont *et al.*, 2008; Grau-Roma *et al.*, 2008; Wang *et al.*, 2009; Cortey *et al.*, 2011).

The rise of PCV2a and the subsequent shift to PCV2d broadly corresponds to incidences of PCVAD and to significant increase in frequency and severity of

clinical PCVAD outbreaks respectively (Cheung *et al.*, 2007; Gagnon *et al.*, 2007; Carman *et al.*, 2008; Dupont *et al.*, 2008). The results of this study matched the current occurrence of a second major PCV2 genotype shift, where the emergence and spread of PCV2d strains is set in motion to replace PCV2b genotype (Xiao, Halbur & Opriessnig, 2015). Both natural infection and vaccine-induced responses were considered, given the ubiquitous nature of PCV2 and the widespread awareness of PCV2 vaccination practice. In Malaysia, PCV2 vaccination is a commonly accepted practice. Currently available commercial PCV2 vaccines, including Circovac® (Ceva), Ingelvac CircoFLEX® (Boehringer Ingelheim), Fostera® PCV (Zoetis) and Porcilis® (MSD), are all inactivated vaccines based on PCV2a genotype. During prolonged circulation in a highly immune population, PCV2 continuously mutates its antigenic features to evade from immune responses (Franzo *et al.*, 2016a).

The majority of Malaysian PCV2d strains were sequestered into seven clusters of their own. Some of the clusters shared clades with PCV2d strains from numerous countries, including Canada, Denmark, France, Sweden, South Korea and U.S., with whom Malaysia has trade relationships involving breeder importation as early as back in 2000 (DVS, 2022). Hence, it may be speculated that the phylogenetic relatedness might be a result of live breeder and semen stock movement. Phylogenetic relationship was also shared among Malaysian PCV2 strains with Thailand, Vietnam and China, possibly due to geographical factors and trade activities involving porcine products (DVS, 2022).

4.5 Conclusion

This study reported PCV2 molecular detection rates of 83.78% and 83.54% at farm and sample population level respectively, close to the last published update on Malaysian PCV2 status. High molecular detection rate of 94.74% was reported in clinically healthy finishers. Given these high detection rates at farm and abattoir levels, it could be beneficial to investigate both subclinical and clinical PCVAD more thoroughly at the farms. Further, this study also confirmed the circulation of PCV2 in wild boar population roaming Peninsular Malaysia. Malaysian wild boar PCV2 sequences were found to be interspersed among clades of domestic pig sequences, sharing at least 80.7% of nucleotide identity similarities. Although the pathogenesis and clinical manifestation of PCV2 in wild boars have yet to be elucidated, potential of wild boars as PCV2 reservoir should be considered. Most notably, an apparent genotype shift away from the sole PCV2b genotype reported ten years ago in Malaysia was observed. This study revealed that 96.2% majority of the latest Malaysian sequences (76 / 79 sequences) were identified as genotype PCV2d. Taken together, findings of continual high molecular detection rates in farms; PCV2 antigen detection in clinically healthy abattoir samples and in wild boar population; and most importantly, a new wave of genotype shift from PCV2b to PCV2d — warrant further research on Malaysian PCV2 and PCVAD situation to facilitate direction of control and management applicable to local situation.

CHAPTER 5

MOLECULAR DETECTION AND CHARACTERISATION OF PORCINE CIRCOVIRUS 3 (PCV3)

Article 4: First molecular detection and complete sequence analysis of Porcine Circovirus type 3 (PCV3) in Peninsular Malaysia

5.1 Introduction

Circoviruses of swine comprise of porcine circovirus type 1 (PCV1), porcine circovirus type 2 (PCV2) porcine circovirus type 3 (PCV3) and most recently reported porcine circovirus type 4 (PCV4). PCV1 was discovered in 1974 as a cell culture contaminate (Tischer *et al.*, 1982). In contrast, PCV2 is associated with a group of complex multi-factorial diseases classified under the umbrella term of Porcine circovirus associated diseases (PCVAD) (Harding, 2007). The novel detection of PCV4 was described in a herd with severe clinical signs of respiratory disease, enteritis and porcine dermatitis and nephropathy syndrome (PDNS) (Zhang *et al.*, 2020b). PCV3 is a newly emerging virus in the swine industry, first reported in 2016 in the United States (Palinski *et al.*, 2017). PCV3 assembles into a 1999 – 2001 bp circular genome; slightly larger than other known porcine circoviruses which ranged from 1758 – 1760 bp (PCV1) and 1766 – 1769 bp (PCV2) (Meehan *et al.*, 1997; Fenaux *et al.*, 2000; Zhang *et al.*, 2015; Fux *et al.*, 2018). PCV4, very recently reported in Hunan province, China, was described to be 1770 bp (Zhang *et al.*, 2020b). Albeit their different lengths, genome of all porcine circoviruses encodes for three known proteins: replication-associated (Rep) protein, capsid (Cap) protein and open reading frame (ORF) 3, which function has yet to be determined.

International Committee on Taxonomy of Viruses (ICTV) defines a distinct *Circovirus* species based on sequence similarity: a novel circovirus must share < 75% nucleotide (nt) identity over its entire genome and < 70% amino acid (aa) identity of its Cap protein with other species in the genus (Rosario *et al.*, 2017). PCV1 and PCV2 shares < 80% similarity of overall nt identity, 86% and 65% similarity of aa identity in their ORF1 and ORF2 respectively (Meehan *et al.*, 1998; Morozov *et al.*, 1998; Gibbs & Weiller, 1999). In a complete genome of PCV3, ORF1 and ORF2 genes encode for 296 – 297aa Rep protein and 214aa Cap protein respectively (Palinski *et al.*, 2017; Fux *et al.*, 2018). PCV3 shares even lower similarity of only < 50% of overall nt identity, 48% and 26 – 36% aa identity of ORF1 and ORF2 respectively with PCV2 (Phan *et al.*, 2016; Palinski *et al.*, 2017; Rosario *et al.*, 2017). The genome of PCV4 showed 50.3%, 51.5% and 43.2% nt similarities to PCV1, PCV2 and PCV3 respectively. Most strikingly, aa identities of Cap protein of PCV3 and PCV4 differ by 75.5% (Zhang *et al.*, 2020b). While the conserved nonanucleotide stem loop motif (T/n)A(G/t)TATTAC representing the origin of replication can be found in all porcine circoviruses, their motif differ. PCV1 and PCV3 have an identical motif of TAGTATTAC whereas the motif on PCV4 *rep* gene was shown to be CAGTATTAC (Li *et al.*, 2010; Palinski *et al.*, 2017; Rosario *et al.*, 2017; Zhang *et al.*, 2020b). PCV2 has a nonamer sequence unique among circovirus species – AAGTATTAC (Li *et al.*, 2010).

PCV3 was associated with a PDNS outbreak in North Carolina, where there was an increase in sow mortality rate and decrease in conception rate, presented with skin and kidney lesions suggestive of PDNS (Palinski *et al.*,

2017). Several other clinical presentations have been associated with PCV3, including reported reproductive failures, neonatal congenital tremor, myocarditis and multi-organ inflammation (Phan *et al.*, 2016; Chen *et al.*, 2017; Ku *et al.*, 2017). Role of PCV3 in porcine respiratory disease complex (PRDC) has also been discussed (Phan *et al.*, 2016; Kedkovid *et al.*, 2018a). Although many common swine pathogens especially PCV2, and others such as porcine reproductive and respiratory syndrome virus (PRRSV) and ungulate protoparvovirus 1 (PPV) have been ruled out in these reports, there could still be other co-infections that elude the role of PCV3 in pathogenesis. A successful reproduction of PDNS-like clinical disease following experimental inoculation of 4- and 8-week-old specific-pathogen-free piglets with infectious PCV3 DNA clone has been demonstrated, thus implying that PCV3 may have a direct role in disease process (Jiang *et al.*, 2019a). Considering the presence of PCV3 in increasing number of countries including Thailand and U.S. with whom Malaysia shares trade and geographical relationship; and that PCV3 is associated with several clinical presentations that affect productivity, molecular prevalence of PCV3 in Malaysia and molecular characteristics of Malaysian PCV3 strains reported in this study may contribute practical knowledge to the Malaysian swine farming industry (Palinski *et al.*, 2017; Phan *et al.*, 2016; Kedkovid *et al.*, 2018a; Sukmak *et al.*, 2019).

5.2 Materials and Methods

5.2.1 Sample Collection

Commercial swine farms involved in this study were located in Perak, Selangor, Melaka and Johor states representing different regions of Malaysia. From these 24 farms, 123 pigs were subjected to convenience sampling method. From three abattoirs and retail shops, 18 clinically healthy finishers were sampled by random selection. A grand total of 141 pigs were included in this study. The sampled animals were also categorized by age group (foetuses, piglets, weaners, growers or finishers and sows), health status (clinically healthy or clinically ill), standing sow population of origin farm (< 800 or > 800 heads) and distance between origin farm and neighbouring farms (< 1km, 1 – 10km, > 10km). The 281 organ samples collected comprised of 49 archived lung samples of 2016 – 2017, 18 lung samples from clinically healthy finishers and 214 tissue samples of various organs from clinically ill pigs of 2018 – 2019. For each animal sampled, at least lung and/or inguinal lymph nodes was collected. Other organs (spleen, tonsil, kidney, heart, mesenteric lymph nodes, liver and brain) were collected on the basis of availability. Clinically ill pigs were those showing various clinical signs such as wasting, moribund, dyspnea, neurological signs and sudden death. Majority of the sampled pigs were of weaner and grower stage between the age of 4 – 12 weeks old. Detailed breakdown of each sampled farm and pig is provided in Supplementary Table 1 and 2. In addition, 28 archived wild boar lung samples of year 2018 – 2019 were also included in this study.

This study was granted approval from the Universiti Putra Malaysia (UPM) Institutional Animal Care and Use Committee (IACUC) under AUP Code UPM/IACUC/AUP-R030/2019 and was conducted adhering to the guidelines as stated in the Code of Practice for Care and use of Animals for Scientific Purposes as stipulated by Universiti Putra Malaysia. All samples were collected under the supervision of veterinarians from the Faculty of Veterinary Medicine, Universiti Putra Malaysia.

5.2.2 Molecular Prevalence of PCV3

DNA extraction was performed using the DNeasy Blood & Tissue Kit extraction kit (Qiagen, Germany) in accordance with the manufacturer's instructions. Conventional PCR was performed to amplify the ORF2 region of PCV3, by using MyTaq™ Red Mix 2X (Bioline, United Kingdom) and published primers, KF–5'– TTACTTAGAGAACGGACTTGTAAC G–3' and KR–5'–AAATGAGACACAGAGCTATATTCAG–3' (Ku *et al.*, 2017). Briefly, 12.5 µL of Taq DNA polymerase master mix and 1.0 µM each from the primer pair were used in a 25 µL total PCR reaction volume. Cycling conditions of the conventional PCR were as described by Ku *et al.* (2017). PCR products were stained using RedSafe™ nucleic acid staining solution (iNtRON Biotechnology, South Korea) and analysed by agarose electrophoresis. Expected PCR product was a 649 bp band indicating PCV3 *cap* gene sequence spanning from nt position 1343 – 1987. Samples that showed a positive band at the 650 bp region as marked by GelPilot 100 bp Plus Ladder (Qiagen, Germany) were further sequenced (Macrogen, South Korea).

To test for association between PCV3 molecular detection status and age group, health status, farm standing sow population and distance from neighbouring farms, Chi-square tests were performed with statistical significance level set at $p < 0.05$. Fisher's exact tests were run in place of Chi-squared tests for variables with expected count of less than five. Post hoc tests of cell residuals were ran on statistically significant chi-square and Fisher's exact test values, with p-values adjusted with Bonferroni correction. Animals that tested positive for PCV3 in lung and/or inguinal tissues, with at least one other organ sample (spleen, tonsil, kidney, heart, mesenteric lymph nodes, liver and/or brain) tested for PCV3 were identified. Molecular detection rate comparison across tissues from different organs was then similarly evaluated as described above. All statistical tests were performed using IBM SPSS Statistics for Windows v23 software programme (SPSS, I. B. M., 2016).

5.2.3 Complete Genome Nucleotide Sequence Generation

Upon confirmation of the nt sequences as *cap* gene of PCV3 by NCBI Nucleotide BLAST®, the positive samples were subjected to PCR with another three pairs of primers (Figure 5, Table 13) to generate the complete 2000 bp genome of PCV3 (Altschul *et al.*, 1990). Two pairs of published sequencing primers were run under modified cycling conditions, chiefly adjusting annealing temperature and final extension time as detailed in Table 1 (Palinski *et al.*, 2017). Primer pair V4F/V4R were designed based on PCV3 strain KY075986 using Primer-BLAST® (Ye *et al.*, 2012; Ku *et al.*, 2017). All PCR assays were performed using the same reagents, reaction volumes and primer concentration as described in the method of PCV3 *cap* gene detection. PCR

protocols used in this study are accessible at http://dx.doi.org/10.17504/protocols.io.bdd9i296 [PROTOCOL DOI].

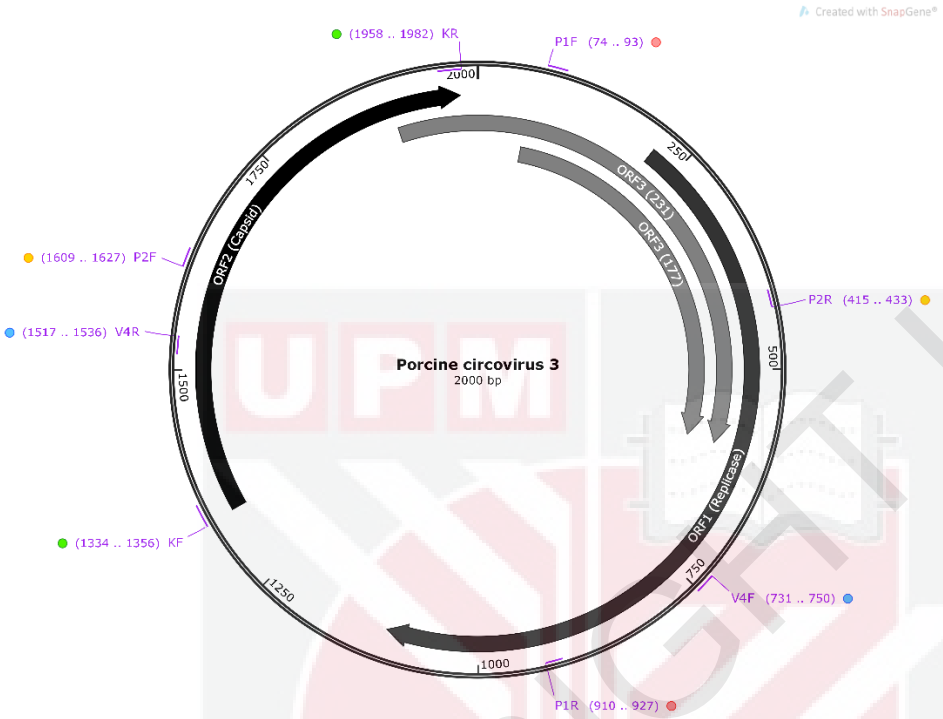

Figure 5: Schematic Representation of Primer Pairs Used in This Study to Generate Complete Genome Sequence of PCV3

Table 13: PCR Cycling Condition and Primers Used in This Study to Generate Complete Genome Sequence of PCV3

Primer Pair	Nucleotide Sequence (5'– 3')	Product Length (bp)	PCR Cycling Condition (Temperature / Time)						Reference
			Initial Denaturation	Number of Cycle	Denaturation	Annealing	Extension	Final Extension	
P1F P1R	CACCGTGTGAGTGGATATAC CAAACCCACCCTTAACAG	854	94ºC/ 4 min	35	94ºC/ 20 s	55ºC/ 30 s	72ºC/ 30 s	72ºC/ 5 min	Palinksi *et al.*, 2017

Table 13: Continued

Primer Pair	Nucleotide Sequence (5'– 3')	Product Length (bp)	PCR Cycling Condition (Temperature / Time)						Reference
			Initial Denaturation	Number of Cycle	Denaturation	Annealing	Extension	Final Extension	
P2F	GTCGTCTTGGAGC CAAGTG	807	95ºC/ 5 min	35	94ºC/ 30 s	62ºC/ 30 s	72ºC/ 1 min	72ºC/ 7 min	Palinksi *et al.*, 2017
P2R	CGACCAAATCCGG GTAAGC								
KF	TTACTTAGAGAACG GACTTGTAACG	649	94ºC/ 5 min	35	94ºC/ 30 s	55ºC/ 30 s	72ºC/ 1 min	72ºC/ 10 min	Ku *et al.*, 2017
KR	AAATGAGACACAGA GCTATATTCAG								
V4F	GAAAACGCGGGAA GCTTGTG	806	95ºC/ 5 min	35	94ºC/ 30 s	56ºC/ 30 s	72ºC/ 1 min	72ºC/ 10 min	Designed in this study
V4R	CCACTTCTGGCGG GAACTAC								

5.2.4 Phylogenetic Analysis

Sequencing was done to confirm the identity of the PCV3 nt sequences. Sequence assembly and multiple sequence alignment were generated using MEGA v7.0.26 software programme (Kumar, Stecher & Tamura, 2016). The resulting sequences were analysed using NCBI Nucleotide BLAST® for a final identity confirmation as PCV3 by comparing their similarity with reference PCV3 sequences deposited in the GenBank (Ye *et al.*, 2012). Maximum likelihood (ML) phylogenetic trees were constructed with MEGA7 programme, using 1000 bootstrap replicates with either General Time Reversible (GTR) model for species-specific circoviruses comparison or Tamura-Nei model for

porcine circoviruses analyses. Pairwise distance analysis with the p-distance model was performed using the same software, similarly with 1000 bootstrap replicates. Both transitions and transversions nt substitutions were included. After the number of base differences per site was computed, percentage nt identities were calculated by subtracting the computed p-distance values from 1.0 and multiplying by 100.

5.2.5 Nucleotide Sequence Polymorphism Analysis

The same 45 PCV3 strains included in the phylogenetic methods were further analysed. Tajima's D, Fu and Li's D, and Fu and Li's F statistical tests of neutrality were performed on nt sequences of PCV3 ORF1 and ORF2 genes using DnaSP v6.12.03 software programme (Tajima, 1989; Fu & Li, 1993; Rozas *et al.*, 2017). DNA polymorphism data of pairwise nt differences were analysed to measure balancing selection and negative selective processes over the sequences. Statistical significance was set at $p \leq 0.05$ for all three tests. Shannon's entropy H(x) values were calculated with BioEdit software v7.2.5 using the Shannon entropy formula: $-(\sum_{j=1}^{4} p_{ij} \log_2 p_{ij})$ where *i; j* is equal to 1, 2, 3 and 4, corresponding to A, C, G and T nt and *pij* being the proportion of nt *j* in site *i*. Entropy plots of aa sequences of the ORF1 and ORF2 genes were constructed to plot the diversity of aa residues at a given position (Shannon, 1948; Hall, 1999). Range, mean and standard error of mean (SEM) were calculated using IBM SPSS Statistics for Windows v22 software programme. Positive and negative selective pressures acting specifically on each codon of the ORF1 and ORF2 nt sequences were estimated based on

calculated difference between non-synonymous (dN) and synonymous (dS) substitution rates per codon. Single-likelihood ancestor counting (SLAC), fixed-effects likelihood (FEL), internal branches fixed-effects likelihood (IFEL), fast, unconstrained Bayesian approximation (FUBAR) and mixed effects model of evolution (MEME) selection pressure methods were run in the DataMonkey web server (http://www.datamonkey.org/) (Kosakovsky Pond & Frost, 2005; Murrell *et al.*, 2012; Murrell *et al.*, 2013; Weaver *et al.*, 2018). To infer dN and dS rates, FUBAR uses Bayesian approach; FEL, IFEL and MEME utilize ML approach; while SLAC incorporates additional counting approaches. FEL, IFEL, SLAC and FUBAR detects both positive and negative selection, but MEME aims to detect aa sites evolving under positive selection. Comparison between rates of dN and dS substitutions were expressed as dN – dS < 0, = 0 and > 0 (dN / dS < 1, = 1 and > 1 for MEME method) and interpreted as indication of negative selection, neutral evolution and positive selection respectively. Statistical significance was set at $p \leq 0.05$ for FEL and IFEL methods and $p \leq 0.1$ for SLAC and MEME methods. The FUBAR method was run with posterior probability of 0.9. The dN – dS and H(x) entropy values for every codon were plotted against their respective aa positions along the ORF1 and ORF2 genes.

5.2.6 Clarification on Nucleotide Sequences

Out of the 24 PCV3-positive samples identified in this study, 15 were randomly selected for further sequencing, which was outsourced to Macrogen, Inc., South Korea. This sequencing effort successfully produced 12 complete

genome sequences, each approximately 2000 bp in length, and three partial cap gene sequences of 645 bp.

For the construction of the phylogenetic tree in Figure 6, only the complete genome sequences (2000 bp) of PCV3 strains from this study were utilized. Comparison strains representing other circovirus species were included, using whole genome sequences downloaded from GenBank, based on the exemplar isolates listed by ICTV. This approach ensured comprehensive representation of PCV3 within the broader context of the circovirus genus.

In Figure 8, the phylogenetic analysis focused specifically on the capsid gene. Complete capsid sequences (645 bp) from the study's PCV3 strains were included, with all sequences verified to ensure they were complete. Relevant comparison sequences from public databases, also confirmed to be 645 bp, were incorporated into the analysis. By specifying the sequence lengths and verifying completeness, this section enhances transparency and ensures reproducibility in the presented phylogenetic studies.

5.3 Results

5.3.1 Molecular Prevalence of PCV3

Out of the 141 pigs, 24 pigs from 10 farms were positive for PCV3 based on PCR detection (Table 14), representing a molecular prevalence of 17.02% of PCV3 in Peninsular Malaysia. Notably, all 18 samples from clinically healthy pigs and all 28 lung samples from the wild boar population were tested negative for PCV3. Statistical significance was observed between PCV3

molecular detection status and age group (p: 0.022), as well as health status (p: 0.043) of the test animals. In the age group set of variables, only the weaners group (p: 0.0007) was shown to be statistically significant at adjusted $p < 0.005$. No statistical differences in the frequency of animals molecularly positive for PCV3 were observed across farms with different standing sow populations (p: 0.180) and distance from neighbouring farms (exact p: 0.512). Statistical significance for all Chi-square and Fisher's exact tests is summarized in Table 15.

Table 14: Distribution of PCV3 Positive Farms and Animals in Peninsular Malaysia as Detected by PCR Assay

Region	State	No. of Positive Farm(s) / Tested Farms	No. of Positive Animal(s) / Tested Animals
Northern	Penang	1 / 4	1 / 16
	Perak	3 / 5	4 / 30
Central	Selangor	4 / 9	16 / 81
Southern	Melaka	1 / 3	2 / 9
	Johor	1 / 3	1 / 5
Total		10 / 24	24 / 141

Table 15: Statistical significance for Chi-square and Fisher's exact tests evaluating association between PCV3 molecular detection status and age group, health status, farm standing sow population, distance from neighbouring farms, and across different organs. Statistically significant values are highlighted with grey boxes and bold typeface

Comparison Variable	Standing Sow Population	Neighbouring Farm Proximity Radius	Clinical Health Status	Age Group		Organ	
Pearson Chi-Square ($p < 0.05$)	0.180	0.449	0.040	0.024		0.049	
Fisher's Exact Test ($p < 0.05$)	0.180	0.512	0.043	0.022		0.047	
Bonferroni correction adjusted p-value	N/A	N/A	N/A	0.005		0.003	
Cell residuals adjustment post hoc ($p <$ adjusted)	N/A	N/A	N/A	T	0.271	L	0.029
				P	0.317	I	0.0002
				W	0.0007	S	0.855
				G	0.134	T	0.450
				F	0.036	K	0.807
						H	0.105
						M	0.077
						V	0.008
						B	0.567

Key:

- Age group: T – foetus, P – piglet, W – weaner, G – grower, F – finisher and sow
- Tissue type: L – lung, I – inguinal lymph node, S – spleen, T – tonsil, K – kidney, H – heart, M – mesenteric lymph node, V – liver, B – brain
- N/A: Not applicable

The molecular detection rate across different organs (lung, inguinal lymph node, mesenteric lymph node, spleen, tonsils, kidney, heart, liver, brain) was tabulated for the positive animals (Table 16). For an accurate representation of the distribution, positive animals with only one organ sample type tested were excluded from the analysis. The presence of PCV3 genetic material was

detected in all nine groups of organ samples included in this study. The highest frequency of PCR positive results was observed in inguinal lymph node and lung samples, with detection rates of 81.82% and 71.43% respectively. For other organs, the positive PCR detection rate ranged from 12.5% to 54.55%. PCR detection rate was moderately high in five organs, namely tonsil, spleen, kidney, mesenteric lymph node and brain. The lowest detection rates were seen in heart and liver. In terms of statistical significance, it was found that only the inguinal lymph nodes group (p: 0.0002) was statistically significant at adjusted $p < 0.003$, as shown in Table 15.

Table 16: PCV3 PCR Detection Rate Across Different Organs Types

Organ	No. of Positive Sample(s) / Tested Sample	PCR Detection Rate (%)
Lung	10 / 14	71.43[a]
Inguinal lymph node	9 / 11	81.82[b]
Mesenteric lymph node	4 / 12	33.33 [a]
Spleen	7 / 14	50.00 [a]
Tonsil	6 / 11	54.55 [a]
Kidney	5 / 11	45.45 [a]
Heart	1 / 6	16.67[a]
Liver	1 / 8	12.50[a]
Brain	1 / 4	25.00[a]

[a, b] Different superscript letters indicate significant differences (adjusted p-value < 0.003) among different organ samples.

5.3.2 Nucleotide Sequence and Complete Genome Analysis

Twelve complete genome sequences and three *cap* gene sequences were obtained successfully. The local PCV3 sequences showed over 99% similarity with PCV3 sequences recorded in GenBank. The genome of Malaysian PCV3 strains in this study is 2000 bp in length, encoding a 296 aa Rep protein and a 214 aa Cap protein.

ML phylogenetic trees were constructed to analyze Malaysian PCV3 strains in relation to known member species of *circovirus* genus using nt sequences of exemplar isolates listed by ICTV (ICTV, n.d.). PCV1 and PCV2, as well as PCV3 strains from different countries were also included in the analysis. The phylogenetic relationship among species in *Circoviridae* was inferred using the ML method based on the GTR model (Figure 6). PCV1, PCV2 and PCV3 were clustered into the same clade with >50% bootstrap value. Supported by a lower bootstrap value of 30%, Malaysian strains of PCV3 were phylogenetically related to bat associated circovirus 7 (BatACV7) and starling circovirus (StCV).

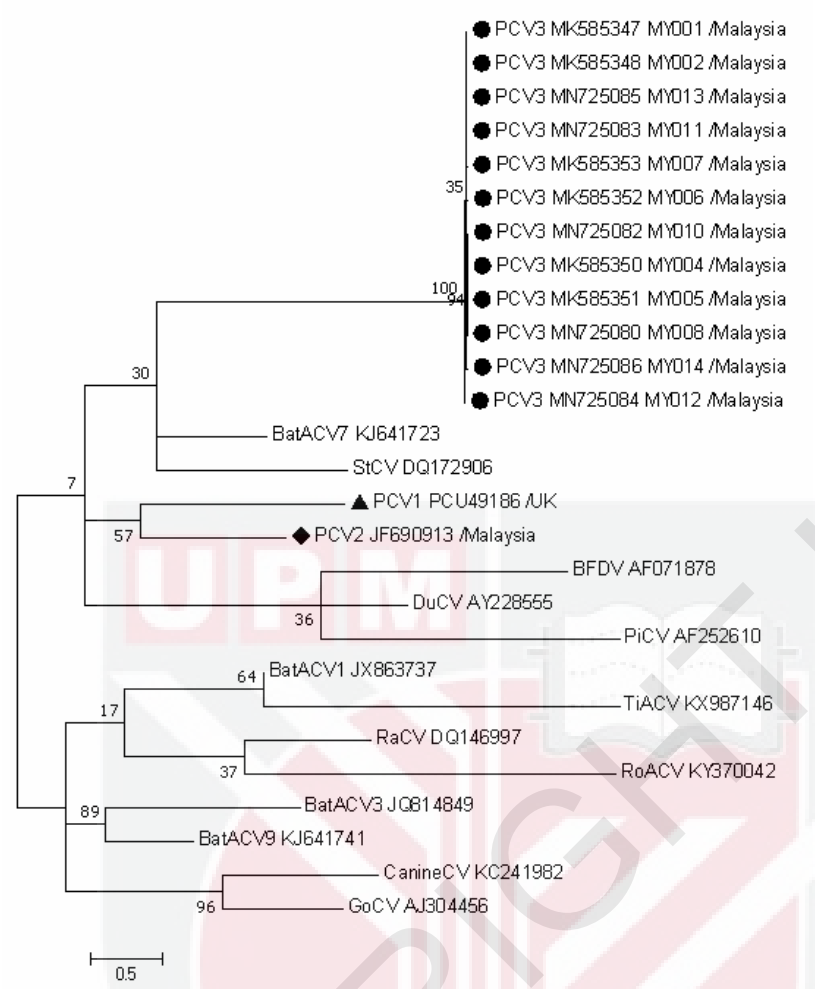

Figure 6: Phylogenetic analysis of complete genome sequences of PCV3 and known member species of circovirus genus.Twelve Malaysian PCV3 strains (●) were compared with PCV1 (▲), PCV2 (◆) and 13 circovirus member species (Bat associated circovirus, BatACV; beak and feather disease virus, BFDV; canine circovirus, CanineCV; duck circovirus; DuCV; goose circovirus, GoCV; pigeon circovirus, PiCV; raven circovirus, RaCV; rodent associated circovirus, RoACV; starling circovirus, StCV; tick associated circovirus, TiACV). GenBank accession numbers are indicated at the end of each species strain. The tree was constructed using ML method, GTR model with gamma distribution and invariant sites with tree topology evaluated with 1000 bootstrap replicates. The scale bar indicates branch length measured in number of substitutions per site

The phylogenetic relationship among porcine circoviruses was inferred using the ML method based on the Tamura-Nei model (Figure 7). All PCV3 strains analyzed in this study were clustered in a distinct clade with the longest branch length, separated from PCV1 and PCV2.

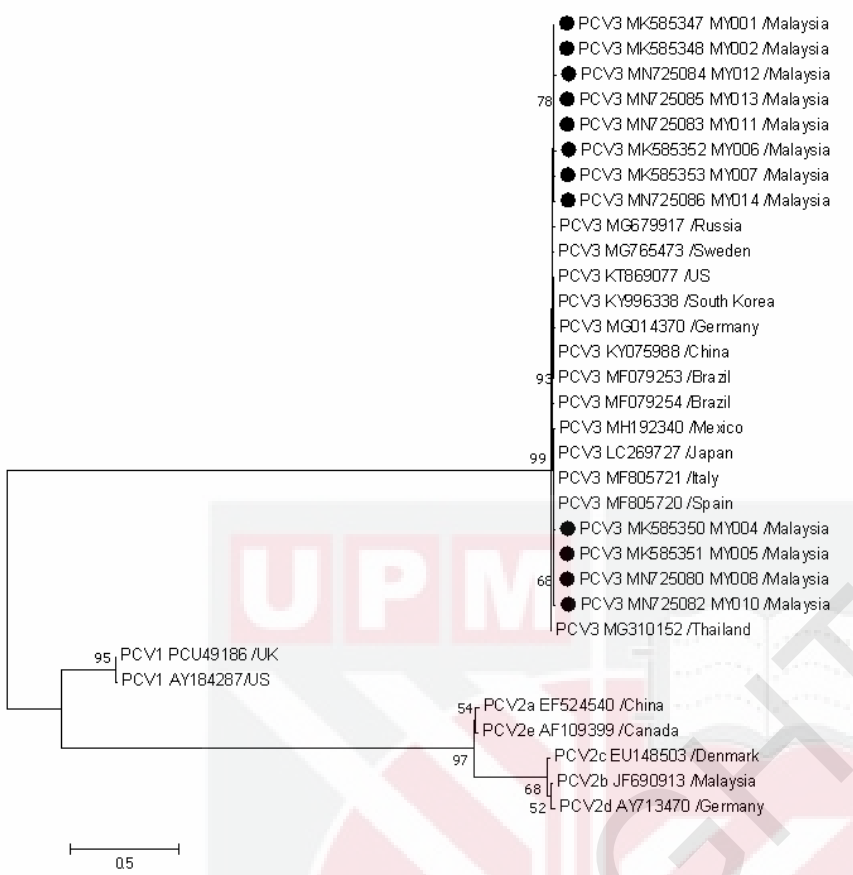

Figure 7: Phylogenetic analysis of complete genome sequences of PCV1, PCV2 and PCV3.Twelve Malaysian PCV3 strains (●) were compared with 5 PCV2 strains and 2 PCV1 strains. GenBank accession numbers and origin country are as indicated. Malaysian PCV3 strains were additionally labelled with strain ID. The tree was constructed using the ML method and the Tamura-Nei model evaluated with 1000 bootstrap replicates. The scale bar indicates branch length measured in number of substitutions per site

Further, percentage nt identities between 42 PCV3 sequences (12 Malaysian sequences and 30 GenBank reference strains) were compared using Pairwise Distance method with p-distance model. All 2000 nt positions were included in the final dataset. Overall, all 42 PCV3 sequences in the comparison dataset are closely related to each other, given that only 0.58% (5 / 861) of the p-distance values are ≥ 0.020. The p-distance values range from 0.002 to 0.021, averaging at 0.010. This is equivalent to a shared percentage nt identities of 97.9% – 99.8%. Among the 12 Malaysian sequences, while the p-distance range maintains, the average is slightly higher at 0.012.

A similar pairwise distance analysis was run to compute the p-distance values among *cap* gene sequences of 15 Malaysian PCV3 strains and 30 PCV3 GenBank reference strains. The range of p-distance values widens from 0.000 to 0.026, with an increased average of 0.013. This is equivalent to a shared percentage nt identities of 97.36% – 100%. Compared to the complete genomes, the *cap* gene sequences are less closely related to each other, given that 8.28% (82 / 990) of the p-distance values are ≥ 0.020. Among the 15 Malaysian sequences, the p-distance range is slighter smaller at 0.000 to 0.020, and the average is slightly lower at 0.012.

Based on the phylogenetic analysis of PCV3 *cap* gene sequences (Figure 8), Malaysian PCV3 strains appear to be grouped into two main clusters within one clade. In the first cluster, Malaysian PCV3 strains were grouped with PCV3 strains from Italy (GenBank accession no.: MF805721, MF162298, MF162299), Thailand (MF589652, MH229786), Brazil (MF079254), Japan (LC269727, LC383840, LC383841), Germany (MG014364) and Spain (MF805720). Particularly, Malaysian strain MY006 (MK585352) showed a high bootstrap value of >50% with a Spanish strain. The second cluster consisted entirely of Malaysian PCV3 strains, with only one U.S. strain (KX458235) and one Mexico strain (MH192340).

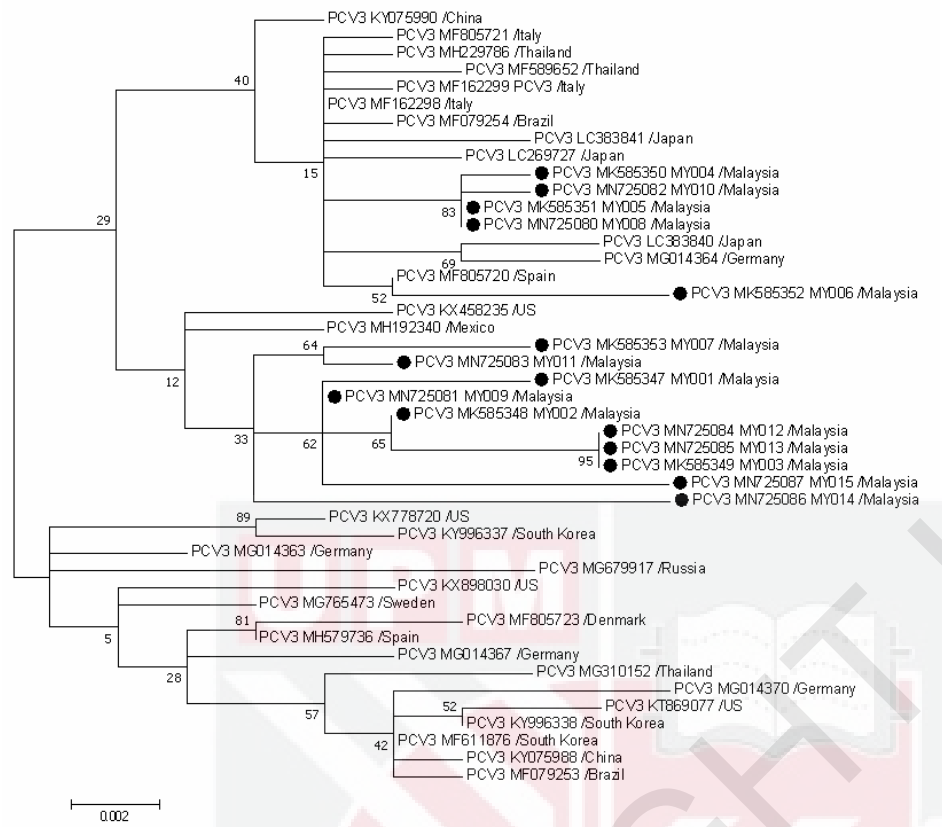

Figure 8: Phylogenetic analysis of cap gene sequences of PCV3. Fifteen Malaysian PCV3 strains (●) were compared with 30 other PCV3 strains. GenBank accession numbers and origin country are as indicated. Malaysian PCV3 strains were additionally labelled with strain ID. The tree was constructed using ML method, Tamura-Nei model evaluated with 1000 bootstrap replicates. The scale bar indicates branch length measured in number of substitutions per site

5.3.3 Nucleotide Sequence Polymorphism Analysis

To determine positive and/or negative selections in the ORF1 and ORF2 genes of PCV3 sequences, neutrality test values, dN – dS values and H(x) entropy values of the two genes were evaluated. Results from the statistical tests of neutrality ran on PCV3 ORF1 and ORF2 gene sequences were summarized in Table 17. All three tests showed negative neutrality values of statistical significance.

Table 17: Results Tabulation of PCV3 ORF1 and ORF2 Tests of Neutrality

Gene Sequence	Tests of Neutrality					
	Tajima's D		Fu and Li's D		Fu and Li's F	
	Test Statistic	Statistical Significance, p-value	Test Statistic	Statistical Significance, p-value	Test Statistic	Statistical Significance, p-value
ORF1	-2.41372	< 0.01	-3.86411	< 0.02	-3.98503	< 0.02
ORF2	-2.04422	< 0.05	-3.75641	< 0.02	-3.73811	< 0.02

H(x) entropy values for each aa position of PCV3 ORF1 and ORF2 are plotted in the secondary bar charts of Figs 5 and 6 respectively. Among the ORF1 gene sequence, 5.41% (16 / 296) of aa sites showed H(x) entropy values of > 0.0. The 16 H(x) values ranged from 0.113 to 0.683, with a mean of 0.170 ± 0.036. ORF2 gene sequences have slightly more aa sites with H(x) entropy > 0.0, at 7.94% (17 / 214). The 17 H(x) values ranged from 0.107 to 0.663, with a mean of 0.247 ± 0.046.

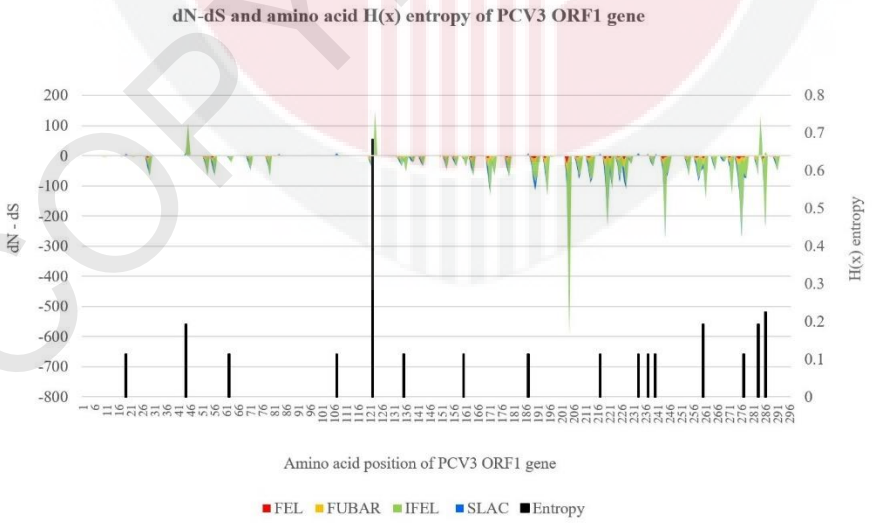

Figure 9: dN−dS and amino acid H(x) entropy values plot for PCV3 ORF1 gene sequences. Left y-axis corresponds to the dN – dS values of the primary stacked line graph, where values obtained by four different selection pressure methods were identified with four different primary colours. Right y-axis corresponds to H(x) entropy values of the

secondary bar chart. X-axis marks the aa sites of the ORF1 gene sequence. The graphs in Figs 5 and 6 were scaled identically

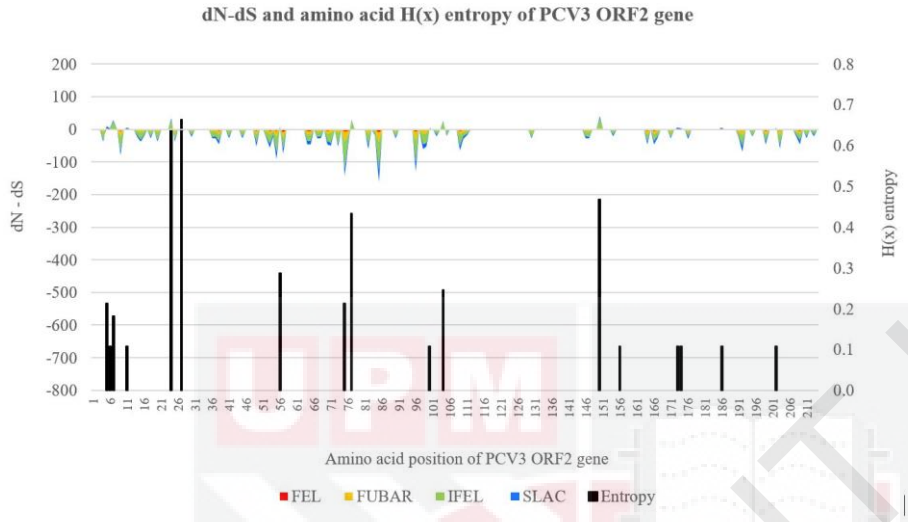

Figure 10: dN−dS and amino acid H(x) entropy values plot for PCV3 ORF2 gene sequences. Left y-axis corresponds to the dN – dS values of the primary stacked line graph, where values obtained by four different selection pressure methods were identified with four different primary colours. Right y-axis corresponds to H(x) entropy values of the secondary bar chart. X-axis marks the aa sites of the ORF2 gene sequence. The graphs in Figs 5 and 6 are scaled identically

Analysis of the selective pressures revealed minor differences between ORF1 and ORF2 genes at both global and site levels. Global dN – dS values of ORF1 and ORF2 were 0.015 and 0.091 respectively, as determined by SLAC method. At site levels, dN – dS values for every codon were determined to identify aa positions under positive or negative selection pressure, as tabulated in Table 18. Large majority of the calculated dN – dS values were < 0, indicating negative selection. At the statistical significance thresholds applied in this study, no positive selection was reported. Within the ORF1 and ORF2 gene, 24 / 296 (8.11%) and 26 / 214 (12.15%) aa sites respectively were identified as negatively selected sites. Among the four selection pressure methods used, descending detection rates were observed from FUBAR, FEL,

SLAC to IFEL method. Only 3 sites, codon 203 of the ORF1 gene, codon 75 and 85 of the ORF2 gene, were identified as negative selection sites across all four methods.

Table 18: Codon selection pressures on amino acid sites of PCV3 ORF1 and ORF2. Comparison between dN and dS is expressed as dN – dS (dN / dS for MEME). Values of dN – dS < 0, = 0 and > 0 (dN / dS < 1, = 1 and > 1 for MEME method) indicate negative selection, neutral evolution and positive selection respectively. Significance level was set to p-value ≤ 0.1 for SLAC and MEME method, p-value ≤ 0.05 for FEL and IFEL methods, posterior probability > 0.9 for FUBAR method. dN – dS values of statistical significance are highlighted in boldface. Statistical method consensus (n) indicates number of different test methods generating significant value for one same aa site

ORF1 (*rep* gene)											
Site	FEL (dN– dS)	p-value	IFEL (dN– dS)	p-value	SLAC (dN– dS)	p-value	FUBAR (dN– dS)	Posterior probability	MEME (dN/dS)	p-value	Statistical method consensus (n)
28	-8.337	0.051	0.000	1.000	-17.871	0.207	**-12.027**	**0.911**	0.018	0.670	1
55	**-8.456**	**0.050**	0.000	1.000	-17.965	0.196	**-12.134**	**0.909**	0.015	0.670	2
78	-4.772	0.078	0.000	1.000	-10.546	0.333	**-10.729**	**0.907**	0.101	0.670	1
121	-4.890	0.089	0.000	1.000	-10.546	0.333	**-10.926**	**0.904**	0.092	0.670	1
163	-8.344	0.063	0.000	1.000	-17.934	0.227	**-12.018**	**0.907**	0.014	0.670	1
170	**-10.155**	**0.020**	0.000	1.000	**-31.639**	**0.048**	**-26.546**	**0.997**	0.000	0.670	3
178	**-8.838**	**0.048**	0.000	1.000	-17.538	0.211	**-12.505**	**0.913**	0.012	0.670	2
189	-8.227	0.064	-47.052	0.339	-17.872	0.228	**-11.877**	**0.906**	0.016	0.670	1
190	**-7.466**	**0.018**	-64.882	0.295	**-28.201**	**0.062**	**-14.23**	**0.983**	0.032	0.670	3
194	**-9.904**	**0.027**	0.000	1.000	-21.092	0.111	**-23.947**	**0.992**	0.006	0.670	2
203	**-28.651**	**0.004**	**-591.414**	**0.027**	**-39.881**	**0.040**	**-32.089**	**0.996**	0.000	0.670	3
208	-4.637	0.077	-50.053	0.304	-12.299	0.329	**-10.508**	**0.966**	0.099	0.670	1
212	-8.221	0.064	0.000	1.000	-17.719	0.229	**-11.875**	**0.906**	0.0158	0.670	1
219	**-12.379**	**0.017**	0.000	1.000	**-34.335**	**0.054**	**-25.356**	**0.993**	0.000	0.670	3
221	**-9.682**	**0.012**	0.000	1.000	-24.609	0.108	**-22.337**	**0.998**	0.000	0.670	3
227	**-14.120**	**0.005**	0.000	1.000	**-42.946**	**0.020**	**-25.231**	**0.998**	0.000	0.670	3
243	**-12.271**	**0.042**	0.000	1.000	-19.919	0.191	**-17.718**	**0.937**	0.000	0.670	2
253	-4.996	0.075	0.000	1.000	-10.546	0.333	**-11.489**	**0.913**	0.090	0.670	1

Table 18: Continued

ORF1 (*rep* gene)											
Site	**FEL (dN– dS)**	**p-value**	**IFEL (dN– dS)**	**p-value**	**SLAC (dN– dS)**	**p-value**	**FUBAR (dN– dS)**	**Posterior probability**	**MEME (dN/dS)**	**p-value**	**Statistical method consensus (n)**
257	-8.344	0.063	0.000	1.000	-17.934	0.227	**-12.018**	**0.907**	0.014	0.670	1
271	-7.898	0.059	0.000	1.000	-15.615	0.226	**-15.186**	**0.927**	0.020	0.670	1
274	**-6.698**	**0.031**	0.000	1.000	-21.092	0.111	**-16.482**	**0.986**	0.040	0.670	2
275	**-12.365**	**0.042**	-69.403	0.215	-19.961	0.191	**-17.762**	**0.937**	0.000	0.670	2
277	-5.142	0.247	-35.504	0.410	-15.819	0.259	**-15.862**	**0.925**	0.266	0.670	1
285	**-12.098**	**0.043**	0.000	1.000	-19.899	0.192	**-17.618**	**0.937**	0.000	0.670	2

Method	FEL	IFEL	SLAC	FUBAR	MEME	**Statistical method consensus (n)**	n = 1	n = 2	n = 3	n = 4
Positively Selected Sites	0	0	0	0	0	**Positively Selected Sites**	0	0	0	0
Negatively Selected Sites	13 / 296 (4.39%)	1 / 296 (0.34%)	5 / 296 (1.69%)	24 / 296 (8.11%)	N/A	**Negatively Selected Sites**	11 / 296 (3.72%)	8 / 296 (2.70%)	4 / 296 (1.35%)	1/296 (0.34%)
						Total	24 / 296 (8.11%)			
						Global dN – dS (SLAC)	0.115			

ORF2 (*cap* gene)											
Site	**FEL (dN– dS)**	**p-value**	**IFEL (dN–dS)**	**p-value**	**SLAC (dN–dS)**	**p-value**	**FUBAR (dN– dS)**	**Posterior probability**	**MEME (dN/dS)**	**p-value**	**Statistical method consensus (n)**
9	-4.885	0.013	-46.208	0.122	-13.110	0.091	-15.807	0.990	0.117	0.670	3
38	-5.990	0.043	-21.962	0.285	-9.710	0.224	-8.683	0.909	0.067	0.670	2
49	-4.965	0.021	-23.684	0.234	-12.778	0.086	-10.704	0.981	0.101	0.670	3
53	-4.362	0.029	-28.375	0.224	-11.220	0.111	-11.830	0.983	0.128	0.670	2
55	-4.647	0.011	-49.553	0.120	-21.439	0.034	-16.465	0.998	0.116	0.670	3
57	-8.992	0.004	-33.177	0.171	-17.495	0.046	-15.624	0.990	0.028	0.670	3
64	-5.930	0.035	-21.793	0.275	-9.691	0.193	-8.628	0.912	0.074	0.670	2

Table 18: Continued

ORF2 (*cap* gene)											
Site	**FEL (dN– dS)**	**p-value**	**IFEL (dN–dS)**	**p-value**	**SLAC (dN–dS)**	**p-value**	**FUBAR (dN– dS)**	**Posterior probability**	**MEME (dN/dS)**	**p-value**	**Statistical method consensus (n)**
65	-5.917	0.045	-21.793	0.251	-9.691	0.193	-8.624	0.910	0.064	0.670	2
70	-5.929	0.032	-21.802	0.281	-9.691	0.193	-8.633	0.912	0.079	0.670	2
71	-2.998	0.051	-30.396	0.187	-8.722	0.218	-8.511	0.915	0.237	0.670	1
73	-3.099	0.049	-31.292	0.183	-8.802	0.216	-8.767	0.917	0.229	0.670	2
75	-7.484	0.020	-83.637	0.022	-22.428	0.046	-29.419	0.989	0.168	0.670	4
76	-4.740	0.024	-22.087	0.249	-12.070	0.096	-10.055	0.978	0.110	0.670	3
82	-3.048	0.029	-32.518	0.176	-14.240	0.077	-10.571	0.981	0.230	0.670	3
85	-8.198	0.000	-87.539	0.029	-35.932	0.002	-29.124	1.000	0.037	0.670	4
96	-6.907	0.004	-68.891	0.056	-28.051	0.004	-26.277	1.000	0.038	0.670	3
98	-3.022	0.038	-32.233	0.207	-14.253	0.106	-10.478	0.979	0.225	0.670	2
99	-3.065	0.078	-30.977	0.251	-8.782	0.264	-8.654	0.908	0.219	0.670	1
109	-4.260	0.050	-35.045	0.180	-14.789	0.126	-10.349	0.924	0.141	0.670	2
110	-3.495	0.042	-12.861	0.331	-8.051	0.232	-5.068	0.881	0.201	0.670	1
164	-5.881	0.045	-21.609	0.252	-9.672	0.193	-8.550	0.910	0.065	0.670	2
166	-5.917	0.045	-21.793	0.251	-9.691	0.193	-8.623	0.910	0.064	0.670	2
192	-3.360	0.039	-34.412	0.167	-16.831	0.037	-13.208	0.996	0.185	0.670	3
199	-5.969	0.024	-21.961	0.236	-9.710	0.193	-8.703	0.917	0.087	0.670	2
203	-4.874	0.024	-30.061	0.214	-11.220	0.111	-12.632	0.985	0.102	0.670	2
209	-4.175	0.020	-20.208	0.268	-11.220	0.111	-9.486	0.977	0.141	0.670	2

Method	FEL	IFEL	SLAC	FUBAR	MEME	**Statistical method consensus (n)**	n = 1	n = 2	n = 3	n = 4
Positively Selected Sites	0	0	0	0	0	**Positively Selected Sites**	0	0	0	0
Negatively Selected Sites	24 / 214 (11.21%)	2 / 214 (0.93%)	10 / 214 (4.67%)	25 / 214 (11.68%)	N/A	**Negatively Selected Sites**	3 / 214 (1.40%)	13 / 214 (6.07%)	8 / 214 (3.74%)	2 / 214 (0.93%)
						Total	26 / 214 (12.15%)			
						Global dN – dS (SLAC)	0.091			

5.4 Discussion

PCV3 has been detected in various tissue samples with varying positive rates (Ku *et al.*, 2017; Fan *et al.*, 2017; Fu *et al.*, 2018; Hayashi *et al.*, 2018; Li *et al.*, 2018e; Klaumann *et al.*, 2019a) Our findings are in agreement with reports from Fan *et al.* (2017), Fu *et al.* (2018) and Li *et al.* (2018e), that PCV3 mainly infect lungs and lymphoid tissues such as lymph nodes and tonsil. PCV2 is known to have tropism for lymphoid tissues in pigs, with lymphoid depletion and histiocytic replacement of lymphoid tissues being hallmark lesions of PCV2 infection (Opriessnig, Meng & Halbur, 2007; Segalés, 2012). Interstitial pneumonia and bronchiolitis with mononuclear infiltration are also part of PCV2 disease manifestation, which correlate with clinical sign of respiratory distress (Harms *et al.*, 2001; Kim, Chung & Chae, 2003). Since high molecular detection rates of 81.82% and 71.43% respectively were found in inguinal lymph nodes and lungs, with demonstrated statistical significance in molecular detection rate of PCV3 in the inguinal lymph nodes group, further research may be focused on these tissues to study potential tissue tropism and relationship with PCV3 pathogenesis. Weaners have been shown to have the highest prevalence of PCV3 across different production stages and statistical results of this study supports this finding (Kwon *et al.*, 2017; Stadejek *et al.*, 2017; Fux *et al.*, 2018). PCV3 has been reported in pigs with various pathological conditions including respiratory, reproductive, neurological and gastrointestinal disorders. Statistics calculated in this study also showed a high PCV3 molecular detection rate in the clinically ill group. Nevertheless, the virus has also been detected in clinically healthy animals (Zhai *et al.*, 2017; Franzo *et al.*, 2018a; Klaumann *et al.*, 2018b). Notably, in this study, all lung samples

of clinically healthy pigs sourced from abattoir and retail shops were tested negative for PCV3 in spite of their origins in PCV3 positive farms. This 100% negative result may not be accurate, considering the low sample number which constitutes only 12.76% (18 / 141 pigs) of the study population, and that sampling healthy animals solely from finishers stage may not be representative for the clinically healthy population.

Apart from domestic pigs, PCV3 was found to infect wild boar population at rates of 23% – 42.66% (Franzo *et al.*, 2018c; Klaumann *et al.*, 2019a; Prinz *et al.*, 2019). Notably, all 28 lung samples from the wild boar population in Peninsular Malaysia were tested negative for PCV3. This finding may suggest that spillover infection from wild boar reservoir hosts is not implicated in introduction of PCV3 into Malaysian commercial swine population. PCV3 infection susceptibility has been suggested to be associated with the age of wild boar, with juvenile animals showing statistically lower detection rates, unlike reports described in domestic pigs (Franzo *et al.*, 2018c; Klaumann *et al.*, 2019a). In our study, 46.42% (13 / 28 animals) were identified as adults of > 12 months old, with another 17.86% (5 / 28 animals) categorized as 6 – 12 months old subadults, thus eliminating the concern of age group bias. High PCV3 prevalence of 33.15% was reported by Franzo *et al.* (2018c), despite sampling mostly apparently healthy wild boars (60 / 62 animals), suggesting that PCV3 may be unlikely to cause overt clinical diseases in wild boars. If such is the case, serum samples might be more suitable for study of PCV3 prevalence in wild boar population, as compared to lung samples used in this study. Nevertheless, 57.14% (20 / 35 samples) and 54.29% (19 / 35 samples)

detection rates in lung and spleen samples respectively have been reported (Klaumann *et al.*, 2019a). Possibility of false negative prevalence of PCV3 in wild boar population in this study due to low sample number still need to be considered.

PCV3 sequences from different years and countries analysed to date showed nt similarities ranging from 97 to 100% (Klaumann *et al.*, 2018a). The complete genome sequences of Malaysian PCV3 strains in this study showed similarities of 97.9% – 99.8%, concurring with the summarized global findings. In the case of PCV2, ORF1 is the most conserved region of the circovirus genome spanning the entire genome sequence (Mankertz *et al.*, 2004). In contrast, ORF2 has been identified as the most variable and most immunogenic viral protein (Fenaux *et al.*, 2000; Nawagitgul *et al.*, 2000; Olvera, Cortey & Segales, 2007). The assumption that the PCV3 *cap* gene is also a variable region like its PCV2 counterpart was supported as the 45 PCV3 *cap* gene sequences analyzed in this study showed higher p-distance values, when compared to the complete genomes in pairwise distance analysis. This indicates that the *cap* genes show higher variability than the complete genomes, in terms of number of base differences per site.

Tajima's D, Fu and Li's D, and Fu and Li's F statistical tests of neutrality share a common foundation of utilizing the frequency distribution of mutations. The unanimous negative values of all three neutrality tests signify an excess of low frequency polymorphisms relative to expectation, an effect resulting from either purifying selection or expansion of population size (Tajima 1989; Fu & Li, 1993). Considering the general negative trend of dN – dS values as shown

in Figs 5 and 6, we suggest that the ORF1 and ORF2 genes of PCV3 may be heavily influenced by negative selection pressure. In this study, up to 12.15% (26 / 214) of ORF2 codons were predicted to be evolving under negative selection pressure. ORF1 has a lower percentage of 8.11% (24 / 296). A recent study predicted up to 17% (37 / 214) of PCV3 ORF2 codons were negatively selected and suggested a strong negative selection acting over ORF2 gene of PCV3, which will likely cause the gene to be subjected to strong restrictions and thus unable to tolerate high levels of variation on its sequence (Saraiva *et al.*, 2018). In the context of diversity of aa residues at a given position, entropy H(x) values range from 0.0 (single residue present) to 4.322 (all 20 residues equally represented). As the H(x) value increases, it would be more likely to observe different aa residue diversity at the same codon position. Aa with H(x) $\geq$ 2.0 are considered variable, while highly conserved aa would be expected to have H(x) of $\leq$ 1.0 (Litwin & Jores, 1992). For PCV3, just as ORF2 has higher dN – dS values as compared to ORF1, ORF2 also have slightly more aa sites with H(x) entropy > 0.0, at 7.94% (17 / 214). This again suggests that ORF2 has higher genetic variability, in terms of number of base differences per site. However, all the H(x) values were well below $\leq$ 1.0, supporting the conjecture of PCV3 ORF2 being under strong purifying selection processes (Nawagitgul *et al.*, 2000). The highest H(x) value of ORF1 was seen at aa position 122, with a value of 0.683. The highest H(x) values of ORF2 ranged from 0.432 – 0.663, seen at aa position 24, 27, 77 and 150. The aa substitutions resulting from nt mutations at these five codons were suggested as criteria to classify PCV3 into different strains (Fux *et al.*, 2018). Apart from being a component of classification criteria, ORF2 aa position 24 was the

convergence of several studies, proposed as a potential epitope region and determinant of antigen effect (Li *et al.,*2018b; Li *et al.*, 2018e; Zou *et al.*, 2018; Qi *et al.*, 2019). Since the *cap* genes are much more divergent, differences over nt sequences may be resolved by phylogenetic comparison of the *cap* gene coding sequence, ORF2. Hence, the genomic variability of Malaysian PCV3 strains were compared by having their 645 bp long *cap* gene sequences phylogenetically analysed. Though the Malaysian PCV3 strains are rather closely related to each other, as reflected by p-distance values of < 0.021, they were grouped into two distinct clades.

This study suggests a homogeneous PCV3 frequency among farms with different size of standing sow population and proximity with neighbouring farms, since no statistically significant differences were found among those tested groups. There was no apparent geographical distribution, strains from central and southern Malaysia are present in both clades. However, it was observed that strains from the same farm are closely related, as shown by strains MY008 (GenBank Accession no.: MN725080) and MY010 (MN725082); MY001 (MK585347), MY002 (MK585348), MY009 (MN725081), MY012 (MN725084) and MY013 (MN725085). Interestingly, from the same farm, strains MY012 and MY013 which were obtained on a later time point in 2018/2019 showed further phylogenetic distance from strains MY001, MY002 and MY009 obtained in 2016/2017. In another farm, a phylogenetic distance gap was seen between strain MY008 (MN725080) and strain MY010 (MN725082) collected five months apart. This may suggest that PCV3 may have the tendency to mutate rapidly, as what was seen with PCV2. High

mutation rates of PCV2 were widely reported (Cortey *et al.*, 2011; Kekarainen *et al.*, 2014; Xiao, Halbur & Opriessnig, 2015). Over the years, new PCV2 antigenic variants have evolved with the variability of PCV2 attributed to high evolutionary rates of $1.2x10^{-3}$ substitution/site/year, higher than expected for a DNA virus and instead resembling RNA viruses (Firth *et al.*, 2009). In addition, low frequency mutations that allow rapid adaptation of the virus in changing environments had been identified in PCV2 genomes (Kekarainen *et al.*, 2014).

In the first clade, notably, Malaysian strain MY006 (MK585352) was singled out in its own cluster with a Spanish strain (MF805720) at a bootstrap value of 52%. The singling out of MY006 strain may be attributed to the isolated location and strict biosecurity practice of the origin farm that possibly reduced the likelihood of introducing circulating PCV3 strains from other local farms. Given that PCV3 can be found in semen of healthy animals and that potential persistent infection nature of PCV3 has been suggested, the phylogenetic relationship between Malaysian and Spanish strains might be related to semen and breeder importation (Ku *et al.*, 2017; Klaumann *et al.*, 2019a). Malaysian PCV3 strains also showed phylogenetic relationship with PCV3 strains from Italy, Thailand, Brazil, Japan and Germany, though with lower bootstrap values. However, of these countries, only Thailand and Germany have trade activities involving porcine products with Malaysia in the past 10 years (DVS, n.d.). In the second clade, Malaysian PCV3 strains were closely related to a U.S. strain (KX458235) and a Mexico strain (MH192340). Since 2001, Malaysia imports live breeders from the U.S., from 30 to 200 heads annually

though not consistently (DVS, n.d.). The U.S. is also the main provider of live swine imports for Mexico, with a 72% share of the Mexican import market (Lara & Kuypers, 2019). Hence, it may be speculated that the phylogenetic relatedness among Malaysian, Spanish, U.S. and Mexico PCV3 strains might be a result of live breeder and semen stock movement.

The first and second clades discussed above are evidently grouped together in a larger cluster. This observation is in accordance with the PCV3 strain classification system proposed by Fux *et al.* (2018). Fux *et al.* observed a specific aa motif which divides the PCV3 strains into two main groups. On aa position 122 of ORF1; aa position 24, 27, 77, 150 of ORF2 and aa position 1, 4, 227 of ORF3, two distinct patterns were observed: A – V K S I – F D G and S – A R S I – S G V, which defined group A1 and B1 respectively. Slight modification of motif into S –V K S I – F D V and S – A R T L – S G V gave rise to subgroups A2 and B2 respectively. Based on extrapolation from Fux *et al.*'s categorization, Malaysian PCV3 strains were classified as PCV3 group A1 and A2 (Table 19).

Table 19: Amino Acid Alignments of Selected aa in ORFs 1, 2 and 3 Showing Group Specific Motifs

Strain / ORF		**ORF1**	**ORF2**				**ORF3**		
		122	**24**	**27**	**77**	**150**	**1**	**4**	**227**
KY075990/China	A1	A	V	K	S	I	F	D	G
MF805721/Italy	A1	A	V	K	S	I	F	D	G
MH229786/Thailand	A1	A	V	K	S	I	F	D	G
MF589652/Thailand	A1	A	V	K	S	I	F	D	G
MF162299/Italy	A1	A	V	K	S	I	F	D	G

Table 19: Continued

Strain / ORF		ORF1	ORF2				ORF3		
		122	24	27	77	150	1	4	227
MF162298/Italy	A1	A	V	K	S	I	F	D	G
MF079254/Brazil	A1	A	V	K	S	I	F	D	G
LC383841/Japan	A1	A	V	K	S	I	F	D	G
LC269727/Japan	A1	A	V	K	S	I	F	D	G
PCV3/SL52/UPM/MY004/MK585350	A1	A	V	K	S	I	F	D	G
PCV3/SW35L/UPM/MY010/MN725082	A1	A	V	K	S	I	F	D	G
PCV3/SL56/UPM/MY005/MK585351	A1	A	V	K	S	I	F	D	G
PCV3/SW13L/UPM/MY008/MN725080	A1	A	V	K	S	I	F	D	G
LC383840/Japan	A1	A	V	K	S	I	F	D	G
MG014364/Germany	A1	A	V	K	S	I	F	D	G
MF805720/Spain	A1	A	V	K	S	I	F	D	G
PCV3/JL72/UPM/MY006/MK585352	A2	S	V	K	S	I	F	D	V
KX458235/US	A2	S	V	K	S	I	F	D	V
MH192340/Mexico	A2	S	V	K	S	I	F	D	V
PCV3/KG11/UPM/MY007/MK585353	A2	S	V	K	S	I	F	D	V
PCV3/PG43i/UPM/MY011/MN725083	A2	S	V	K	S	I	F	D	V
PCV3/SL28/UPM/MY001/MK585347	A2	S	V	K	S	I	F	D	V
PCV3/SS31L/UPM/MY009/MN725081			V	K	S	I			
PCV3/SL37/UPM/MY002/MK585348	A2	S	V	K	S	I	F	D	V
PCV3/SW51L/UPM/MY012/MN725084	A2	S	V	K	S	I	F	D	V
PCV3/SW53L/UPM/MY013/MN725085	A2	S	V	K	S	I	F	D	V
PCV3/KL44/UPM/MY003/MK585349			V	K	S	I			
PCV3/KG194L/UPM/MY/015/MN725087			V	K	S	I			
PCV3/MG196L/UPM/MY014/MN725086	A2	S	V	R	S	I	S	D	V
KX778720/US	B1	A	A	R	S	I	S	G	G
KY996337/Korea	B1	A	V	R	S	I	S	D	G
MG014363/Germany	B1	S	A	R	S	I	S	G	V
MG679917/Russia	B1	S	A	R	S	L	S	G	V
KX898030/US	B1	S	A	R	S	I	S	G	V

Table 19: Continued

Strain / ORF		ORF1	ORF2				ORF3		
		122	24	27	77	150	1	4	227
MG765473/Sweden	B1	S	A	R	S	I	S	G	V
MF805723/Denmark	B1	S	A	R	S	I	S	G	V
MH579736/Spain	B1	S	A	R	S	I	S	G	V
MG014367/Germany	B1	S	A	R	S	I	S	G	F
MG310152/Thailand	B2	S	A	R	T	L	S	G	V
MG014370/Germany	B2	S	A	R	T	L	S	G	V
KT869077/US	B2	S	A	R	T	L	S	G	V
KY996338/Korea	B2	S	A	R	T	L	S	G	V
MF611876/Korea	B2	S	A	R	T	L	S	G	V
KY075988/China	B2	S	A	R	T	L	S	G	V
MF079253/Brazil	B2	S	A	R	T	L	S	G	V

Similarly, there was no geographical distribution pattern observed, for both Malaysian and global strains of PCV3 A1, A2, B1 and B2. The arrangement of the motif was reflected in the phylogenetic clade arrangement, with strain A and B each clustered into separate clades (Figure 8). Further clustering into strain 1 and 2 were more evident in the A group, where clade A1 and A2 were clearly branched apart. However, more PCV3 sequences are needed to validate this genotype classification, and further work is required to determine if these genetic differences correlate to specific biological properties of PCV3 (Fux *et al,* 2018). Nevertheless, with the rapid emergence of PCV2 genotype 2d and 2e unfurling the speed of worldwide distribution of a new type of PCV, these phylogenetic and epidemiological data may give us a head start with PCV3 (Opriessnig *et al,* 2013).

In terms of relationship with other species of *Circoviridae,* China strains of PCV3 was reported to share a clade with bat associated circovirus 8 (BatACV8) at a high 82% bootstrap value; while the U.S. strains of PCV3 traced back to a common ancestor bat associated circovirus 2 (BatACV2) (Allan *et al.*, 2000; Li *et al.*, 2018e). Malaysian strains of PCV3 showed phylogenetic relatedness to bat-associated circovirus 7 (BatACV7) and StCV with a 30% bootstrap value. Another instance of involvement of bats in porcine diseases would be fruit bats of *Pteropid* species acting as natural reservoir hosts of Nipah virus, where a Nipah outbreak in the 1990s almost costed the entire Malaysian swine industry (Lam & Chua, 2002; Chua, 2003). As for starlings (*Sturnus vulgaris*), they are native to Europe, Asia and North Africa and have successfully established populations on nearly every continent (Rollins *et al.*, 2009). Starling faecal material has been shown to be one of the transmission sources of transmissible gastroenteritis (TGE) coronavirus, with history of TGE outbreaks in swine farms attributed to starlings as outbreak vector (Linz *et al.*, 2007). Although *Circovirus* genus members are known to infect a wide host range, cases of unspecific cross-species transmission have been reported. PCV1 and PCV2 have been detected in human stool samples, and avian circovirus-like DNA was found in wild chimpanzee faeces samples (ICTV, n.d.; Li *et al.*, 2010). PCV3 has also been detected in chamois, roe deer, cattle and canine (Zhang *et al.*, 2018; Franzo *et al.*, 2019b; Wang *et al.*, 2019). Considering the possibility of cross-species transmission, documented pathogenicity of closely related *Circovirus* species, high mutation and recombination rate of certain ssDNA viruses, and history of bats and starlings transmitting porcine pathogens, further investigation into the relationship

between PCV3 with BatACV and StCV may be warranted (Li *et al.*, 2010). On the other hand, the most recently discovered PCV4, showed the closest genomic and phylogenetic relationship with mink circovirus (MiCV) (Zhang *et al.*, 2020b). The pathogenicity of PCV4 remains unclear and to date, no direct relationship between PCV3 and PCV4 has been reported.

5.5 Conclusion

PCV3 is present in Peninsular Malaysia at a molecular prevalence of 17.02%, with inguinal lymph nodes and lungs showing the highest molecular detection rates of 81.82% and 71.43% respectively. Among the nine organ types tested, only the molecular detection rate in inguinal lymph nodes was statistically significant. Although PCV3 positive samples spanned across all age groups from foetuses to finishers and sows, only the weaner group was shown to be statistically significant. Despite wide reports of PCV3 in healthy animals and wild boars, no positive samples were detected in the clinically healthy finishers and wild boar population in this study. PCV3 strains included in this study were found to be heavily influenced by negative selection pressure. In both ORF1 and ORF2, aa positions with the highest H(x) values correspond with the distinct mutation patterns included in the current PCV3 strain classification system. Malaysian PCV3 strain A1 and A2 were phylogenetically related to Spanish, U.S. and Mexico strains.

CHAPTER 6

DIAGNOSTIC DEVELOPMENT FOR PORCINE CIRCOVIRUS 3 (PCV3)

Article 5: Chromogenic In-Situ Hybridisation Technique for Detecting Porcine Circovirus 3 in Lung and Lymphoid Tissues

6.1 Introduction

In the past two decades, porcine circovirus type 2 (PCV2), the key etiological agent in PCV2-associated disease (PCVAD), has caused significant economic losses in the pig industry. While the impact of PCVAD on pig production is still being mitigated, a novel PCV species has been discovered. Porcine circovirus 3 (PCV3) was first reported in 2015 in a US sow herd with increased mortality and decreased conception rate. The sows in the index case presented clinical signs similar to porcine dermatitis and nephropathy syndrome (PDNS), and reproductive failure, which were often associated with PCV2. Metagenomic analysis revealed a novel PCV, designated PCV3 (Palinski *et al.*, 2017). Another report described PCV3 cases in weaners showing myocarditis with multisystemic inflammation (Phan *et al.*, 2016). Although PCV3 infection is associated with multiple clinical signs, the three most consistent clinical presentations are reproductive failure, multisystemic inflammation, and subclinical infection (Kroeger *et al.*, 2022). While many field reports indicated coinfections of PCV3 with other common swine pathogens, the pathogenicity of PCV3 as a single etiological agent was demonstrated through successful reproduction of PDNS-like clinical disease following experimental inoculation of 4- and 8-week-old specific-pathogen-free piglets with infectious PCV3 DNA clone (Jiang *et al.*, 2019a). Recently, another novel species was identified in

China, South Korea, Thailand, and Malaysia, sequentially named PCV4 (Nguyen *et al.*, 2022; Zhang *et al.*, 2020b; Sirisereewan *et al.*, 2023; Tan 2023b). Although the infectious PCV4 clones induced systemic pathological changes in piglets, it may be too early to corroborate the clinical significance of PCV4 in swine husbandry (Niu *et al.*, 2022). PCV3 infection continues to be reported in more countries, showing the prevalence of PCV3 coinfection with other ubiquitous porcine pathogens (Ouyang *et al.*, 2019b). Numerous polymerase chain reaction (PCR) and quantitative-PCR (qPCR)-based PCV3 diagnostic assays have been developed, with detection limits of $<10^2$ copies/µL sample (Kroeger *et al.*, 2022). Using conventional PCR, PCV3 was detected in the Malaysian commercial pig population from samples collected over 2016 to 2019 with molecular prevalence rates of 41.67% and 17.02% in farms and domestic pig populations, respectively. The molecular detection rate was highest in the inguinal lymph nodes and lungs, at 90% and 73.33%, respectively (Tan *et al.*, 2020; Chapter 5 of thesis). At present, the development of PCV3 antibodies and their subsequent application for direct antigen detection is limited to studies involving immunohistochemistry (IHC) and immunofluorescence assay (IFA) (Kroeger *et al.*, 2022). Under these circumstances, *in situ* hybridisation (ISH) assay might be a valuable adjunct to the current molecular antigen detection methods for investigating tissue tropism and pathogenesis of PCV3. In general, ISH involves fixation and pre-treatment of tissue samples to preserve and present the target nucleic acid optimally. Then, the labeled probes are hybridized using complementary base pairing to the target, which is detected using microscopic visualization of the hybrid (Warford, 2016). Successful localization of PCV3 antigen within the

tissue environment is crucial for elucidating its pathogenesis. Although ISH has been used in several PCV3 studies, it has not yet been thoroughly described. Furthermore, most PCV3 ISH studies employed the RNAScope assay, which might not be economically feasible for small-scale research or diagnostics laboratories (Phan *et al.*, 2016; Arruda *et al.*, 2019; Saporiti *et al.*, 2021). This study aimed to describe a chromogenic ISH technique using digoxigenin (DIG)-labeled cloned PCV3 open reading frames 1 (ORF1) fragment DNA to detect the localization of PCV3 antigen in formalin-fixed, paraffin-embedded (FFPE) lung, and lymphoid tissue specimens.

6.2 Materials and Methods

6.2.1 Sample Collection

A sample subset with ten corresponding sets of lung and lymphoid tissues from a previous Malaysian PCV3 study based on the PCV3 PCR results on fresh tissues and the availability of FFPE was selected tissues (Tan *et al.*, 2020). All the PCV3-positive and -negative samples were also positive for PCV2 and porcine reproductive and respiratory syndrome (PRRS) and negative for PCV4. Porcine circovirus 2, PCV3, and PCV4 antigens were detected using PCR, as described previously by (Tan *et al.*, 2020; Tan *et al.*, 2022; Tan *et al.*, 2023). Polymerase chain reaction was done to detect the PRRS viral antigen using the RealPCR PRRSV Type 1 and Type 2 Multiplex RNA Mix (IDEXX, US) according to the manufacturer's instructions. The fresh tissues were kept at −80°C (Haier Thermo Scientific TDE60040LD Ultra-Low Temperature Freezer, China) in the Clinical Research Laboratory, Faculty of Veterinary

Medicine, Universiti Putra, Malaysia. A part of each sample was preserved as FFPE tissues. The tissue samples were cut into small pieces (1 cm × 1 cm), fixed in 10% (w/v) neutral buffered formalin for 48 h, and paraffin-embedded according to standard histopathological procedures. Then, 4 µm-thick sections were cut using a microtome (Leica HI1210, Germany) and floated on a 45°C water bath. The resulting tissue section ribbons were mounted on poly-L-lysine coated glass slides for ISH (PorLab Scientific, China).

6.2.2 Preparation of DIG-Labelled Probe

Two PCV3-positive samples from the previous Malaysian PCV3 study were subjected to conventional PCR to amplify a 69 bp region of the PCV3 ORF1 spanning from nucleotide (nt) positions 282 – 350. The PCR was performed using the same reagents and reaction volumes described in the PCV3 cap gene detection methodology (Tan *et al.*, 2020). Table 20 provides the details of the primer pair and cycling conditions.

Table 20: Primers and PCR Cycling Conditions Used in This Study for Amplification of PCV3 ORF1 Fragment

Primer Pair	Nucleotide Sequence (5'– 3')	Product Length (bp)	PCR Cycling Condition (temperature/time)	
PCV3-69F	CGAGTGGGAATCTATTGTGGA	69	Initial denaturation	95°C/3 min
			Number of cycles	40
			Denaturation	98°C/20 s
			Annealing	50°C/30 s
PCV3-69R	CCAACCTCTTTGCCGATAAT		Extension	72°C/50 s
			Final extension	72°C/10 min

Agarose electrophoresis of the PCR products showed a positive band between 50 and 100 bp region as marked by GelPilot 50 bp Plus Ladder (Qiagen®, Germany). These bands were purified using the MEGAquick-spin™ Total Fragment DNA Purification Kit (Intron®, Korea) and sequenced (Macrogen Inc., South Korea). The MEGA v7.0.26 software program was used for sequence assembly and multiple sequence alignment (Kumar, Stecher & Tamura, 2016). The resulting sequences were analyzed using NCBI Nucleotide BLAST® for confirming PCV3 by comparing their similarity with reference PCV3 sequences from GenBank (Altschul *et al.*, 1990).

Using the purified PCR product as the insert DNA, PCR cloning was done using the StrataClone PCR Cloning Kit according to the manufacturer's instructions with some modifications (Agilent®, US). First, the ligation reaction mixture was prepared by gently mixing 3 µL of cloning buffer, 2 µL of the aforementioned purified PCR product (50 ng), and 1 µL of pSC-A-amp/kan cloning vector in that order. Individual tubes of StrataClone SoloPack competent cells were thawed on ice immediately after removing them from the freezer. On 20 min of incubation at room temperature of 24 – 26°C, 1 µL of the ligation mixture was gently mixed by swirling it into the thawed competent cells. After incubating for 20 min on ice, the transformation mixture was heat-pulsed at 42°C for 45 s and promptly kept on ice for 2 min. Next, 200 µL of super optimal broth with catabolite repression (SOC) medium, pre-warmed at 42°C, was added to the tubes containing the transformation mixture (Sigma-Aldrich, US), which were placed horizontally in an incubator shaker for 1 h at 37°C with 1 x*g* agitation. The media was prepared using 20 g of Miller's LB broth powder

(Huankai Microbial, China) and 20 g of nutrient agar powder (HiMedia, U.S.) in 1L of deionized water. After the media was autoclaved and cooled down to 55°C, 10 mL of 10 mg/mL filter-sterilized ampicillin (ampicillin: Bio Basic, Canada; 25 mm/0.22 µm syringe filter: CNW, China) was added into the media. For each 100 mm Petri dish, 25 mL of agar was poured and cooled to room temperature of 24 – 26°C for 30 min.

For the blue-white screening, 2% X-gal was prepared by mixing 0.2 g X-gal in 10 mL IPTG (Sangon Biotech, China) and adding it onto the LB-ampicillin plates at 40 µL per plate. For each transformation mixture, 5 µL and 100 µL volumes were plated separately. The 5 µL mixture was plated by pipetting it into 50 µL of SOC media to facilitate spreading. The X-gal and transformation mixture were uniformly plated using 15 autoclaved glass plating beads for each Petri dish (Zymo Research, US). The plates were gently shaken in different directions to evenly roll the beads. After the agar surface dried, the beads were removed, and the plates were incubated for 24 h at 37°C. After the transformation, the white colonies were inoculated in LB-ampicillin broth overnight in a shaker incubator at 37ºC and 3 x*g*. After the incubation, 10 mL of culture from each incubated cloning reaction was collected for plasmid isolation. Plasmid DNA isolation was performed using ISOLATE II Plasmid Mini Kit, according to low-copy plasmid isolation protocol (Meridian Life Science, US).

Next, 25 pg of the purified plasmid DNA was used as the template DNA for DIG-dUTP (DIG-dUTP) labeling. The reaction mix using the PCR DIG Probe Synthesis Kit (Sigma-Aldrich®, US) according to the manufacturer's

instructions using the cycling conditions shown in Table-1. The end reaction mixture was verified for successful DIG-labeled probe synthesis by agarose gel electrophoresis with 5 µL of PCR product stained with GelPilot DNA loading dye as molecular weight marker (Qiagen, Germany).

6.2.3 *In situ* Hybridisation

In situ hybridisation was performed using the BioSpot ISH Kit for the DIG probe with the modified protocol (BioTnA, Taiwan). The slides were heated on a heating plate at 60°C for 20 min until the wax dissolved. For deparaffinization of the tissue sections, the slides were rinsed thrice with absolute xylene for 5 min each and rehydrated for 5 min each through a 100%, 90%, 70%, 50%, and 30% ethanol gradient. After washing with distilled water, hydrogen peroxide block was dropped liberally on the slides, and coverslips were placed on the tissue sections. After incubation for 20 min at 25°C, the slides were rinsed with tris-buffered saline solution, which was used for all subsequent rinsing steps. Next, the slides were immersed in a heat pre-treatment solution pre-warmed to 100°C and maintained in an isothermal water bath for 30 min. The slides were cooled for 5 min and then rinsed. Next, pepsin solution pre-warmed to room temperature was dropped on the slides, covered with coverslips, incubated in a moist humidity chamber at 37°C for 10 min, and rinsed. After pre-treatment, the ISH probe was prepared as a 1/150 dilution: 1 µL probe to 150 µL of hybridisation buffer (Roche, Switzerland). After heating the diluted probe in a 100°C water bath for 15 min, 100 µL of the heated probe was added to each slide, and coverslips were placed over the tissue sections. The slides were incubated in a moist humidity chamber at 55°C for 16 h, immersed in

saline sodium citrate solution pre-warmed to 55°C, and maintained in an isothermal water bath for 5 min. After rinsing, a blocking solution was added to the slides, covered with coverslips, incubated in a moist humidity chamber at 37°C for 30 min, and rinsed. The step was repeated using a mouse anti-DIG antibody for a longer incubation period of 60 min. Horseradish peroxidase was applied on the slides, incubated at 25°C for 30 min, and rinsed. The slides were stained with DAB for 20 s and counterstained with nuclear blue stain for 15 min. Finally, they were dehydrated for 5 min each through 30%, 50%, 70%, 90%, and 100% ethanol and evaluated using light microscopy evaluation using an imaging microscope (Motic Image plus 2.0, China).

6.3 Results and Discussion

Nucleotide sequence analysis of the PCR products showed the targeted ORF1 region from nt position 282 to 350 for positive samples L43 and L53. Agarose electrophoresis results indicated a positive band between the 50 and 100 bp region for the unlabeled control probes, while the corresponding DIG-labeled probes were closer to 100 bp (Figure 11). The DIG-labeled probes were expected to migrate slower during electrophoresis as the DIG molecules increased the overall molecular weight. Hence, the development of a PCV3-specific ISH DIG probe targeting the 69 bp region of PCV3 ORF1 was confirmed.

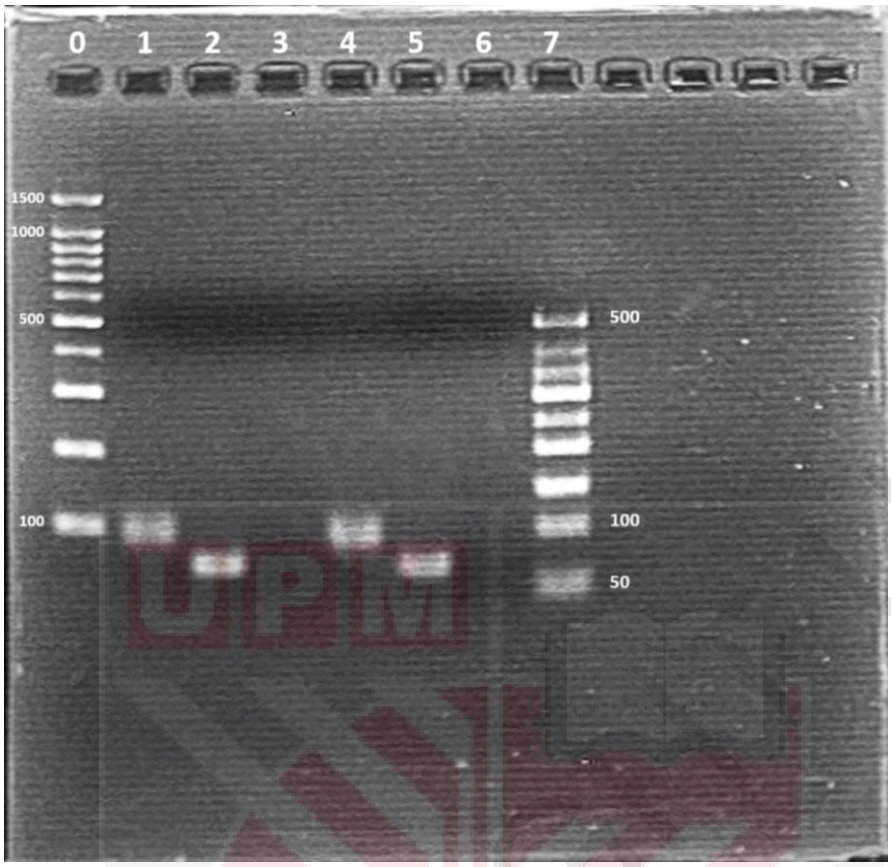

Figure 11: Verification of *in situ* hybridisation (ISH) digoxigenin (DIG) probe development by agarose electrophoresis. DIG-labeled probes were successfully developed using porcine circovirus 3 (PCV3) positive samples L43 and L53 as template. Unlabeled probes were expected to be 69 bp, while corresponding labeled probes were expected to appear larger. The generated probes were used in subsequent ISH staining protocol. Lane 0: DNA ladder (Qiagen® GelPilot 50 bp ladder). Lane 1: Positive band for unlabeled experimental PCV3 probe from sample L43. Lane 2: Positive band for DIG-labeled experimental PCV3 probe from sample L43. Lane 3: Negative control. Lane 4: Positive band for unlabeled experimental PCV3 probe from sample L53. Lane 5: Positive band for DIG-labeled experimental PCV3 probe for sample L53. Lane 6: Negative control. Lane 7: DNA ladder (Qiagen® GelPilot 100 bp plus ladder)

Light microscopic observation of PCV3 antigens that were stained brown indicates positive ISH results. Figures 12a to 12d showed positive ISH staining results in the lung, inguinal lymph node, and mesenteric lymph node tissue sections under magnification, while Figures 13a and 13b showed negative ISH staining results.

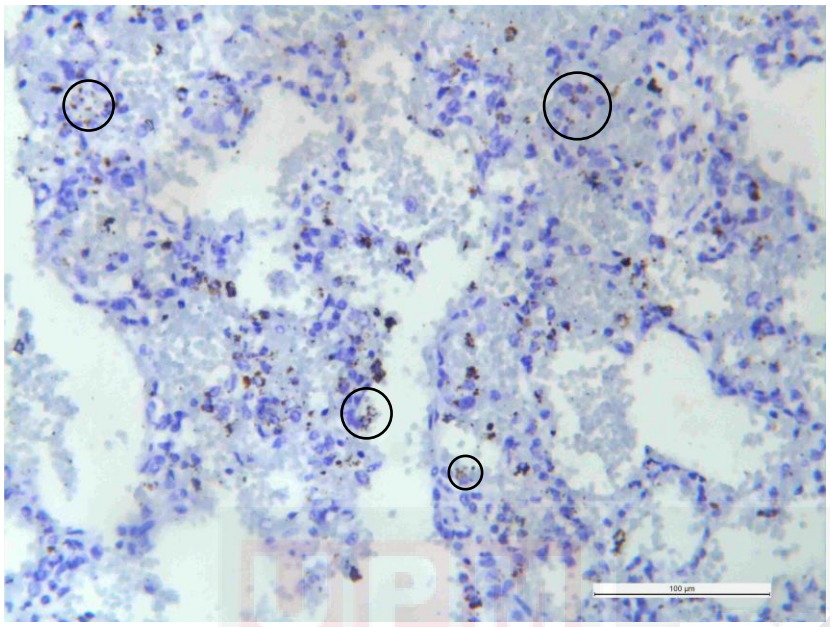

Figure 12(a): Positive *in situ* hybridisation (ISH) results from lung tissues. Multifocal ISH positive signals were observed as brown granules of chromogenic staining, indicating localization of PCV3 antigen present within cytoplasm of pneumocytes. Circles highlight several of the many brown stains. 200× magnification with scale bar equivalent to 100 µm

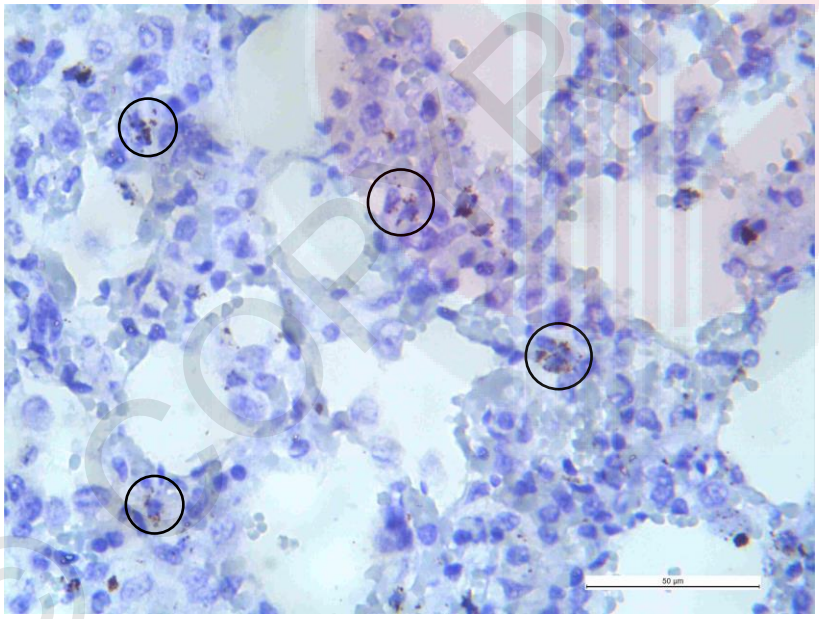

Figure 12(b): Positive ISH results from lung tissues. Multifocal ISH positive signals were observed as brown granules of chromogenic staining, indicating localization of PCV3 antigen present within cytoplasm of pneumocytes. Circles highlight several of the many brown stains. 400× magnification with scale bar equivalent to 50 µm

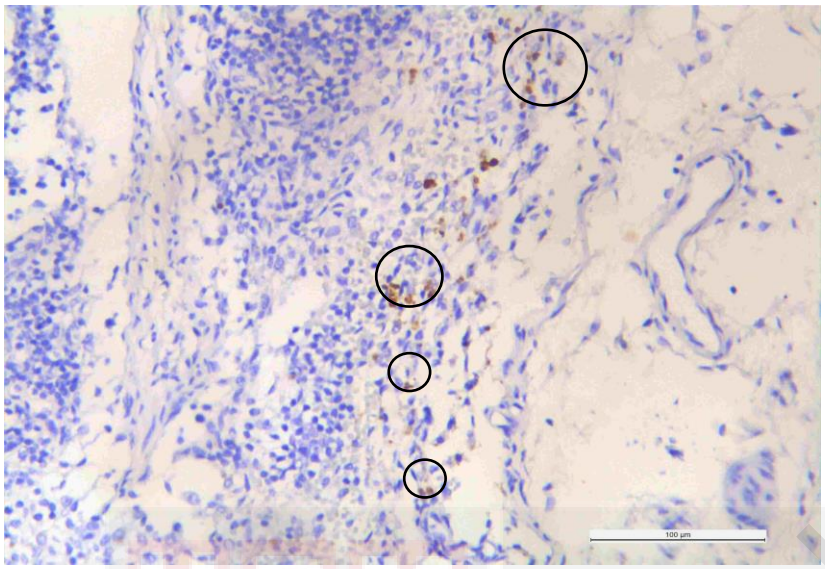

Figure 12(c): Positive ISH results from inguinal lymph node tissue. Multifocal ISH positive signals were observed as brown granules of chromogenic staining, indicating localization of PCV3 antigen present within cytoplasm of lymphocytes. Circles highlight several of the many brown stains. 200× magnification with scale bar equivalent to 100 µm

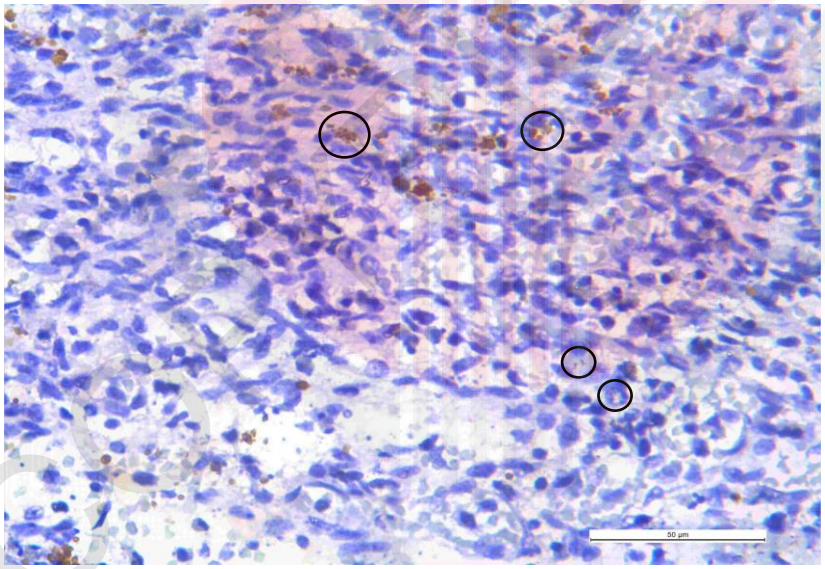

Figure 12(d): Positive ISH results from mesenteric lymph node tissues. Multifocal ISH positive signals were observed as brown granules of chromogenic staining, indicating localization of PCV3 antigen present within cytoplasm of lymphocytes. Circles highlight several of the many brown stains. 400× magnification with scale bar equivalent to 50 µm

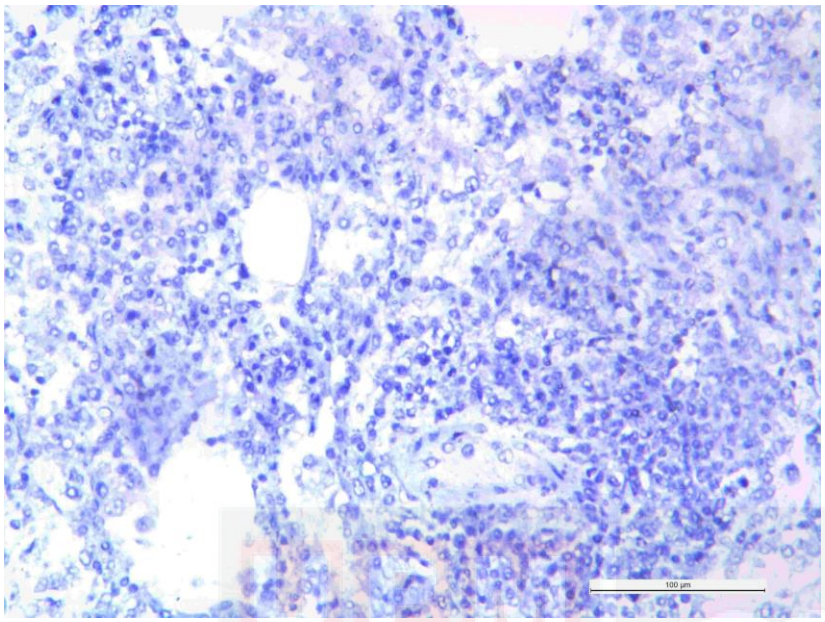

Figure 13(a): Negative in situ hybridisation (ISH) results from lung tissues. ISH positive signals were not observed, indicating absence of PCV3 antigen. 200× magnification with scale bar equivalent to 100 µm

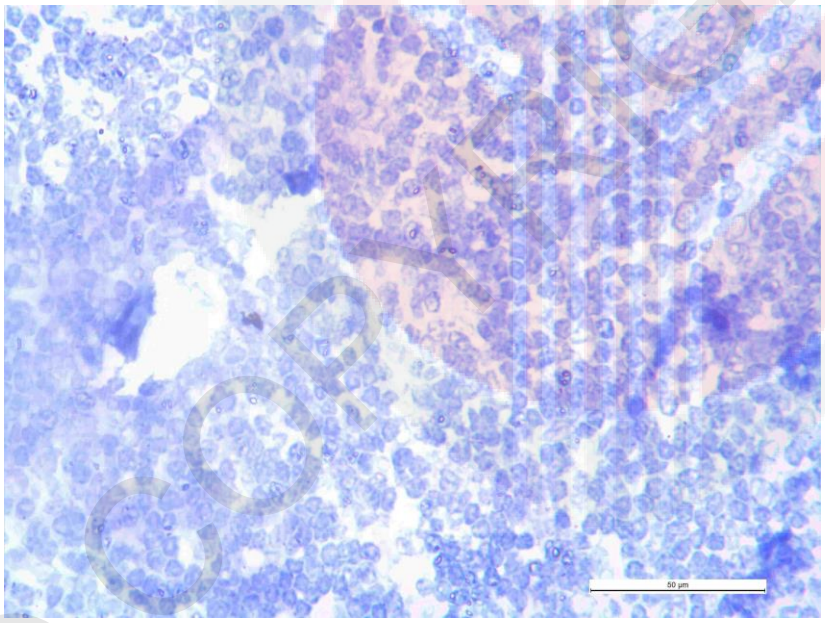

Figure 13(b): Negative ISH results from inguinal lymph node tissues. ISH positive signals were not observed, indicating absence of PCV3 antigen. 400× magnification with scale bar equivalent to 50 µm

As the previous Malaysian PCV3 study reported that PCV3 was mainly detected in the inguinal lymph nodes and lung tissues, these tissues were targeted in this study (Tan *et al.*, 2020*)*. Tissue samples from these organs for

the cases with PCV3-suspected disease were also collected, together with heart tissues (De Conti *et al.*, 2021). All the positive hybridisation signals observed during PCV3 ISH were intracytoplasmic with a predominantly multifocal distribution pattern, consistent with the previous ISH studies (Kedkovid *et al.*, 2018a; Saporiti *et al.*, 2021). Apart from lung and lymph node tissues, positive ISH signals were also reported in lesions of various organs, including myocarditis, arteritis, perivasculitis, nephritis, chorioamnionitis, encephalitis, dermatitis and pneumonia from foetuses, weaners, growers, and sows (Phan *et al.*, 2016; Kedkovid *et al.*, 2018a; Arruda *et al.*, 2019; Oh & Chae, 2020; De Conti *et al.*, 2021; Saporiti *et al.*, 2021; Cobos *et al.*, 2022). Table 21 shows the results of PCV3 molecular detection status using PCR and ISH, which were in good agreement.

Table 21: Tabulation of PCV3 ISH Results Compared to PCV3 PCR Results. Samples Were Segregated Based on PCV3 PCR Detection Status to Compare Agreement Between PCV3 PCR and ISH Detection Results

PCV3 PCR Positive Samples (n = 11)			**PCV3 PCR Negative Samples (n = 19)**		
PCV3 PCR Results	**Sample ID**	**PCV3 ISH Results**	**PCV3 PCR Results**	**Sample ID**	**PCV3 ISH Results**
PCV3 positive	43 - ILN	Positive	PCV3 negative	43 - Lung	Negative
	43 - MLN	Negative		208 - Lung	Negative
	51 - Lung	Positive		208 - ILN	Negative
	51 - ILN	Positive		208 - MLN	Negative
	51 - MLN	Positive		209 - Lung	Negative
	53 - Lung	Positive		209 - ILN	Negative
	53 - ILN	Positive		209 - MLN	Negative
	53 - MLN	Positive		210 - Lung	Negative
	199 - Lung	Positive		210 - ILN	Negative

Table 21: Continued

PCV3 PCR Positive Samples (n = 11)			PCV3 PCR Negative Samples (n = 19)		
PCV3 PCR Results	**Sample ID**	**PCV3 ISH Results**	**PCV3 PCR Results**	**Sample ID**	**PCV3 ISH Results**
	199 - ILN	Positive		210 - MLN	Negative
	199 - MLN	Positive		220 - Lung	Negative
				220 - ILN	Negative
				220 - MLN	Negative
				221 - Lung	Negative
				221 - ILN	Negative
				221 - MLN	Negative
				222 - Lung	Negative
				222 - ILN	Negative
				222 - MLN	Negative

ILN=Inguinal lymph node, MLN=Mesenteric lymph node, PCR=Polymerase chain reaction, PCV3=Porcine circovirus 3, ISH=*In situ hybridisation*

Only one out of the 11 samples positive for PCV3 by PCR method was ISH-negative. This was probably because the mesenteric lymph node tissues collected during post-mortem were too scarce, causing imperfect tissue fixation onto the ISH slide and insufficient binding of the DIG probe to the tissues. The PCR and ISH results for all 19 PCV3-negative samples were in agreement. Further, no false positivity of PCV2 and PRRS was observed. No positive ISH signals were observed in the PCV3 negative samples, which were positive for PRRS and PCV2.

At present, PCV3 detection is mostly done using molecular techniques, such as conventional PCR and qPCR, and its Characterisation by Sanger sequencing or next-generation sequencing (NGS) (Klaumann *et al.*, 2018a).

Five years into its discovery, commercial PCV3 antibodies are still not widely available, possibly due to limited virus isolation (Kroeger *et al.*, 2022). This limited availability of PCV3-specific antibodies restricts the development of IHC and IFA methods. Compared to IHC and IFA that require antibodies, ISH can be developed more rapidly using only the sequence information from tissue samples positive for PCV3. Furthermore, ISH can be applied in conjunction with PCR or qPCR, NGS, and histopathology to investigate the association among pathogens, lesions, and clinical outcomes (Resende *et al.*, 2019). Disease investigation or epidemiological study can start with PCV3 detection and/or quantification by PCR or qPCR, followed by ISH to visualize the localization of PCV3 DNA within tissues or cells.

Successful ISH depends on sufficient preservation of the sample tissues' morphologic details, the ISH probe's specificity, proper probe hybridisation, and labeling for accurate antigen detection (Qian & Lloyd, 2003). All the samples in this study were fixed in 10% neutral buffered formalin for 48 h immediately on collection to maximize the detection potential of nucleic acids within FFPE tissues (Maes *et al.*, 2014). The ISH results are also less susceptible to structural alterations in tissue, commonly seen with formalin fixation (Choi & Chae, 1999; Kim & Chae, 2001). Although ISH with RNA probes is more sensitive than DNA probes, given the single-stranded nature of the PCV3 DNA, DNA probes are considered equally sensitive and reliable as RNA probes for diagnostic purposes (Brown, 1998; Choi & Chae, 1999). The ISH probes in this study were designed based on the ORF1 gene of PCV3, considering that it is the most conserved region of the circovirus genome

(Mankertz *et al,* 2004). Further, the probe length was kept to a minimum of 69 bp to ensure the specificity of the probe. The ISH procedure, from cloning to prepare the DIG-labeled probes to probe hybridisation and labeling, was optimized to achieve accurate PCV3 antigen detection. Further, compared to the antibodies used for IHC, the antibodies for the DIG-labeled probes do not show any cross-reactivity with animal tissues (Maes *et al.*, 2014). Thus, by combining DIG-labeled DNA probes and ISH in this study, the possibility of errors caused by antigenic cross-reactivity and/or the alteration of binding sites caused by formalin fixation were minimized (Chae, 2004) Moreover, most of the published PCV3 ISH studies used the RNAScope assay, which is more expensive whereas our DIG-ISH protocol is a more economically feasible and practical option for smaller-scale research or diagnostics laboratories (Phan *et al.*, 2016; Arruda *et al.*, 2019; Saporiti *et al.*, 2021).

6.4 Conclusion

This study reports a chromogenic ISH technique using DIG-labeled cloned PCV3 ORF1 fragment DNA probe that enabled detection and localization of PCV3 antigen in FFPE lung and lymphoid tissue specimens. Here, both PCR and ISH results for detecting PCV3 were in good agreement. Only one lymphoid tissue sample was ISH-negative, presumably due to lower tissue availability. *In situ* hybridisation can complement PCR in detection studies for the localization of antigens in infected tissue sections. The diagnostic application of ISH could remain relevant in investigating PCV3 replication and pathogenesis, especially with the limited development of PCV3 antibodies required by other immunostaining methods.

CHAPTER 7

MOLECULAR DETECTION AND CHARACTERISATION OF PORCINE CIRCOVIRUS 4 (PCV4)

Article 6: First molecular detection of Porcine Circovirus type 4 (PCV4) in Malaysia

7.1 Introduction

Circoviruses have been of veterinary importance especially in the swine and avian field, where beak and feather disease virus (BFDV) and porcine circovirus type 2 (PCV2) related diseases have been extensively researched (Todd, 2004). Porcine circoviruses (PCVs) belong to the *Circoviridae* family, genus circovirus. Historically, the first porcine circovirus was discovered in 1974 as an apathogenic cell culture contaminant, designated as porcine circovirus 1 (PCV1) (Tischer *et al.*, 1982). PCV was given more attention two decades later, when porcine circovirus 2 (PCV2) was associated with the first clinical outbreak of postweaning multisystemic wasting syndrome (PMWS) reported from December 1994 to March 1996. PMWS was described with a characteristic presentation of unthriftiness, jaundice, respiratory distress, diarrhea and increased post weaning mortality rate (Clark, 1996; Harding, 1998). The term porcine circovirus associated diseases (PCVAD) was later coined by the American Association of Swine Veterinarians (AASV) to be more inclusive of complex PCV2-related multifactorial diseases under one umbrella term (AASV, 2017).

Another two decades following the emergence of PCV2, metagenomic sequencing uncovered a third porcine circovirus species. Porcine circovirus 3 (PCV3) was genetically divergent from PCV1 and PCV2, sharing only <50% of overall nucleotide identity (Phan *et al.*, 2016; Palinski *et al.*, 2017; Rosario *et al.*, 2017). The index PCV3 case reported increased sow mortality rate and decreased conception rates with presenting dermal and renal lesions, suggestive of porcine dermatitis and nephropathy syndrome (PDNS) (Palinski *et al.*, 2017). As with PCV2 of the PCVAD, PCV3 has also been associated with a myriad of clinical presentations including respiratory, enteric, myocarditis and nervous signs (Phan *et al.*, 2016; Chen *et al.*, 2017; Ku *et al.*, 2017; Zhai *et al.*, 2017; Arruda *et al.*, 2019; Qi *et al.*, 2019). Successful isolations of PCV3 as well as pathogenicity demonstration through infectious molecular clones have been reported (Jiang *et al.*, 2019; Mora-Díaz *et al.*, 2020; Oh & Chae, 2020). Most recently, yet another new species was identified in China, South Korea and Thailand, sequentially named porcine circovirus 4 (PCV4) (Zhang *et al.*, 2020b; Nguyen *et al.*, 2022; Sirisereewan *et al.*, 2023). Infectious clone of PCV4 had been shown to induce systemic pathological changes in piglets (Niu *et al.*, 2022)

In Malaysia, PCV2 with its myriad of PCVAD has been reported to be endemic in the local pig farming industry since its first detection (Hassuzana *et al.*, 2004). To date, the molecular detection rate of PCV2 remains high at over 80%. Furthermore, a notable genotype shift from PCV2b to PCV2d has been reported (Tan *et al.*, 2022). Porcine circovirus 3 (PCV3) was also reported in

Malaysia, albeit at a lower rate of below 20% (Tan *et al.*, 2020). This study aims to investigate whether PCV4 is present in Malaysia.

7.2 Materials and Methods

7.2.1 Sample Collection

A sample subset consisting of 49 lung tissue samples from the aforementioned Malaysian PCV2 and PCV3 study was used to conduct molecular detection study of PCV4 (Tan *et al.*, 2020; Tan *et al.*, 2022). The aforementioned PCV study was granted approval from the Institutional Animal Care and Use Committee (IACUC) under AUP Code UPM/IACUC/AUP-R030/2019 and was conducted adhering to the guidelines as stated in the Code of Practice for Care and use of Animals for Scientific Purposes as stipulated by Universiti Putra Malaysia. Briefly, the sample subset comprising 30 lung tissue samples was randomly picked from the PCV2/PCV3 positive/negative PCR status quadrant. The selected samples originated from 25 commercial intensive pig farms in northern, central and southern regions of Peninsular Malaysia run on a semi-closed house, farrow-to-finish system, with farm size ranging from 200 to 1500 sows. In addition to that, nine lung samples from the abattoir and ten lung samples from the wild boar population were also included in this study. The samples were collected from 2016 to 2021.

7.2.2 Molecular Detection and Molecular Characterisation

The samples were subjected to conventional PCR for detection of PCV4 capsid (*cap*) using previously published primers (Zhang *et al.*, 2020). The optimized cycling conditions were detailed in Table 22.

Table 22: Primers and PCR Cycling Conditions Used in This Study to Generate Complete Nucleotide Sequence of PCV4 Cap Region

Primer Pair	Nucleotide Sequence (5'– 3')	Product Length (bp)	PCR Cycling Condition (temperature/time)	
Zhang-F	TGAGGGAGGA TGGGCAGTTGT ATG	842	Initial denaturation	95°C/5 min
			Number of cycles	35
			Denaturation	95°C/20 s
			Annealing	58°C/30 s
Zhang-R	CACCACCCACA GATGCCAATCA		Extension	72°C/1 min
			Final extension	72°C/10 min

Positive samples were further sequenced for PCV4 *cap* identification, located at a region spanning from position nucleotide (nt) 1047 – 1733. The nucleotide sequences were analysed with NCBI Nucleotide-BLAST® to verify their similarity with reference PCV4 sequences in the GenBank database (Altschul *et al.*, 1990).

For the subsequent phylogenetic analyses of Malaysia PCV4 *cap* nucleotide sequences, sequence assembly and multiple sequence alignment were generated using MEGA v7.0.26 (Kumar, Stecher & Tamura, 2016). Phylogenetic relatedness of the Malaysian strains in relation to porcine circoviruses and International Committee on Taxonomy of Viruses (ICTV)

exemplar isolates of *circovirus* genus was inferred using maximum likelihood method based on the General Time Reversible (GTR) model with 1000 bootstrap replicates (Nei & Kumar, 2000; Breitbart *et al.*, 2017).

7.3 Results and Discussion

PCV4 was confirmed to be present in Malaysia at a detection rate of 4.08% (2 / 49 samples) (Table 23). Both positive samples were from clinically healthy finishers, originating from a farm in northern Peninsular Malaysia. The detection rate of PCV4 was rather low compared to the most recent Malaysian PCV2 and PCV3 molecular prevalence, reported to be 83.54% and 17.02% respectively (Tan *et al.*, 2020; Tan *et al.*, 2022).

Table 23: Distribution of PCV4 PCR Positive Samples Amongst the Previously Positive PCV2 and PCV3 Samples

Sample Description					PCV4 PCR Detection Status	
PCV2 Status	**PCV3 Status**	**Sample Origin**	**Number of Samples (n)**		**PCV4 Positive (n)**	**PCV4 Negative (n)**
Positive	Positive	Farm	10	11	0	10
		Abattoir	1		1	0
Positive	Negative	Farm	10	17	0	10
		Abattoir	7		1	6
		Wild boar	10	11	0	10
Negative	Positive	Farm	1		0	1
Negative	Negative	Farm	9	10	0	9
		Abattoir	1		0	1
TOTAL			49 samples		2 / 49 (4.08%)	47 / 49 (95.92%)

Both *cap* nucleotide sequences were amplified successfully (GenBank accession numbers: OP588909, OP588910). Nucleotide sequence and phylogenetic analysis of Malaysian PCV4 *cap* gene sequences showed that the strains shared over 99% similarity with PCV4 sequences recorded in GenBank databases. In striking contrast, *cap* gene sequences of Malaysian PCV2 and PCV3 were very distinct from that of Malaysian PCV4, sharing only 25.27% and 28.68% nucleotide identity respectively (Figure 14).

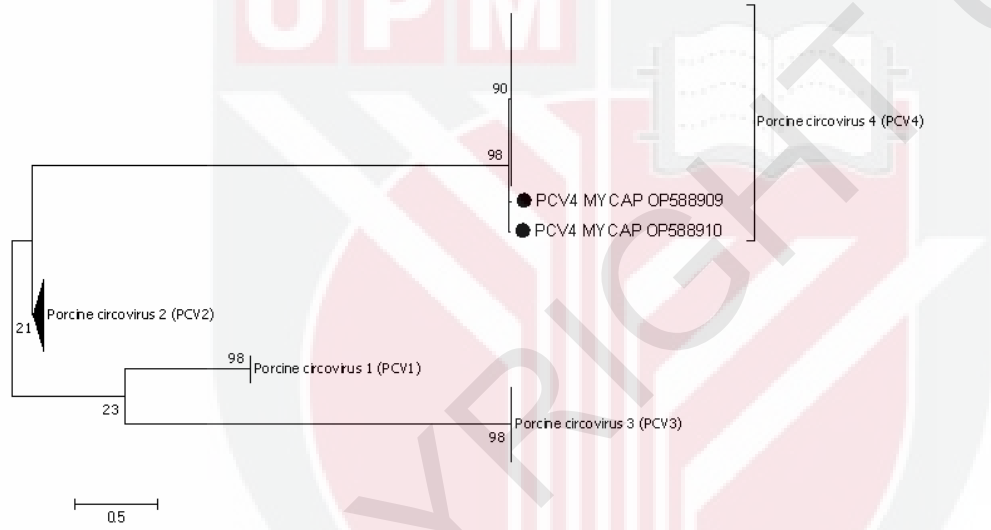

Figure 14: Condensed phylogenetic analysis of Malaysian PCV4 cap gene nucleotide sequences with reference to PCV1, PCV2, PCV3 and PCV4 cap gene nucleotide sequences. Two Malaysian PCV4 strains (●) were compared with 143 GenBank reference sequences which consisted of 11 PCV1 cap gene nucleotide sequences, 30 PCV2 cap gene nucleotide sequences, 30 PCV3 cap gene nucleotide sequences and 72 PCV4 cap gene nucleotide sequences. GenBank accession numbers for Malaysian PCV4 cap gene sequences were labelled. The tree was constructed using the maximum likelihood method, General Time Reversible model with 1000 bootstrap replicates. The scale bar indicates branch length measured in number of substitutions per site

Based on the tentative PCV4 genotype classification, Malaysian PCV4 strains were clustered in genotype PCV4b, with amino acid mutations at aa position 15, 27 and 138 identified as R, N and H respectively (Xu *et al.*, 2022). Our findings were also in agreement with reports from Zhang *et al.* (2020) and Sun

et al. (2021), that PCV4 is more closely related to mink circovirus and bat-associated circovirus (Figure 15).

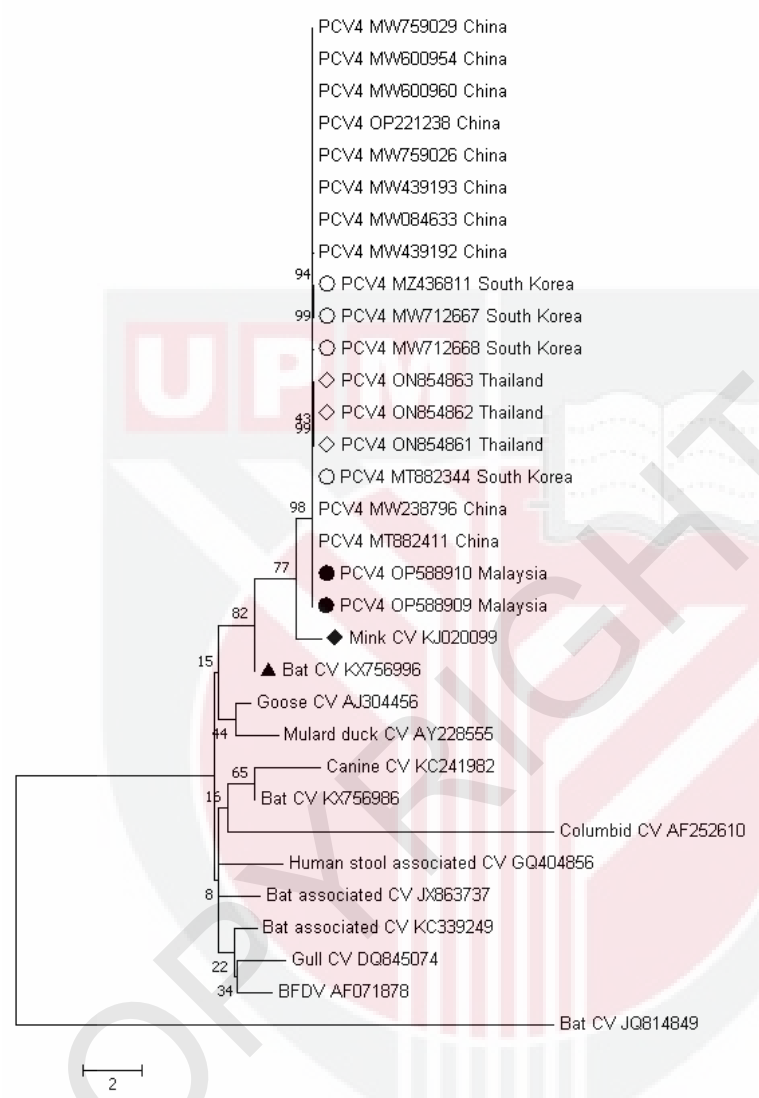

Figure 15: Phylogenetic analysis of Malaysian PCV4 cap gene nucleotide sequences with known member species of circovirus genus. Two Malaysian PCV4 strains (●) were compared with 86 GenBank reference sequences which consisted of 72 PCV4 cap gene nucleotide sequences and 14 circovirus species cap gene nucleotide sequences. Mink circovirus (◆), bat circovirus (▲) and PCV4 strains from South Korea (O) and Thailand (◇) were additionally labelled. GenBank accession numbers for all sequences were listed. The tree was constructed using the maximum likelihood method, General Time Reversible model with 1000 bootstrap replicates. The scale bar indicates branch length measured in number of substitutions per site

It was observed that the Malaysian PCV4 strains were clustered into a distinct clade with the longest branch length, away from China, South Korea and Thailand PCV4 strains (Figure 16).

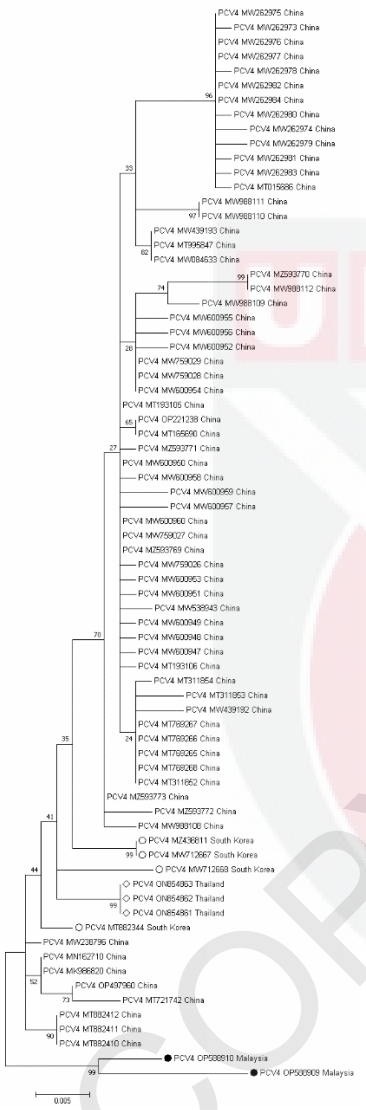

Figure 16: Phylogenetic analysis of Malaysian PCV4 *cap* gene nucleotide sequences with reference PCV4 *cap* gene nucleotide sequences. Two Malaysian PCV4 strains (●) were compared with 72 GenBank reference PCV4 *cap* gene nucleotide sequences. PCV4 strains from South Korea (O) and Thailand (◇) were additionally labelled. GenBank accession numbers for all sequences were listed. The tree was constructed using the maximum likelihood method, General Time Reversible model with 1000 bootstrap replicates. The scale bar indicates branch length measured in number of substitutions per site

The following discussion provide an analysis of the phylogenetic findings presented in the preceding tree, highlighting the potential origins and transmission pathways of PCV4 strains detected in Malaysia. This phylogenetic tree reveals significant genetic similarities between Malaysian PCV4 strains and those previously reported in neighboring countries such as China, South Korea, and Thailand. Following China, South Korea and Thailand, Malaysia is the fourth country to have detected the presence of PCV4. Malaysia does share trading relationships with the three aforementioned countries involving pork products. From 2011 to 2018, Malaysia had imported over 11,000 metric tonnes (MT) of pork products from the three countries (DVS, 2022). At the moment, it could only be suggested that the relationship among PCV4 strains from Malaysia, China and South Korea could be associated with pork product trade activities. Previously, it was speculated that the phylogenetic relatedness among Malaysian, Spanish, America and Mexico PCV3 strains might be a result of live breeder and semen stock movement (Tan *et al.*, 2020). The involvement of imported breeders and semen could not be ruled out from transboundary transmission of PCV4 into Malaysia.

To date, geographical distribution of PCV4 seems to be limited to Asian countries (Franzo *et al.*, 2020; Wang *et al.*, 2022). The current knowledge gap of global PCV4 genetic information and scarce number of Malaysian sequences impede definitive interpretation of this phylogenetic observation. PCV2 and PCV3 have been readily identified in clinically healthy and ill pigs (Patterson & Opriessnig, 2010; Madson *et al.*, 2011; Segalés, 2012; Zhai *et*

al., 2017; Tochetto *et al.*, 2018; Franzo *et al.*, 2019). Similarly, PCV4 had been detected from pigs with a myriad of disease presentation ranging from respiratory, enteric and PDNS signs; and, in clinically healthy pigs including testicles of piglets (Tian *et al.*, 2020; Zhang *et al.*, 2020; Ha *et al.*, 2021; Nguyen *et al.*, 2022). For PCV2 and PCV3 infection, viral loads of statistically significant difference between clinically healthy and ill groups of pigs have been proposed to be part of the diagnostic criterion (Brunborg *et al.*, 2004; Woźniak *et al.*, 2020). In view of that, a study attempted to quantify PCV4 viral titer to infer association with PCV4 infection but it was inconclusive due to limited sample size (Nguyen *et al.*, 2022). In this Malaysian study reporting 4.08% molecular prevalence, both the PCV4 positive samples originated from clinically healthy finishers. For a more accurate epidemiological picture, more field samples from cases of different clinical manifestations need to be included. Further experimental studies are warranted to elucidate the role of PCV4 in health status of pigs; and its interaction with PCV2 and PCV3 during co-infections.

7.4 Conclusion

In conclusion, presence of PCV4 in Peninsular Malaysia has been confirmed at a molecular prevalence of 4.08% (2 / 49 samples). Both Malaysian PCV4 strains originated from clinically healthy finishers and were classified as genotype PCV4b phylogenetically distinct from the China, South Korea and Thailand PCV4 strains. The research progression of PCV4 is following the trajectory of PCV3: establishing diagnostic assays and thereafter recovering infectious clones within three years from the novel discovery. Infectious clone

studies had confirmed pathogenicity of PCV3 and PCV4, making headway for more in-depth clinical research. With the recent findings of PCV2 genotype shift, and detection of both PCV3 and PCV4 strains in Malaysia, more attention needs to be given to the management of PCV diseases. At research level, more epidemiological studies are required to investigate the prevalence of novel porcine circoviruses in commercial pigs both healthy and ill, and also in wild boar population. At farm level, common management strategy of PCV2 challenge in farms applies to potential PCV3 and PCV4 challenges. At national level, biosecurity screening procedures during live breeders and pork products importation may need to be revised to be more inclusive of emerging diseases.

CHAPTER 8

SUMMARY, CONCLUSION AND RECOMMENDATIONS

8.1 Summary and Conclusion

In Malaysia's livestock industry, pigs are the second largest commodity with an estimated standing pig population of close to 1.7 million heads. Of the 1.7 million count, 1.3 million pigs are from farms in Peninsular Malaysia. At a production capacity of over 200,000 tonnes of pork annually, Malaysian local pig industry is able to achieve a 93% self-sufficiency rate (DVS, 2022). The pig livestock subsector is well-developed in terms of production capacity and technology, but it is not without challenges both from within and external. Endemic swine diseases such as Porcine circoviruses (PCVs), salmonellosis, colibacillosis, Glässer's disease and Porcine reproductive and respiratory syndrome (PRRS) issues are inevitable in continuous production cycles. Geographical proximity, international trades and movement risk our local pig industry with transboundary diseases such as African Swine Fever and novel strains of porcine circoviruses.

Porcine circovirus (PCV), a member of the Circoviridae family within the genus Circovirus, is ubiquitous in the worldwide pig population. The hypernym porcine circovirus associated diseases (PCVAD) has been coined to include the myriad of PCV2-related multi-factorial and multisystemic diseases (AASV, 2022). PCVAD poses a significant economic risk to the global swine industry. For decades, ever since the first report of clinical outbreak of postweaning multisystemic wasting syndrome (PMWS), porcine circovirus 2 (PCV2) was the

focus in PCV management with vaccination being one of the effective forefront strategies. To date, another two PCVs have been discovered with reported pathogenicity. Multisystemic manifestation of porcine circovirus 3 (PCV3) infection includes but is not limited to respiratory tract, gastrointestinal, nervous to cardiovascular systems (Phan *et al.*, 2016; Chen *et al.*, 2017; Ku *et al.*, 2017; Zhai *et al.*, 2017; Arruda *et al.*, 2019; Qi *et al.*, 2019; Hou *et al.*, 2020). Porcine circovirus 4 (PCV4) has been associated with respiratory and enteric clinical signs, as well as with porcine dermatitis and nephropathy syndrome (PDNS) (Zhang *et al.*, 2020b).

This thesis reports the latest epidemiological status of PCV in Malaysia, covering PCV2 which is known to be endemic in the local pig farms; and extends to PCV3 and PCV4 which has yet to be studied locally. Through this study, molecular detection rate of porcine circovirus 2 (PCV2) in farm and sampled pig population were reported to be 83.78% (31 / 37 farms) and 83.54% (66 / 79 pigs) respectively in this study. These findings are similar to the previous reports of 83.78% – 88.10%, indicating that molecular detection rate of PCV2 still remain high (Jaganathan *et al.*, 2011). Secondly, PCV2 antigen was also detected in Malaysian abattoir lung samples at 94.74% detection rate (18 / 19 samples). This is the first report of PCV2 detection in local clinically healthy finishers. Detection rates of PCV2 are considered high at both farm and abattoir levels.

Further, this is also the first study to confirm the circulation of PCV2 in wild boar population roaming Peninsular Malaysia, where 28 out of 28 (100%) wild boar lung samples were found positive. Malaysian wild boar PCV2 sequences

were found to share considerable nucleotide identity of at least 80.7% with domestic pig sequences. Most notably, an apparent genotype shift away from the sole PCV2b genotype reported ten years ago in Malaysia was uncovered in this study. One decade earlier, only genotype PCV2b was reported in Malaysia. Now, genotypes PCV2a, PCV2b and PCV2d were found to be present in Malaysia, with a large 96.2% majority of Malaysian PCV2 sequences (76 / 79 sequences) identified as genotype PCV2d. High mutation rate of PCV2 was also evident through phylogenetic divergence across PCV2 strains collected from longitudinal sampling only several months apart.

This research also confirms the presence of PCV3 in Malaysia. Molecular detection rate of PCV3 in farm and sampled pig population were reported to be 17.02% (24 / 141 pigs) and 41.67% (10 / 24 farms) respectively. Organ samples from inguinal lymph node and lung showed the highest molecular detection rates of 81.82% and 71.43% respectively. Further research may be focused on these two organ tissue types to investigate potential tissue tropism and relationship with PCV3 pathogenesis. Unlike PCV2, no PCV3 positive samples were detected in domestic finishers and wild boar population sampled in this study but an increased sample size would be more conclusive. Malaysian PCV3 strains were classified as PCV3 strain A1 and A2, and phylogenetically related to Spanish, U.S. and Mexico strains. It may be speculated that the phylogenetic relatedness among Malaysian, Spanish, the U.S. and Mexico PCV3 strains might be a result of live breeder and semen stock movement.

A chromogenic ISH technique using DIG-labeled probes targeting PCV3 ORF1 was described in this study to detect PCV3 antigens in formalin-fixed, paraffin-embedded tissues. Lung and lymphoid tissues were targeted given their high PCV3 molecular detection rate. Chromogenic staining of PCV3 antigens was visualized within the cytoplasm of pneumocytes and lymphocytes with a predominantly multifocal distribution pattern, consistent with previous ISH studies. As PCV3 is still relatively new and under-researched, diagnostic development as such the economically feasible and practical DIG-ISH protocol described in this study would be useful in the settings of local research and diagnostics laboratories. Currently, limited availability of PCV3-specific antibodies restricting immunostaining methods and high costs of RNAScope assay are cumbering further studies of PCV3 at smaller facilities.

Presence of the novel PCV4 has also been confirmed through this study. Molecular detection rate of PCV4 is very much lower compared to that of PCV2 and PCV3, only at 4.08% (2 / 49 samples). Samples were collected from commercial intensive pig farms, abattoir and wild boar population. It was observed that the only two PCV4 positive samples originated from clinically healthy finishers. Malaysian PCV4 strains were classified as genotype PCV4b, and were found to be phylogenetically distinct from the China, South Korea and Thailand strains. Currently, Malaysia is the fourth country to report detection of PCV4.

8.2 Knowledge Gaps and Recommendation

This research work faced hurdles of COVID-19 pandemic and African Swine Fever outbreak which restricted sampling activities. A few aspects could be considered for future porcine circoviruses research in Malaysia. Firstly, larger sample size should be included in future studies. Farms with different management practice including population size, open versus closed house system and farrow-to-finish versus multi-site rearing should be included in the sampling population. PCV2 vaccination practice of the farms should also be recorded to investigate how it impacts the circulation of circovirus in the herds. Farms in Sabah and Sarawak states should be included to obtain study results that reflect Malaysian pig industry as a whole. Sampled age group in this current study leaned towards weaners due to convenience sampling arrangement. To improve on sample representativeness, higher number of samples from finishers should be collected from abattoirs in different regions of Malaysia. On the other hand, it is especially crucial to increase sample size and sampling region for wild boar population in future studies investigating potential of wild boars as PCV reservoir, in order to reach more conclusive evidence of PCV cross transmission between domestic pigs and wild boar population.

While this study has addressed a number of research objectives, it has also brought up several topics that warrant future investigation. Instances of phylogenetic divergence across PCV2 strains and even genotype shift from longitudinal sampling have been observed in this study. More archived samples dating back from 2016 could be sequenced, and more farms could

be sampled longitudinally to grasp the rate of PCV2 strain mutation in Malaysian local farm setting. Further, Rep gene of PCV2 could be studied for any occurrence of similar genetic divergence. Apart from that, given the high detection rates of PCV2 at farm and abattoir levels, further research could look into both subclinical and clinical PCVAD more thoroughly at the farms.

As for PCV3, research on tissue tropism and disease association still requires a lot of work. Sampling of organ tissues apart from lung and inguinal lymph nodes could be planned. More importantly, including both clinically healthy and ill groups of pigs in the sampling; or running an in vivo inoculation study could contribute greatly to this aspect of knowledge gap. Future PCV3 epidemiological study could also incorporate in situ hybridisation (ISH) to visualize localization of PCV3 DNA within tissues, following PCV3 PCR or qPCR detection. Moving forward, as more genetic information of worldwide PCV3 strains is updated in the GenBank, phylogenetic analyses of Malaysian PCV3 strains should be updated to obtain more accurate information on the potential origin of Malaysian strains.

Guard dogs are commonly kept in pig farms and stray dog packs also often roam in pig farming areas. Given that PCV2, PCV3 and PCV4 have all been reported to be present in canine samples, farm dog population should be investigated for prevalence of PCVs to study the role of canine species in the transmission dynamics. In addition, flies could be included in research samples to similarly establish any epidemiological link as insects including flies, mosquitoes, and ticks have been postulated to be carrier of PCVs. These findings would be useful when planning biosecurity defence against PCVs.

Last but not least, these latest findings of presence of novel PCV4 in Malaysia, and observation of genotype shift from PCV2b to PCV2d in local farms serve as important cues calling for further research and continuous surveillance of PCVs in Malaysia. Collaborative efforts from industry stakeholders, government authorities and research bodies could facilitate steering the direction of PCVAD disease control and management to be aligned with current PCVAD situation.

8.3 Links To Articles

Review Article 1: Porcine circoviruses in Malaysia

[Accepted, pending publication]

Review Article 2: What do We Know About Porcine circovirus 3 (PCV3) Diagnosis So Far?

https://doi.org/10.1111/tbed.14185

Article 3: Genotype Shift of Malaysian Porcine Circovirus 2 (PCV2) from PCV2b to PCV2d within a Decade

https://doi.org/10.3390/ani12141849

Article 4: First molecular detection and complete sequence analysis of porcine circovirus type 3 (PCV3) in Peninsular Malaysia

https://doi.org/10.1371/journal.pone.0235832

Article 5: Chromogenic in-situ hybridization technique for the detection of porcine circovirus 3 (PCV3)

https://doi.org/10.14202/vetworld.2023.1444-1450

Article 6: First molecular detection of porcine circovirus type 4 (PCV4) in Malaysia

https://doi.org/10.47665/tb.40.3.005

CHAPTER 9

FURTHER DISCUSSION

This chapter serves to compile and elaborate on key points raised during the viva and thesis examination, addressing examiner feedback and providing additional insights. It aims to refine interpretations, clarify findings, and enhance the overall understanding of the study's outcomes.

9.1 Method Flow and Sampling Strategy

This section aims to clarify the sampling methodology and the selection process of samples used across the studies on PCV2, PCV3, and PCV4. It provides an overview of the strategies employed to ensure representative sampling and robust data collection. Additionally, it highlights the rationale behind the sample selection to support the study's objectives.

9.1.1 Flow of Thesis Chapters

The thesis chapters are structured to enhance readability, beginning with the PCV2 molecular study, followed by the PCV3 molecular study and the development of the PCV3 in situ hybridization (ISH) technique, and concluding with the PCV4 molecular study. Each section was conducted as an independent study, allowing for focused analysis and timely publication of findings upon completion.

9.1.2 Flow of Study Progression

The research timeline differs from the sequence presented in the thesis. The study began in late 2018 with a molecular prevalence study of PCV3, driven by industry concerns following PCV3's initial identification in 2016. During PCV3 sampling, a notable number of pigs exhibited post-mortem lesions characteristic of PCV2, prompting a timely assessment of PCV2 prevalence on farms, which had not been updated since 2011. As the study progressed, reports from China on a novel PCV4 virus surfaced, prompting its inclusion in the research to evaluate its potential impact on the swine industry. Table 24 summarizes the flow of study progression in this research.

Table 24: Summary of Study Progression

PCV3 →	PCV2 →	PCV4
• First report of PCV3 in 2016 • Study of PCV3 initiated due to industry concern	• Post mortem lesions suggestive of PCV2 observed during PCV3 sampling • Last PCV2 prevalence update in Malaysia was in 2011 • Sampling expanded to additional farms for a comprehensive PCV2 prevalence study	• First report of PCV4 in 2018 • Samples from the PCV2 and PCV3 sets used to investigate PCV4 • Included in the study to provide a comprehensive overview of PCV situation in Malaysia

9.1.3 Sample Collection

It should be noted that the samples were shared across the studies on PCV2, PCV3, and PCV4. The samples were obtained primarily from commercial swine farms across various regions in Malaysia. Due to farm access limitations

and biosecurity constraints, convenience sampling was employed. Sampling targeted clinically ill pigs that exhibited symptoms such as wasting, dyspnea, neurological issues, moribund states, and sudden death. Weaners and growers aged 4–12 weeks constituted the majority of the sample population. Additionally, samples from fetuses and sows were collected sporadically, following incidents such as abortions or sudden deaths in sows. Consequently, the number of samples from these age groups is low, with only a few fetal and sow samples available.

Sampling from a broader production group can provide valuable insights into the molecular detection of PCV2 within this sample population. However, the small sample sizes for certain groups, as shown in Table 6, limit the robustness and generalizability of the conclusions. For instance, piglets (2 samples), sows (2 samples), and foetuses (7 samples) all tested positive for PCV2, resulting in a total of 11 positive samples. Such small sample sizes can introduce sampling bias and reduce the power to detect variability or trends across larger populations, thereby limiting the reliability of the findings. Nonetheless, the high detection rates observed align with the globally recognized prevalence of PCV2 in pig populations, particularly in highly susceptible age groups such as piglets and foetuses, lending biological plausibility to the results. Furthermore, the larger sample sizes for weaner (56 tested) and grower (12 tested) groups strengthen the conclusions for these categories, where high detection rates were similarly observed. To enhance the reliability of future research, larger and more balanced sample sizes across all production age groups should be prioritized to minimize sampling bias and improve statistical power. For this

study, however, the findings should be interpreted with caution, as the small sample sizes in certain groups may overestimate or fail to accurately represent the true prevalence of PCV2 in broader populations.

Post-mortem examinations were conducted for each sampled animal, prioritizing lung and inguinal lymph nodes, as both PCV2 and PCV3 are known to target these tissues. PCV2, in particular, exhibits tropism for lymphoid tissues in pigs, often causing lymphoid depletion and histiocytic replacement, which are hallmark lesions of PCV2 infection (Opriessnig, Meng & Halbur, 2007; Segalés, 2012). Interstitial pneumonia and bronchiolitis with mononuclear infiltration also occur with PCV2 infection, correlating with clinical signs of respiratory distress (Harms *et al.*, 2001; Kim, Chung & Chae, 2003). PCV3 similarly infects the lungs and lymphoid tissues such as lymph nodes and tonsils (Fan *et al.*, 2017; Fu *et al.*, 2018; Li *et al.*, 2018e). Other organs, including the spleen, tonsil, kidney, heart, mesenteric lymph nodes, liver, and brain, were collected based on availability.

In addition, lung samples from clinically healthy finishers obtained from abattoirs and retail shops, as well as samples from wild boar populations, were also included to broaden the study scope. Fresh tissues were stored at -80°C until they were processed for polymerase chain reaction (PCR) analyses, with a portion preserved as formalin-fixed, paraffin-embedded (FFPE) tissue for ISH studies. A summary of the sampling plan detailing location, farm type, and organ type is provided in Table 25.

Table 25: Summary of Sampling Plan

Method	Convenience Sampling
Location	• Northern region: Penang, Perak • Central region: Selangor • Southern region: Melaka, Johor
Farm Criteria	• Commercial swine farms • Semi-closed house, farrow-to-finish system, with a farm size ranging from 200 to 1500 sows
Sampled Population	• Majority: Clinically ill weaner & grower between 4 – 12 weeks old of age • Others: Foetus, piglet, finishers, sows
Sample Age	• Archived samples: 2016 – 2017 • Clinical samples: 2018 – 2021
Sample Organ	• Majority: Lung & inguinal lymph nodes • Others: mesenteric lymph nodes, spleen, tonsil, kidney, liver, heart, brain

9.1.4 Sample Size

Sample sizes were determined using Thrusfield's equation, adjusted for expected prevalence rates and precision requirements (Thrusfield *et al.*, 2017). Calculations ensured that statistical power and sample sizes were representative of the populations under study, providing reliable insights into the molecular prevalence of PCV2, PCV3, and PCV4 across different regions and populations. The Thrusfield equation is as follows:

$$n = \frac{1.96^2 \, Pexp \, (1 - Pexp)}{d^2},$$

where: n = required sample size
P_{exp} = expected prevalence
d = desired absolute precision

The Thrusfield equation calculation were summarized in Table 26 to confirm that the sample sizes in PCV2, PCV3 and PCV4 studies met statistical sufficiency. For all three studies, the level of confidence is set at 95% with desired absolute precision of 10%.

Table 26: Sample Size Calculation Using Thrusfield's Equation

Parameters	PCV2	PCV3	PCV4
P_{exp}, expected prevalence	80%	20%	5%
n, required sample size	61.59	61.59	18.28
Actual included sample size	79	123	30

The sample sizes per study phase align with the progression of research topics. Given that PCV3 was the first phase, fewer farms were sampled initially. However, the number of pigs tested in this phase was sufficient for support a robust epidemiological analysis. As the study expanded in the subsequent PCV2 phase, more commercial farms were recruited, reflecting the goal to update data on the endemic presence of PCV2 in Malaysia, with prior data last reported in 2011. A smaller sample size was tested for PCV2, as the high prevalence of PCV2 in Malaysia allowed sufficient representation to confidently support the findings.

For PCV4 detection, sample size calculations took into account the generally low prevalence rates ranging from 1.6% to 25.4% (Chen *et al.*, 2021; Ha *et al.*, 2021; Tian *et al.*, 2021). With known PCV2 and PCV3 molecular detection statuses, samples for PCV4 detection were randomly selected from PCR-

status quadrants, as detailed in Table 27, employing a targeted sampling approach to capture representative data on PCV4.

Table 27: Sampling Quadrant of PCV4 Molecular Detection Study

PCV2 Status	PCV3 Status	Sample Origin	Number of Samples (n)
+	+	Farm	10
		Abattoir	1
+	–	Farm	10
		Abattoir	7
		Wild boar	10
–	+	Farm	1
–	–	Farm	9
		Abattoir	1

Finally, for PCV3 ISH study, ten corresponding sets of lung and lymphoid tissues were selected based on the PCV3 PCR results on fresh tissues with formalin-fixed, paraffin-embedded (FFPE) tissue availability enabling detailed examination. Table 28 provides an overview of all samples utilized across study sections.

Table 28: Number of Samples Collected for PCV2, PCV3 and PCV4 Studies

	PCV2 Molecular Detection	PCV3 Molecular Detection	PCV3 ISH	PCV4 Molecular Detection
Number of farms sampled	37	24	6	25
Number of pigs sampled (Various organ samples were collected)	79	123	10	30
Number of lungs sampled from abattoir	19	18	0	9
Total number of organ samples sampled	111	214	30	30
Sampling focus / note	Sampling focused on lungs and inguinal lymph nodes given PCV2's known tissue tropism.	Various organs sampled to assess tissue tropism	− Study is focused on lungs as PCV4 has been associated with respiratory clinical signs. − Samples were randomly picked from the PCV2/PCV3 positive/negative PCR status quadrant	Corresponding sets of lung, inguinal and mesenteric lymph node samples, preserved in both frozen and FFPE formats were randomly selected from PCV3-positive and PCV3-negative groups

9.1.5 Ethical Approval

This study received ethical approval from the Universiti Putra Malaysia (UPM) Institutional Animal Care and Use Committee (IACUC) under AUP Code

UPM/IACUC/AUP-R030/2019. All procedures adhered strictly to the guidelines outlined in the UPM Code of Practice for the Care and Use of Animals for Scientific Purposes. The collection of samples was overseen by qualified veterinarians from the Faculty of Veterinary Medicine, Universiti Putra Malaysia.

9.1.6 Recap of Results

To consolidate the results published in various papers, Table 29 below provides a synchronized summary of the data. The prevalence rates are separated for the domestic swine and wild boar populations. For the domestic swine population, prevalence includes detection in pigs from both commercial farms and finishers sampled at abattoirs.

Table 29: Summary of Molecular Prevalence Rates for PCV2, PCV3, and PCV4 in Malaysian Commercial Swine and Wild Boar Populations

Sampling Population		PCV2 Molecular Detection	PCV3 Molecular Detection	PCV4 Molecular Detection
Commercial farms				
Farm	Number of farms sampled	37	24	25
	Positive rate	83.78% (31 / 37)	41.67% (10 / 24)	0% (0/25)
Domestic pigs	Number of pigs sampled	79	123	30
	Positive rate	83.54% (66 / 79)	19.51% (24 / 123)	0% (0 / 30)
Abattoir	Number of lungs sampled	19	18	9
	Positive rate	94.74% (18 / 19)	0% (0 / 18)	22.22% (2 / 9)

Table 29: Continued

Sampling Population		PCV2 Molecular Detection	PCV3 Molecular Detection	PCV4 Molecular Detection
Wild boar population				
Wild boar	Number of lungs sampled	28	28	10
	Positive rate	100% (28 / 28)	0% (0 / 28)	0% (0/10)

The sampling strategy and methodology outlined here provide a comprehensive approach to assessing PCV2, PCV3, and PCV4 prevalence across diverse porcine populations. The plan is streamlined according to the situation, as explained previously, ensuring flexibility in response to emerging research needs. These methods ensure that the data gathered accurately represents Malaysia's swine industry, offering valuable insights to support ongoing management and control efforts for these viruses in swine health.

9.2 Sensitivity and Specificity of Diagnostic Methods

This section evaluates the diagnostic methods utilized in the study, focusing on their sensitivity and specificity in detecting PCV2, PCV3, and PCV4. It discusses the reliability and accuracy of these methods in identifying viral presence. Comparative analyses are presented to highlight strengths and limitations across diagnostic approaches.

9.2.1 Overview of Diagnostic Methods

Each study utilized different diagnostic tools tailored to the detection of various porcine circoviruses (PCV2, PCV3, and PCV4). These include:

i. Conventional PCR for initial molecular detection of PCV2, PCV3 and PCV4
ii. Chromogenic in situ hybridization (ISH) for antigen localization of PCV3
iii. Sequencing for molecular Characterisation and phylogenetic analysis of PCV2, PCV3 and PCV4

Table 30 outlines the rationale for selecting PCR, ISH, and sequencing, their relevance to the studies, and the limitations each method addresses.

Table 30: Summary of Diagnostic Methods: Rationale, Relevance, and Addressed Limitations

	Conventional PCR	**Chromogenic In Situ Hybridization (ISH)**	**Sequencing**
Application	**Initial molecular detection**	**Antigen localization**	**Molecular Characterisation and phylogenetic analysis**
Rationale for Selection	• *Sensitivity and specificity:* PCR offers high sensitivity, capable of detecting minute amounts of viral DNA, even in subclinical infections. Its specificity is assured through the use of primers targeting conserved regions of viral genomes	• *Antigen localization:* ISH enables visualization of viral antigens within specific tissues, providing critical insights into the sites of viral replication and pathogenesis. For PCV3, this is particularly important given its diverse tissue tropism (e.g., lungs and lymphoid tissues)	• *Definitive confirmation*: Sequencing validates PCR results by confirming the exact nucleotide sequence of the detected virus, eliminating doubts about specificity or primer cross-reactivity

Table 30: Continued

	Conventional PCR	Chromogenic In Situ Hybridization (ISH)	Sequencing
Application	**Initial molecular detection**	**Antigen localization**	**Molecular Characterisation and phylogenetic analysis**
	• *Broad applicability:* PCR is *versatile* and can be applied to various sample types, such as fresh tissues, archived samples, and even environmental swabs, making it ideal for diverse diagnostic and surveillance scenarios • *Time efficiency*: PCR is relatively rapid, making it a practical choice for high-throughput screening in both research and field conditions	• *High specificity:* ISH uses labelled probes that hybridize specifically to viral nucleic acids, minimizing false positives and ensuring accuracy in tissue diagnostics. • *Complementary to PCR*: While PCR identifies the presence of viral DNA, ISH confirms its localization, strengthening diagnostic confidence.	• *Genetic insights*: Sequencing reveals genetic variations, enabling phylogenetic analysis to trace viral evolution, understand transmission dynamics, and monitor genotype shifts • *Global comparability*: Sequence data can be compared with international databases (e.g., GenBank) to identify relationships between local and global strains, as seen with PCV2 genotype shifts and PCV4 classification
Relevance to Study	• PCV2: The high detection rate (83.54% in pigs) underscores PCR's effectiveness in capturing true infections in endemic settings like Malaysia	• ISH was applied to PCV3-positive samples to confirm and map the virus in tissue environments. This step provided additional confirmation for PCR results, showing a 90.91% agreement rate	• PCV2: Sequencing revealed a genotype shift from PCV2b to PCV2d in Malaysia, indicating the ongoing evolution of the virus and its implications for vaccine efficacy

Table 30: Continued

	Conventional PCR	Chromogenic In Situ Hybridization (ISH)	Sequencing
Application	**Initial molecular detection**	**Antigen localization**	**Molecular Characterisation and phylogenetic analysis**
	• PCV3 and PCV4: PCR identified these emerging strains at prevalence rates of 17.02% and 4.08%, respectively, facilitating early detection and monitoring of novel pathogens	• The method illuminated the involvement of specific cell types (e.g., pneumocytes and lymphocytes), offering a deeper understanding of viral impact on host tissues.	• PCV3 and PCV4: Sequencing confirmed the presence of these novel strains and classified Malaysian PCV4 as genotype PCV4b, distinct from strains in other countries
Limitations Addressed	• While PCR alone cannot confirm infection activity or localization, it provides a reliable foundation for further confirmatory or complementary tests	• ISH's labour-intensive nature and lower sensitivity (compared to PCR) are mitigated by its unique capability to provide spatial context, which is invaluable for pathogenesis research	• Sequencing addresses the inability of PCR to identify viral variants and offers comprehensive data to guide epidemiological and control strategies.

In summary, all the methods were chosen to complement each other, providing a comprehensive diagnostic approach. PCR establishes the presence of the virus with high sensitivity and specificity, suitable for routine detection and surveillance. ISH confirms viral localization within tissues, offering insights into the sites and effects of viral replication. Sequencing provides definitive confirmation and genetic Characterisation, which is crucial for understanding viral evolution and guiding control measures. Together, these methods form a

robust diagnostic framework for addressing both endemic and emerging porcine circoviruses in Malaysia.

9.2.2 Evaluation of Diagnostic Methods Sensitivity and Specificity

Sensitivity and specificity are key metrics for evaluating diagnostic methods. Sensitivity measures the proportion of true positives correctly identified by a test and is calculated using the formula:

$$\textbf{Sensitivity} = \frac{\textbf{True Positives}}{\textbf{True Positives + False Negatives}} \times \textbf{100}$$

True positives (TP) refer to the number of samples accurately identified as positive by the diagnostic method, typically verified against a gold standard such as sequencing or clinical diagnosis. False negatives (FN) are cases that the diagnostic method failed to identify as positive but were confirmed by the gold standard. In the context of the published studies, sensitivity is evaluated using data comparing PCR or ISH results to sequencing confirmation.

Specificity, on the other hand, measures the proportion of true negatives correctly identified by a test and is calculated as:

$$\textbf{Specificity} = \frac{\textbf{True Negatives}}{\textbf{True Negatives + False Positives}} \times \textbf{100}$$

True negatives (TN) are samples correctly identified as negative by the diagnostic method and confirmed by the gold standard, while false positives (FP) are samples erroneously identified as positive by the diagnostic method

despite being negative according to the gold standard. In the studies reviewed, specificity of ISH is assessed through validation of results against PCR results. Table 31 compiles in detail the specificity and sensitivity, and briefly on predictive value in this study

Table 31: Diagnostic Performance Metrics of PCR and ISH: Specificity, Sensitivity, and Predictive Values

	Conventional PCR	Chromogenic In Situ Hybridization (ISH) of PCV3
Sensitivity	• High sensitivity in detecting viral genomes, especially for well-conserved regions like the capsid (cap) genes • PCV2: 100% (From PCR and sequencing comparison) − High molecular detection rates of 83.54% (66/79) in domestic pigs and 100% (28/28) in wild boar population may suggest potential high sensitivity for PCR under field conditions − Sequencing was performed on 51 positive samples from domestic pigs and all 28 positive samples from wild boars, with all 79 sequences confirmed as PCV2 − Total PCV2-positive samples by PCR: 79 − Total PCV3-positive samples by sequencing: 79 • PCV3: 100% − (From PCR and sequencing comparison) − Molecular detection rate of 17.02% (24/141) in domestic pigs − Sequencing was performed on 15 positive samples, with all 15 sequences confirmed as PCV3	• Detected PCV3 antigens in fixed tissue samples with 90.91% agreement compared to PCR results, indicating high specificity • High concordance (90.91%) with PCR, but some false negatives might occur due to variability in tissue quality or antigen expression levels.

Table 31: Continued

	Conventional PCR	Chromogenic In Situ Hybridization (ISH) of PCV3
Specificity	− Total PCV2-positive samples by PCR: 15 − Total PCV3-positive samples by sequencing: 15 • PCV3: 90.91% (From ISH and PCR comparison) − Total PCV3-positive samples by PCR: 11 − Total PCV3-negative samples by PCR: 19 − ISH failed to identify 1 PCR-positive sample (FN = 1) • PCV4: − Low detection rate of 4.08% (2/49 lungs) due to the low prevalence of PCV4 in the population may affect sensitivity calculations − This may not fully reflect the assay's true capability • Specificity depends on primer design; cross-reactivity may occur with closely related circoviruses • PCV2: High − Cannot be explicitly calculated in the absence of sequencing for negative sample − Strong indirect evidence including validated primers and consistent sequencing confirmation of positives supports the assumption of high specificity • PCV3: 100% (From ISH and PCR Comparison) − Total PCV3-positive samples by PCR: 11	• Relatively lower sensitivity for cases with limited viral antigen expression in tissues and constrained by tissue condition • Close to 100%, as no false positives were reported in the study due to the precise hybridization method

Table 31: Continued

	Conventional PCR	Chromogenic In Situ Hybridization (ISH) of PCV3
	− Total PCV3-negative samples by PCR: 19 − ISH identified all 19 PCR-negative samples correctly (TN = 19) − No false positives were observed for ISH (FP = 0) • PCV4: − Cannot be explicitly calculated in the absence of sequencing for negative sample − Similarly as commented for sensitivity, low prevalence of PCV4 in the population may affect sensitivity calculations and results may not reflect the assay's true capability	
Predictive value	• Positive predictive value (PPV) refers to the probability that a subject tested positive for a condition truly has the condition • High PPV for PCR due to Malaysia's known endemic status for PCV2 and increasing prevalence of PCV3 • PCR's ability to detect low-copy targets also supports high PPV	• Negative predictive value (NPV) refers to the probability that a subject who tests negative for a condition truly does not have the condition • Stronger NPV for ISH, as it cross-verifies localization in the tissue, minimizing false negatives

9.2.3 Limitations and Recommendations for Diagnostic Methods in Malaysia

Limitations of Current Diagnostic Methods

Polymerase chain reaction (PCR) is a highly sensitive and specific diagnostic tool; however, it has certain limitations that affect its utility in some contexts.

One major issue is the risk of false positives due to contamination, particularly in high-prevalence areas where viral DNA may inadvertently transfer between samples during handling. Additionally, PCR is limited by its inability to confirm active infection, as it detects DNA and cannot distinguish between viable viruses and residual or non-infectious viral DNA. Another challenge is its reduced sensitivity in cases of low viral loads, such as during subclinical or early infections, where the amount of viral DNA may fall below the detection threshold. These limitations can lead to diagnostic errors, particularly in cases where precise infection status is crucial. Addressing these challenges involves implementing rigorous contamination control protocols. Laboratories should establish separate areas for pre- and post-PCR processes and routinely incorporate no-template controls and extraction blanks in each run to detect and mitigate contamination. To enhance PCR's ability to detect low viral loads, quantitative PCR (qPCR) assays can be developed, allowing for the detection of even minimal amounts of viral DNA and the quantification of infection severity. Multiplex PCR is another promising solution, enabling simultaneous detection of multiple pathogens and reducing the likelihood of false positives due to cross-reactivity.

Chromogenic in situ hybridization (ISH) is another valuable diagnostic tool, but its limitations reduce its practicality in some settings. ISH is labor-intensive and time-consuming, requiring skilled personnel and specialized equipment, which limits its scalability for routine diagnostics. The technique also relies on high-quality tissue samples, which must be properly fixed and preserved to yield reliable results. In field settings or resource-limited farms, obtaining such high-

quality samples can be a significant challenge due to suboptimal preservation or delays in processing. Furthermore, the cost of ISH, including materials and labor, makes it a less accessible option for widespread use. To mitigate these limitations, efforts should focus on simplifying ISH protocols to reduce labor and costs. Rapid and cost-effective alternatives, such as RNAScope, could replace traditional chromogenic ISH if budgets allow. Additionally, investing in the development of specific monoclonal antibodies for PCV3 and PCV4 would enable complementary techniques, such as immunohistochemistry (IHC) or immunofluorescence assays, which are less dependent on tissue quality. Combining ISH with PCR can also enhance diagnostic confidence by confirming ambiguous cases and providing localization data that PCR alone cannot offer.

Emerging Strain Challenges

The detection of emerging strains, such as PCV4, presents unique challenges due to the limited availability of diagnostic tools tailored to novel viruses. The lack of routine surveillance exacerbates the under-detection of these strains, particularly in low-prevalence areas where their presence may go unnoticed. This gap in monitoring is evident in the studies, where PCV4 was detected at a low prevalence of 4.08%, highlighting the need for enhanced diagnostic strategies and surveillance systems.

To address these challenges, implementing regular surveillance programs in commercial farms, abattoirs, and wild populations is essential for the early detection of emerging strains. Cross-validation of PCR results through

sequencing can further ensure the specificity of diagnostics while simultaneously expanding the genetic databases needed for assay validation. These measures would improve the detection and management of novel strains like PCV4, reducing their potential impact on animal health and productivity.

Impact in the Malaysian Context

The limitations of current diagnostic methods have significant implications for disease surveillance and management in Malaysia. The low detection rate for PCV4 underscores the gaps in monitoring emerging strains, which can hinder timely intervention and containment. Diagnostic deficiencies, such as the inability to identify co-infections accurately, may also compromise vaccine efficacy and lead to suboptimal disease management strategies. Strengthening diagnostic accuracy is critical to overcoming these challenges. Combining PCR with ISH can enhance diagnostic robustness by addressing sensitivity and specificity gaps. Additionally, developing targeted serological tests, such as ELISA, for emerging strains like PCV4 can complement molecular methods and provide a more comprehensive diagnostic toolkit. By addressing these limitations and adopting innovative solutions, Malaysia can improve its capacity to detect and manage porcine circoviruses effectively, safeguarding the health and productivity of its swine populations.

9.3 Implications of PCV Research for the Malaysian Swine Industry

This study's findings on PCV2, PCV3 and PCV4 carry critical implications for the Malaysian swine industry and potentially the broader Southeast Asian

region. Firstly, this study provides several novel insights that are pivotal to the understanding of PCV epidemiology. The detection of a major genotype shift from PCV2b to PCV2d in Malaysia marks a significant finding, indicating the potential for changes in viral virulence and challenges in vaccine efficacy. The detection of novel PCV3 and PCV4 strains in Malaysia introduces new complexities for disease management, as these previously unrecognized viruses may require updated health protocols and research focus.

From a practical perspective, the high prevalence of PCV2, combined with rising detection rates of PCV3 and PCV4, could lead to economic losses due to subclinical infections, reduced productivity and increased veterinary costs. Severe outbreaks of these viruses could result in large-scale losses within farms and disrupt supply chains. The increased costs for disease prevention and control measures may affect the competitiveness of the Malaysian swine industry on both regional and global levels. Hence, this study also serves as a critical alert to stakeholders: farmers, veterinarians and researchers, regarding the evolving PCV situation in Malaysia and dynamic nature of circoviruses. This awareness is essential as it underlines the urgency of continual surveillance and adaptive disease control strategies in mitigating emerging threats posed by PCVs.

The study also points to the role of international trade in the spread of PCV strains. Genetic similarities between Malaysian PCV strains and those from countries like Thailand and the United States suggest that the global movement of livestock and animal products could facilitate virus transmission. With increasing global movement of livestock and animal products, there is a

potential for these viruses to spread beyond Southeast Asia. The swine industry may therefore need to strengthen biosecurity and regulatory frameworks for imports and exports of pigs and pork products to minimize transboundary transmission. This calls for international collaboration in surveillance, data sharing and development of common diagnostic and biosecurity standards. Furthermore, findings of PCV2 in all wild boar samples in this study and the phylogenetic overlap between wild and domestic PCV2 strains underscore the importance of strong biosecurity measures. Similarly, the detection of PCV4 strains linked to wildlife, such as bats, highlights the need to prevent transmission from wild animals. Strengthening biosecurity protocols, such as tighter controls on animal movement, enhanced sanitation, and quarantine measures for new animals entering farms, can reduce the risk of viral introduction. Enhanced monitoring of wildlife populations and farm-level surveillance are also recommended.

Furthermore, the study underscores the need for advanced diagnostic techniques, particularly for PCV3 and PCV4. While commercial diagnostic services for PCV2 are well-established, the detection of PCV3 and PCV4 strains is still emerging. The development of in situ hybridization (ISH) for PCV3 detection in this study represents a significant advance, as it not only aids in the localization of antigens within infected tissues but also provides a more cost-effective and accessible diagnostic tool.

The ISH technique facilitates a better understanding of the tissue tropism and pathogenesis of PCV3, enhancing diagnostic capacity and supporting targeted control strategies within the Malaysian swine industry. Encouraging ongoing

research to elucidate the epidemiology, pathogenicity and host interactions of PCV3 and PCV4 is crucial. This study provides essential baseline information on the molecular prevalence and strain diversity of PCVs in Malaysia, laying the groundwork for future research on their clinical impacts and potential vaccine development.

In summary, this study's findings emphasize the need for enhanced surveillance, improved diagnostics, updated management practices and strengthened biosecurity measures. As these viruses pose risks to farm productivity and the broader swine industry, proactive research and international cooperation are essential for minimizing their impact. Swift adaptation to these evolving challenges will be key to the long-term sustainability and economic resilience of the swine industry.

9.4 Updated Global Report on Porcine Circoviruses

There has been an increasing number of reports concerning porcine circovirus 2 (PCV2), porcine circovirus 3 (PCV3), and porcine circovirus 4 (PCV4) across the globe. Current trend indicates that PCV2 persists to circulate endemically within swine herds. Table 24 compiles references pertaining to the worldwide occurrence of PCV2, detailing its continuous presence and the ongoing research into its genetic and immunological characteristics.

Table 32: Continued Worldwide Occurrence of PCV2

Country		Reference
Asia	China	Ge *et al.* (2012)
	India	Barman *et al.* (2018)
	Indonesia	Manokaran *et al.* (2008)
	Japan	Mori *et al.* (2000)
	Malaysia	Tan *et al.* (2022)
	Myanmar	Min Thein Maw *et al.* (2014)
	Philippines	Dela Cruz *et al.* (2021)
	South Korea	Chae (2012)
	Taiwan	Tsai *et al.* (2019)
	Thailand	Jittimanee *et al.* (2011)
	Vietnam	Nguyen *et al.* (2023)
North America	Canada	Harding (2007)
	Costa Rica	Liu *et al.* (2002)
	Cuba	Pérez *et al.* (2011)
	Dominican Republic	Gainor *et al.* (2022)
	Mexico	Ramírez-Mendoza *et al.* (2009)
	United States	Shen *et al.* (2012)
South America	Argentina	Pereda *et al.* (2011)
	Brazil	Rodrigues *et al.* (2024)
	Chile	Neira *et al.* (2017)
	Colombia	Rincón Monroy *et al.* (2014)
	Ecuador	Bermeo *et al.* (2009)
	Uruguay	Ramos *et al.* (2018)
Europe	Austria	Weissenbacher-Lang *et al.* (2020)
	Belgium	Wei *et al.* (2019)
	Bosnia and Herzegovina	Lukač *et al.* (2016)

Table 32: Continued

Country		Reference
	Bulgaria	Mortrovski *et al.* (2009)
	Croatia	Jemeršić *et al.* (2004)
	Estonia	Jarveots *et al.* (2016)
	Germany	Reiner *et al.* (2015)
	Greece	Papatsiros *et al.* (2022)
	Hungary	Dán *et al.* (2003)
	Italy	Franzo *et al.* (2020c)
	Ireland	Allan *et al.* (2007)
	Latvia	Piginka-Vjačeslavova *et al.* (2018)
	Netherlands	Dieste-Pérez *et al.* (2018)
	Norway	Brunborg *et al.* (2010)
	Poland	Woźniak *et al.* (2019a)
	Portugal	Henriques *et al.* (2011)
	Romania	Cadar *et al.* (2007)
	Serbia	Toplak *et al.* (2012)
	Slovakia	Csank *et al.* (2011)
	Slovenia	Toplak *et al.* (2012)
	Sweden	Brunborg *et al.* (2010)
	Switzerland	Staebler *et al.* (2004)
	Spain	Sibila *et al.* (2021)
	Ukraine	Rudova *et al.* (2022)
	United Kingdom	Grierson *et al.* (2004)
Eurasia	Kazakhstan	Mambetaliyev *et al.* (2018)
	Turkey	Karaoğlu *et al.* (2011)
	Russia	Raev *et al.* (2021)

Table 32: Continued

Country		Reference
Africa	Burkina Faso	Franzo *et al.* (2022c)
	Cape Verde Cameroon Congo Cote d'Ivoire Ethiopia Senegal Tanzania Zambia	Dundon, Molini & Franzo (2024)
	Mozambique	Laisse *et al.* (2018)
	Namibia	Molini *et al.* (2021)
	Nigeria	Afolabi *et al.* (2024)
	South Africa	Afolabi *et al.* (2019)
	Tanzania	Chang'a *et al.* (2023)
	Uganda	Wilfred *et al.* (2018)
Australia		Raye *et al.* (2005)
New Zealand		Neumann *et al.* (2007)

Furthermore, PCV3 has demonstrated global spread and PCV4 has recently been detected beyond the Asian region. Table 25 and Table 26 summarize the initial reports of PCV3 and PCV4 across various global regions respectively. These two tables present updated information that was not available when the review articles in the literature review chapter were produced. Additionally, the tables enhance information clarity by grouping the reports by region.

Table 33: Initial Reports of PCV3 Across Various Global Regions

Country			First Report of PCV3	
			Positive Rate	Sample Type
Asia	China	Ku *et al.* (2017)	34.68% (77/222)	Stillborn, organ tissues, semen, serum
	India	Bera *et al.* (2019)	Not reported	Stillborn, sow blood and nasal swabs, tissues, serum
	Japan	Hayashi *et al.* (2018)	9.59% (7/73)	Organ, foetal tissues
	Malaysia	Tan *et al.* (2020)	17.02% (24/141)	Organ tissues
	South Korea	Kim *et al.* (2018)	42.86% (6/14)	Aborted foetus, suckling piglet organ tissues
	Taiwan	Zeng (2018)	4.02% (17/423)	Aborted foetus
	Thailand	Kedkovid *et al.* (2018)	62.50% (5/8)	Organ tissues
	Vietnam	Nguyen *et al.* (2018)	4.44% (6/135)	Organ tissues
North America	Canada	Zhang *et al.* (2022)	Not reported	Organ tissues, oral, processing and thoracic fluids, faeces
	Dominican Republic	Gainor *et al.* (2023)	21% (21/100)	Faeces
	United States	Phan *et al.* (2016)	Not reported	Organ tissues
		Palinski *et al.* (2017)	93.75% (45/48)	Organ tissues
			12.55% (34/271)	Respiratory disease diagnostic samples
			55.42% (46/83)	Serum
	Costa Rica	Chaverri *et al.* (2022)	57.14% (32/56)	Blood

Table 33: Continued

Country			First Report of PCV3	
			Positive Rate	**Sample Type**
South America	Argentina	Serena *et al.* (2020)	93.75% (15/16)	Mummified and stillborn piglet pools
			2.29% (3/131)	Foetal tissues
	Brazil	Tochetto *et al.* (2018)	Not reported	Serum pool
	Chile	Rubilar *et al.* (2020)	Not reported	Mummified stillbirths
	Colombia	Vargas-Bermudez *et al.* (2019)	25.00% (1/4)	Mesenteric ganglia
			16.67% (1/6)	Serum
	Mexico	Dal Santo *et al.* (2020)	97.83% (270/276)	Mummified foetus
Europe	Belgium	Saporiti *et al.* (2020b)	13.00% (13/100)	Serum
	Denmark	Franzo *et al.* (2018a)	56.41% (44/78)	Lung, lymph nodes, serum
	Germany	Fux *et al.* (2016)	75.47% (40/53 farms)	Serum pool
	Hungary	Deim *et al.* (2019)	89.09% (49/55)	Aborted, stillborn, weak born piglets
	Ireland	Collins *et al.* (2017)	20.00% (48/240)	Organ tissues, faeces
	Italy	Faccini *et al.* (2017)	12.50% (2/16)	Aborted foetuses, stillborn piglets
	Netherlands	Saporiti *et al.* (2020b)	14% (7/50)	Serum
	Poland	Stadejek *et al.* (2017)	36.00% (9/25)	Foetal tissues, stillborn piglets
	Serbia	Savic *et al.* (2020)	84.85% (28/33)	Organ tissue
	Slovenia	Plut *et al.* (2020)	20.98% (64/305)	Oral fluid, faeces, serum

Table 33: Continued

Country			First Report of PCV3	
			Positive Rate	**Sample Type**
	Spain	Klaumann *et al.* (2018)	11.47% (75/654)	Serum
	Sweden	Ye *et al.* (2018)	20.41% (10/49)	Lymph nodes
	United Kingdom	Collins *et al.* (2017)	20.00% (48/240)	Organ tissues, faeces
Africa	Mozambique	Anahory *et al.* (2021)	7.69% (7/91)	DNA samples
	Namibia	Molini *et al.* (2024)	54.54% (42/77) pig	Tonsil
			21.82% (12/55) warthog	
	Nigeria	Luka *et al.* (2022)	Not accesible	DNA samples
	Tanzania	Chang'a *et al.* (2023)	11.29% (14/124)	Organ tissues, blood, serum
Russia		Yuzhakov *et al.* (2018)	Not reported	Organ tissue, serum, pleural effusion, peritoneal cavity fluid

Table 34: Initial Reports of PCV4 Across Various Global Regions

Country			First Report of PCV3	
			Positive Rate	**Sample Type**
Asia	China	Zhang *et al.* (2020)	12.80% (24/187)	Organ tissues, faeces, nasal swab, serum
	South Korea	Nguyen *et al.* (2022)	3.28% (11/335)	Organ tissues, testicles
	Thailand	Sirisereewan *et al.* (2023)	0.41% (3/734)	Organ tissues
	Malaysia	Tan *et al.* (2023)	4.08% (2/49)	Lung

Table 34: Continued

Country			First Report of PCV3	
			Positive Rate	**Sample Type**
	Taiwan	Hung *et al.* (2024)	0.78% (1/128)	Organ tissues
North America	United States	Kroeger *et al.* (2024)	8.59% (44/512)	Organ tissues, faeces, foetal tissues
Europe	Spain	Holgado-Martín *et al.* (2023)	Spanish wild boar: 34.34% (57/166)	Organ tissues, faeces, rectal swab
			Semi-extensive bred Iberian pig: 4.04% (9/223)	

Figure 17 maps the global distribution of PCV2, PCV3 and PCV4. The distribution patterns of PCV3 and PCV4 may mirror that of PCV2, as evidenced by the frequent co-occurrence of two or more porcine circoviruses within a single country. These findings highlight the critical need for continuous surveillance and strengthened biosecurity measures.

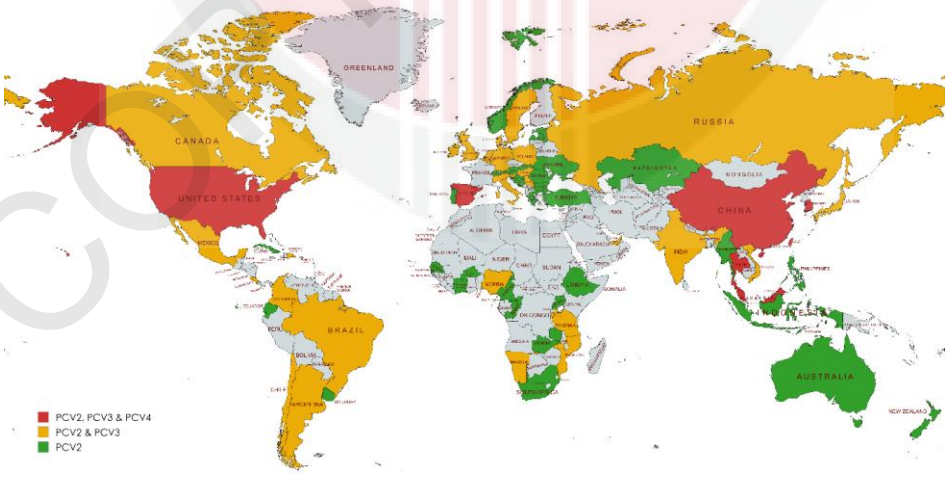

Figure 17: Global distribution of PCV2, PCV3 and PCV4. Countries marked in red indicate the presence of all three porcine circoviruses. Yellow regions signify that both PCV2 and PCV3 have been reported, while green regions denote the presence of only PCV2

9.5 Global Phylogenetic Clustering and Regional Diversity of PCV3

The phylogenetic tree in Figure 8 (Chapter 5) reveals clear phylogenetic clustering patterns, reflecting both localized evolution and global transmission pathways. The major clusters are defined by country-specific branches, which reflect regional adaptations and potential viral dissemination pathways. The phylogenetic clusters will be discussed further.

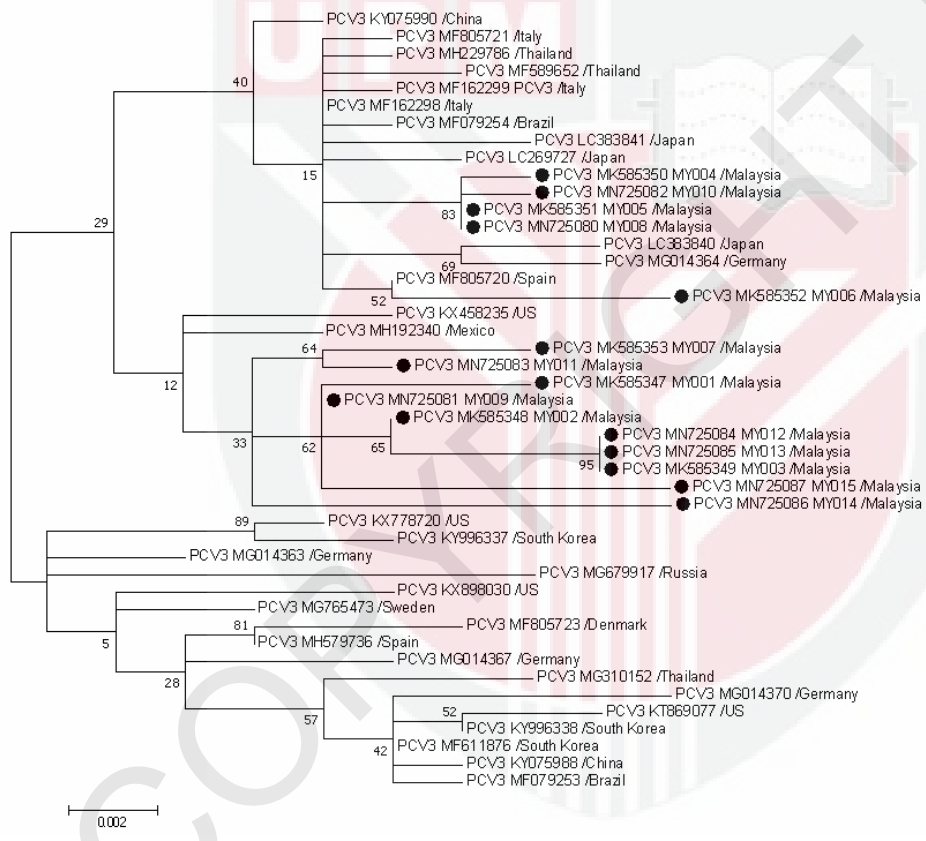

Figure 8 (Retrieved from Chapter 5): Phylogenetic analysis of cap gene sequences of PCV3. Fifteen Malaysian PCV3 strains (●) were compared with 30 other PCV3 strains. GenBank accession numbers and origin country are as indicated. Malaysian PCV3 strains were additionally labelled with strain ID. The tree was constructed using ML method, Tamura-Nei model evaluated with 1000 bootstrap replicates. The scale bar indicates branch length measured in number of substitutions per site Malaysian Strains: Tight Clustering

The Malaysian PCV3 sequences form tightly grouped clusters, particularly in branches with high bootstrap support values (e.g.: 83% for MK585350, MK585351, MN725080, MN725082; 95% for MK585349, MN725084 – MN725087). These high bootstrap values affirm the reliability and robustness of these clusters. This close-knit pattern suggests localized evolution within Malaysia, likely due to consistent transmission within the swine population without significant external introductions. These strains are genetically similar, pointing to limited genetic drift or mutation over time within this population. The clustering pattern indicates limited genetic divergence among Malaysian strains, likely due to localized transmission and minimal external introductions. Additionally, the tight clustering could imply that PCV3 has undergone relatively recent diversification within the region, remaining largely confined to its point of origin.

Korean Strains: Dispersed Evolution

The Korean strains do not cluster as tightly as the Malaysian strains. Instead, form distinct branches within the phylogenetic tree. These strains show a closer relationship with sequences from other regions, such as strain KY996338 and strain KY996337 with the U.S. at a bootstrap value of 52% and 89% respectively. Strain MF611876 shares a subclade with strains from China and Brazil. This dispersed clustering also indicates that PCV3 in Korea may have multiple sources of introduction or more complex transmission dynamics. It may reflect trade-related movement of live animals or pork products.

European, Brazilian and Chinese Strains: Diverse Grouping

Sequences from European sequences (e.g.: Italy, Germany, Spain, and Denmark), Brazil and China are scattered throughout the tree rather than forming a compact cluster. The broad distribution suggests higher genetic diversity in Europe, signifying multiple introductions of the virus or a longer history of viral evolution in the region. This diversity may be attributed to broader international trade networks, larger pig populations and differences in farming practices across European countries. The scattered pattern also suggests that PCV3 has undergone significant genetic divergence within Europe, potentially driven by varied environmental and epidemiological pressures.

American Strains: Multiple Introductions

Sequences from the U.S. appear in several distinct clusters, alongside strains from Korea, Europe, and Malaysia. For instance, strain KX458235 clusters with Mexico and Malaysian sequences, while strains KX77870 and KT869077 cluster with a Korean sequence. The clustering indicates multiple introductions of PCV3 into or from the USA. It suggests that international trade, especially involving breeding stocks, semen or pork products, could be contributing to transboundary viral dissemination.

Conclusion

The clustering patterns of PCV3 in the phylogenetic tree highlight the interplay between regional transmission dynamics, international trade and genetic

diversity. Regions like Malaysia exhibit localized evolution, while others like Korea and the USA show dispersed clustering indicative of complex introduction routes. These insights emphasize the importance of ongoing surveillance and biosecurity measures to monitor and mitigate PCV3 spread globally.

9.6 PCV4 in Malaysia: Insights and Implications of Detection

The detection of PCV4 in Malaysian swine populations, as presented in this study, holds important implications for swine health management and warrants careful consideration. While the pathogenicity of PCV4 has been demonstrated through infectious clone studies, the full clinical significance and impact of PCV4 in pigs remain unclear. PCV2, a well-studied circovirus, is known to cause severe health issues, including postweaning multisystemic wasting syndrome (PMWS). Should PCV4 prove to have similar pathogenic potential, it may pose new health challenges for the Malaysian swine industry. This underscores the importance of incorporating PCV4 monitoring into routine disease surveillance and developing proactive strategies to manage potential outbreaks effectively.

The genetic diversity observed in the Malaysian PCV4 strains, clustered within genotype PCV4b and showing a distinct genetic profile from PCV2 and PCV3, is noteworthy (Tan *et al.*, 2023; Chapter 7). This finding suggests that the circovirus population in Malaysia is diverse and evolving. As new strains of PCV, such as PCV3 and PCV4, emerge, their genetic variation could potentially lead to different clinical outcomes and diagnostic challenges.

Continued genetic monitoring of circoviruses will be essential for identifying mechanisms of viral adaptation and evolution, which may influence both transmission dynamics and pathogenicity in pigs, impacting herd health management practices. The Malaysian PCV4 strains identified in this study have been classified as belonging to the PCV4b genotype, based on phylogenetic analysis that includes sequences from ORF1, ORF2, and the complete genome. Although the three phylogenetic trees are consistent in placing Malaysian strains within the PCV4b clade, it's important to note that this classification is derived from a relatively limited dataset of 35 known PCV4 strains globally (Xu *et al.*, 2023). As such, while the trees currently agree across different genomic regions, a larger dataset is necessary to confirm the stability of this classification and to understand potential sub-lineages within PCV4b. Expanding the phylogenetic sampling will be crucial to refine PCV4 genotyping and to monitor its evolution, especially as the virus continues to emerge in new geographic regions.

Further, the close phylogenetic relationship of Malaysian PCV4 strains with bat-associated raises the possibility that wildlife may play a role in the introduction and dissemination of this virus (Tan *et al.*, 2023; Chapter 7). This potential cross-species transmission suggests that wildlife, particularly bats, may serve as reservoirs for the virus, especially in regions where contact between swine and wildlife is possible. Understanding the ecology of PCV4 in wildlife populations and the environmental pathways for its transmission may help to identify risk factors for its introduction into commercial swine farms, aiding in the development of biosecurity measures.

The detection of PCV4 in domestic pigs in Malaysia, Thailand, China and Korea, but not in other regions despite ongoing surveillance, raises further points for discussion (Zhang *et al.*, 2020; Nguyen *et al.*, 2022; Sirisereewan *et al.*, 2023). This geographic discrepancy could be attributed to environmental and agricultural practices that influence viral spread. Malaysia and its neighboring countries share similar swine farming practices, which may create favorable conditions for the virus to thrive. On the other hand, the limited detection of PCV4 outside certain Asian regions may indicate that the virus is in the early stages of global spread, not yet widely circulating within the global swine population. Its absence in the United States and other Western countries could also suggest a restricted geographic distribution, potentially with PCV4 remaining primarily endemic to Southeast and East Asia.

Malaysia has established trading relationships with Thailand, China and South Korea, involving imports of pork products. From 2011 to 2018, Malaysia imported over 11,000 metric tonnes of pork products from these countries (DVS, 2022). This trade activity raises the possibility that the phylogenetic relatedness observed among PCV4 strains from these countries may be linked to pork product imports. Previously, it was speculated that the genetic similarities among Malaysian, Spanish, American and Mexican PCV3 strains could have resulted from the international movement of live breeders and semen stocks (Tan *et al.*, 2020; Chapter 5). Similarly, the involvement of imported breeders and semen in the transboundary transmission of PCV4 into Malaysia cannot be ruled out.

In conclusion, these findings suggest that PCV4 has the potential to become a significant pathogen in swine populations. Its evolving genetic diversity, probable involvement of wildlife reservoirs, and currently limited geographic spread highlight the need for ongoing research, enhanced surveillance, and robust biosecurity practices to manage potential outbreaks and understand its impact on global swine health.

9.7 Recent Findings on Pathogenesis and Pathogenicity of PCV3 and PCV4

Initial Detection and Clinical Manifestations of PCV3

PCV3 was initially identified in a sow herd exhibiting reproductive issues and symptoms consistent with Porcine Dermatitis and Nephropathy Syndrome (PDNS). The affected herd showed a 0.6% decrease in conception rates and a 10.2% increase in mortality (Palinski *et al.*, 2017). Another early report identified PCV3 in pigs with cardiac and multisystemic inflammation (Phan *et al.*, 2016). Other clinical and pathological manifestations of PCV3 infection include respiratory, gastrointestinal, neurological disorders and systemic wasting (Chen *et al.*, 2017; Ku *et al.*, 2017; Zhai *et al.*, 2017; Arruda *et al.*, 2019; Qi *et al.*, 2019; Hou *et al.*, 2020). The detection of PCV3 in apparently healthy animals has raised questions regarding its pathogenicity. Moreover, many field reports are confounded with co-infection of PCV2 and/or other common swine pathogens (Zheng *et al.*, 2017; Franzo *et al.*, 2018b). Numerous detection studies reported presence of PCV3 viral genome in pigs exhibiting diverse clinical signs, yet comprehensive diagnostic studies to

establish a causative role for PCV3 in the observed conditions are still lacking (Klaumann *et al.*, 2018a).

Experimental Studies and Pathogenesis of PCV3

To investigate the pathogenicity of PCV3, researchers have employed methods similar to those used in PCV2 studies. Fenaux *et al.* (2002) utilized in vitro-constructed recombinant plasmids of infectious PCV2 molecular DNA clones and an in vivo transfection system to study PCV2's pathogenicity. In a similar approach, Jiang *et al.* (2019a) conducted a study in which specific-pathogen-free (SPF) piglets, aged four and eight weeks, were intranasally inoculated with an infectious molecular clone of PCV3. The study recorded PCV3 viremia and seroconversion of anti-PCV3 Cap antibodies, along with clinical signs that progressed from anorexia, coughing, sneezing, and diarrhoea to increased respiratory rates, lethargy, skin lesions, shivering, hyperesthesia, and, in some cases, death. Pathological lesions were observed in the lungs, liver, spleen, and lymph nodes (Jiang *et al.*, 2019a). Another study involving an infectious PCV3 DNA clone revealed notable changes in the alveolar and cardiac tissues of intraperitoneally infected mice, particularly interstitial pneumonia (Jiang *et al.*, 2020). Furthermore, Mora-Díaz *et al.* (2020) conducted an inoculation experiment in six weeks old CD/CD pigs using PCV3-whole virus isolated and cultured in the study. Viremia and IgG seroconversion was detected in the inoculated pigs throughout the study. Histopathological analysis revealed ongoing multisystemic inflammation, including enteritis, nephritis, myocarditis, arteritis and periarteritis. Viral replication was observed within myocardiocytes, arterial tunica media and

endothelial cells, as well as in inflammatory cells. These findings underscored the pathogenicity of PCV3 as a single etiological agent, capable of inducing multisystemic clinical disease. Sirisereewan, Thanawongnuwech, and Kedkovid (2022) summarized field observations and experimental infections, concluding that PCV3 infection typically results in multi-organ inflammation. Proposed mechanisms of pathogenesis include virus-induced apoptosis, immune-response-mediated type IV hypersensitivity and immune-complex-mediated type III hypersensitivity (Sirisereewan, Thanawongnuwech & Kedkovid, 2022).

Emerging Evidence of PCV4 Pathogenicity

Despite being a relatively recent discovery, studies on infectious clones have already been conducted to demonstrate the pathogenicity of PCV4 (Niu *et al.*, 2022; Chen *et al.*, 2024). Pathological lesions and PCV4-specific antigens were observed in the lungs, kidneys, lymph nodes, spleen, and liver of inoculated pigs (Chen *et al.*, 2024). Notably, despite the marked pathological changes, no obvious clinical symptoms were observed in either study (Niu *et al.*, 2022; Chen *et al.*, 2024). Additionally, Chen *et al.* (2024) suggested that PCV4 replicates more efficiently in the lungs and lymph nodes, as evidenced by the higher genomic copy numbers detected in these tissues.

9.8 Influence of Genetic, Environmental and Host Factors on Progression and Severity of PCV3 Clinical Disease

Genetic Factors and Viral Adaptability

As discussed earlier, studies which conducted infectious molecular clone experiments have confirmed that PCV3 can induce clinical disease (Jiang *et al.*, 2019a; Mora-Díaz *et al.*, 2020). Additionally, Sirisereewan, Thanawongnuwech, and Kedkovid (2022) have highlighted potential pathogenesis pathways. Looking from the perspective of epidemiological triad, several factors might influence the development of PCV3 infection and clinical manifestations.Firstly, in the aetiological agent component, the genetic makeup of PCV3 plays a critical role in its ability to cause disease. The estimated mean substitution rate of PCV3 strains was 1.22 × 10-4 to 2.10 × 10−3 substitution/site/year (ssy) (Li *et al.*, 2018a, Saraiva *et al.*, 2018). Although the rates fall within the expected for a single single stranded DNA virus, they are higher than that of PCV2 which were estimated to be 3.05 × 10-4 to 1.59 × 10-3 ssy (Franzo *et al.*, 2016). This higher substitution rate might enhance the virus's adaptability to diverse biological conditions. Additionally, the calculated effective reproduction number (Re) of PCV3, ranging from 1.82 to 3.08, suggests a high potential for widespread transmission and the risk of becoming endemic in pigs (Li *et al.*, 2018a). PCV3 also has mechanisms for evading the host immune response, such as modulating the type I interferon response and inducing autophagy to enhance viral replication and persistence (Shen *et al.*, 2020; Zhang *et al.*, 2020c; Kroeger, Temeeyasen, & Piñeyro, 2022).

Host Susceptibility and Disease Progression

In terms of host factor, PCV3 can infect both healthy and sick pigs, making it difficult to control transmission. Healthy pigs can act as asymptomatic carriers, contributing to the spread of the virus. Infected pigs which only exhibit mild respiratory and/or enteric symptoms could also aid as intermediates for the virus transmission (Chen *et al.*, 2021). PCV3 can infect pigs of all ages, from one-day-old piglets to adult sows, regardless of health condition, gender, or age (Ouyang *et al.*, 2019b; Chen *et al.*, 2021). These characteristics opened up a wide range of potential hosts, contributing to PCV3's effectiveness in establishing clinical disease. The adaptability of PCV3 in different hosts increase its ability to persist and spread across swine populations. On the other hand, previously, host genetics including breed type have been shown to influence susceptibility to PCV2, for instance increased clinical lesions in Landrace breeds, and higher level of PCV2 uptake and disintegration in monocytic cells of purebred and hybrid Piétrain pigs (Opriessnig *et al.*, 2006; Wei *et al.*, 2018). The genetic factors influencing the resistance or susceptibility to PCV3 remain unclear.

Environmental Influences on PCV3 Disease Dynamics

Environmental factors, particularly stressors such as poor hygiene, overcrowding, and inadequate ventilation, should be investigated for their roles in exacerbating PCV3 disease especially in regions with tropical climates like Malaysia where such stressors are pronounced. Synergistic effects of co-infection of PCV3 with other common swine pathogens are also not well understood yet. Co-infections with pathogens like PCV2, PRRSV and

Mycoplasma hyopneumoniae may intensify the clinical outcomes of PCV3, though this interplay is not yet fully understood. This is important to understand especially in regions where biosecurity measures may be suboptimal, which is a relevant scenario in some Malaysian swine farms.

Transmission Mechanisms and Global Dissemination

Although the specific routes of PCV3 transmission are still being clarified, viral shedding in bodily fluids and detection in various swine tissues have been reported, indicating both horizontal and vertical transmission. Vertical transmission is supported by the detection of PCV3 antigen in colostrum, placentas, and stillborn foetuses (Ouyang *et al.*, 2019b; Chen *et al.*, 2021). PCV3 persistence in the environment, such as in sponges collected after truck sanitization, suggests the potential role of fomites in transmission (Franzo *et al.*, 2018d). Global transmission could also be fuelled by the detection of PCV3 DNA in semen from healthy commercially imported boars from the United States and western European countries (Feng *et al.*, 2019; Zhang *et al.*, 2019b). In addition, wild boars are suspected to be important reservoirs for PCV3, as high prevalence rates have been observed in these populations. The long-term, persistent infections noted in wild boars suggest that PCV3 may have a chronic nature, which may facilitate its persistence and ongoing transmission within both wild and domestic swine populations (Franzo *et al.*, 2018c; Klaumann *et al.*, 2019a). Although PCV3 has been detected in ticks and mosquitoes, there is currently no strong evidence to support vector-borne transmission (Franzo *et al.*, 2019b; Ha *et al.*, 2020).

In conclusion, the development of PCV3 infection and exacerbation of the clinical disease manifestation are influenced by a combination of viral genetics, host factors, and environmental conditions.

9.9 Vaccine Progress in PCV2 and Vaccine Development in PCV3

This section reviews the advancements in vaccine development for PCV2, including efficacy and implementation progress. It also explores the current status and challenges in developing vaccines for PCV3. Comparative insights are provided to highlight gaps and future directions in PCV vaccine research.

9.9.1 PCV2 Vaccination

Before the advent of PCV2 vaccines, PCVAD had a significant impact on the swine industries worldwide (Gillespie *et al.*, 2009). Morbidity rates as high as 50–60% and post-weaning mortality rate between 4 and 20% were reported in Europe during the epidemic stage of the disease in 1996 – 2004 (Madec *et al.*, 2000; Segales and Domingo, 2002). The development and deployment of PCV2 vaccines have significantly reduced the economic losses caused by PCV2-associated diseases (PCVAD). Current PCV2 vaccines are primarily subunit or inactivated vaccines targeting the capsid protein. While they effectively reduce clinical disease, improve weight gain, and decrease viral load, their effectiveness varies across different genotypes (Maity *et al.*, 2023). Efficacy of commercial PCV2 vaccines had been widely studied – with a general consensus that the vaccines are, under both experimental and field conditions, capable of reducing PMWS incidence, lesion severity, viremia level

and number of co-infections (Grau-Roma *et al.*, 2011). Viral clearance in cases of natural PCV2 infection is thought to be mediated by a combination of neutralizing antibodies (NA) and cell-mediated responses (Pogranichnyy *et al.*, 2000): pigs with high NA and interferon-γ (IFN-γ) responses demonstrated lower levels of viral replication (Meerts *et al.*, 2005). IFN-γ secreting cells (IFN-γ SCs) develop specifically during the adaptive response to PCV2, thus are likely to contribute to viral clearance in infected pigs (Fort *et al.*, 2009). In line with that, the participation CD4+ and CD8+ T-cells in virus-specific IFN-γ responses is deduced from weakening of IFN-γ responses after depletion of the T-cell subsets (Steiner *et al.*, 2009).

PCV2 exhibits a high mutation rate, contributing to the emergence of diverse genotypes, which have significant implications for vaccine efficacy. The primary genotypes: PCV2a, PCV2b, and PCV2d, have dominated at different times, with genotype shifts often correlating with outbreaks of PCVAD. These shifts have challenged the effectiveness of existing vaccines and highlighted the need for continuous monitoring and adaptation in vaccine strategies. Early commercial PCV2 vaccines, developed in the mid-2000s, primarily targeted PCV2a, the predominant genotype at the time. These vaccines proved effective in reducing clinical disease, viremia, and economic losses (Maity *et al.*, 2023). However, around 2004–2005, PCV2b emerged as the dominant genotype globally, replacing PCV2a in many regions. Phylogenetic studies suggest that PCV2b's higher replication efficiency and pathogenicity might have contributed to its increased prevalence (Franzo *et al.*, 2016b). Although PCV2a-based vaccines provided cross-protection against PCV2b, some

studies reported suboptimal outcomes, including incomplete protection against viral shedding and transmission (Opriessnig *et al.*, 2021). Since 2012, PCV2d has become the predominant genotype worldwide, coinciding with reports of increased vaccine failures in field conditions. PCV2d, initially referred to as "mutant PCV2b," exhibits amino acid variations in the capsid protein, the primary target of neutralizing antibodies induced by vaccines (Maity *et al.*, 2023). These variations potentially reduce the binding affinity of antibodies generated by PCV2a-based vaccines, leading to diminished efficacy.

Although current PCV2 vaccines are designed to induce broad cross-protection, their effectiveness varies across genotypes. A meta-analysis by Opriessnig *et al.* (2021) demonstrated that vaccines based on PCV2a effectively reduced PCVAD across genotypes but were less effective in completely suppressing viral replication in pigs infected with PCV2d. The antigenic drift observed in PCV2d poses a challenge to vaccine-mediated immunity, emphasizing the need for genotype-specific vaccine formulations (Franzo *et al.*, 2016b). The simultaneous circulation of multiple genotypes within herds adds another layer of complexity to vaccination strategies. Studies have identified co-infections of PCV2a, PCV2b, and PCV2d in pigs, which may lead to genetic recombination and the emergence of novel genotypes (Franzo *et al.*, 2016b). These recombination events could further undermine the efficacy of existing vaccines, necessitating ongoing genomic surveillance and vaccine adaptation. The genetic shifts from PCV2a to PCV2b and subsequently to PCV2d have highlighted the adaptability of PCV2 and its ability to evade immune responses induced by vaccines. While PCV2a-based

vaccines remain effective in reducing clinical disease, their limited efficacy against emerging genotypes like PCV2d underscores the need for updated vaccine formulations. Strategies such as incorporating multivalent vaccines or developing genotype-specific solutions will be critical for maintaining effective control of PCV2 and minimizing its economic impact.

9.9.2 Vaccination Practices and History in the Sample Population

All the farms sampled in this study reported implementing PCV2 vaccination programs as part of their herd health management strategies. However, specific details regarding the vaccine brands used and the exact vaccination schedules practiced were not recorded. Commercial PCV2 vaccines widely available in Malaysia, along with typical vaccination routines, are detailed in Section 8.8.3. Despite the lack of precise documentation, the adoption of PCV2 vaccination across all sampled farms reflects its critical role in controlling porcine circovirus-associated diseases (PCVAD) and underscores the widespread recognition of its efficacy in improving herd health and productivity.

9.9.3 PCV2 Vaccination in Malaysia

Currently, there are five prophylactic vaccines against PCV2 that are available commercially. The details of the vaccines are summarized below (European Medicines Agency, 2007, 2008, 2009):

Table 35: Commercially Available Vaccines in Malaysia

Vaccine / Company	Antigen / Adjuvant	Licensed For	Administration Dosage, Route & Protocol	Immunity Onset & Duration
Circovac® Ceva	Inactivated PCV2a expressed in inactivated baculovirus → Light paraffin oil	• Gilts, sows • Piglets ≥3 weeks of age	• Gilts: 2mL deep IM/dose → Second injection 3 to 4 weeks later, at least 2 weeks before mating → Third injection, at least 2 weeks before farrowing • Sows: 2mL deep IM/dose → Second injection 3 to 4 weeks later, at least 2 weeks before farrowing • Revaccination: One injection at each gestation, at least 2 to 4 weeks before farrowing • Piglets: 0.5mL deep IM single dose	Up to 5 weeks after transfer of passive antibodies through colostrum intake Onset 2 weeks, last up to 14 weeks
Ingelvac CircoFLEX® / Boehringer Ingelheim	PCV2 ORF2 protein expressed in inactivated baculovirus → Aqueous polymer	• Piglets ≥3 weeks of age • Not to use during pregnancy and lactation if combined with MycoFLEX	• 1 mL IM single dose	Onset 2 weeks, last up to 17 weeks

Table 35: Continued

Vaccine / Company	Antigen / Adjuvant	Licensed For	Administration Dosage, Route & Protocol	Immunity Onset & Duration
Porcilis® / MSD	PCV2a ORF2 protein expressed in inactivated baculovirus → Dl-α-tocopheryl acetate & liquid paraffin	• Piglets ≥3 weeks of age • Not to use during pregnancy and lactation	• 2mL IM • Low MDA: Single dose at ≥3 weeks of age • High MDA: First injection at 3 – 5 day of age → Second injection 2 – 3 weeks later	Onset 2 weeks, last up to 22 weeks
Fostera® / Zoetis	Inactivated recombinant PCV1 expressing PCV2 ORF2 protein (inactivated PCV1–2 chimera) → Sulfolipo-cyclodextrin (SLCD) & squalene	• Piglets ≥3 weeks of age • Not to use during pregnancy and lactation	• 2 mL IM single dose	Onset 3 weeks, last up to 19 weeks
Fostera® Gold PCV / Zoetis	Inactivated PCV1–2a chimera & PCV1–2b chimera → Squalene	• Piglets ≥3 weeks of age	• IM • Single-dose protocol: Single 2mL dose intramuscularly, pigs ≥3 weeks of age • Two-dose protocol: Two 1mL doses, three weeks apart, pigs ≥3 days of age	Onset 3 weeks, last up to 23 weeks

The respective immunity induction mechanism of the vaccines is discussed on the basis of their antigen component.

Circovac® (Ceva) is the first PCV2 vaccine on the market, using the classical approach of an inactivated oil-adjuvanted vaccine. Since both capsid and replicase contribute to the cell-mediated immune response (Fort *et al.*, 2010; Stevenson *et al.*, 2006), a vaccine that incorporate the entire PCV2 particle may have its own advantage. Designed for use in sows and gilts, Circovac® aims to confer passive immunity to piglets via the colostrum ingestion, after active immunization of sows and gilts. Piglets from vaccinated sows had shown evidence of higher levels of passively derived PCV2-IgG and NA antibodies (Opriessnig *et al.*, 2010; Pejsak *et al.*, 2010). Moreover, apart from humoral immunity, cell-mediated immunity can also be transferred via colostrum. In a study by Oh *et al.* (2012), delayed type hypersensitivity and lymphocyte proliferation responses observed in newborn piglets from vaccinated sows proved the presence of PCV2 antigen specific maternal colostral lymphocytes in the piglet circulation, indicating anti-PCV2 specific adaptive cellular responses. Further, PCV2-specific gamma interferon secreting cells (IFN-γ-SCs) were detected at high levels in the peripheral blood mononuclear cells (PBMCs) of piglets from vaccinated sows even before PCV2 challenge; while challenged piglets from non-vaccinated sows took 14 – 21 days post-challenge to produce a significant level of IFN-γ-SCs. Given that the appearance of both serum virus NA and PCV2-specific IFN-γ-SCs plays important role in reducing PCV2 viremia load (Fort *et al.*, 2009), the piglets will benefit from protection against PCV2 infection. In terms of efficacy, the protection level is shown to last up to 5 weeks after transfer of passive antibodies through colostrum intake. Under field conditions, piglets from vaccinated sows do show improved pre-weaning average daily weight gains

(ADWGs) compared to piglets actively immunized with PCV2 (Opriessnig *et al.*, 2010; Pejsak *et al.*, 2010). However, this is very subjective as it relies upon the piglet's access to sufficient amounts of colostrum during the initial 24 – 48hr after birth. It should be noted that there are studies that suggest sow vaccination using vaccines marketed for sows induce higher levels of maternal-derived antibodies (MDA), as compared to using vaccines licensed for piglets (Opriessnig *et al.*, 2010; Oh *et al.*, 2014). On the other hand, in terms of efficiency, sow vaccination is much less labour intensive than vaccination of piglets – vaccinating one sow versus vaccinating one litter of 10 – 14 piglets.

Ingelvac CircoFLEX® (Boehringer Ingelheim) and Porcilis® (MSD) are subunit vaccines derived from the capsid protein of PCV2 and expressed by baculovirus systems using insect cell line derived from the ovaries of *Spodoptera frugiperda*. These vaccines, as well as all subsequent vaccines discussed in this essay, are licensed for the direct induction of active immunity in piglets 3 – 4 weeks of age or older. Although vaccination of sows may be beneficial in herds where newborn piglets are naturally infected with PCV2 at an early age as previously discussed, in contrast, vaccination of piglets may be more suitable for herds where PCV2 infection occurs between the late-nursery and early-finisher phase – when MDA is unlikely to be protective. Seroconversion is observed around 3 weeks post vaccination (Opriessnig *et al.*, 2010; Fraile *et al.*, 2012; Trible *et al.*, 2012). Although both *cap* and *rep* proteins of PCV2 elicit host immune response, *cap* is regarded as the most immunogenic protein of PCV2 (Blanchard *et al.*, 2003; Pogranichnyy *et al.*, 2000). Three monoclonal antibodies with neutralizing capability directed

against PCV2 *cap* epitopes, 3F6, 6H9 and 7F5, has been identified (Shang *et al.*, 2009). Specifically, vaccination against PCV2 induces immunity by preventing the diversion of host humoral response away from a protective epitope. Although vaccinated and naturally infected pigs possessed similar levels of PCV2-specific antibodies, vaccination increased PCV2 neutralizing activity by 4-fold. Antibodies from vaccinated pigs preferentially recognized CP43 – 233, the largest polypeptide fragment on the *cap*; while clinically diseased pigs futilely produced antibodies against a short oligopeptide, CP169 – 180, a highly conserved 'immunological decoy' among all PCV2 isolates (Trible *et al.*, 2011; Trible *et al.*, 2012).

CircoFLEX® and Porcilis® are adjuvanted with aqueous polymer, and liquid paraffin with Dl-α-tocopheryl acetate respectively. CircoFLEX® are marketed to be safe for simultaneous use with MycoFLEX® in a single dose for of both PCV2 and *Mycoplasma hyopneumoniae* (M. hyo) infection. However, in herds with endemic PCVADs, the benefits and efficacies of different vaccines adjuvants and antigen combination should be carefully evaluated, as studies suggest the potential negative effect that certain vaccines may have on PCV2 replication. Hoogland and Halbur (2006) found that pigs vaccinated with PCV2 vaccine with oil-in-water adjuvants had an increased length of PCV2 viremia, increased amount of PCV2 in serum and tissue, and increased severity of lymphoid depletion compared to pigs vaccinated with aqueous and aluminum hydroxide vaccines. Allan *et al.* (2000) detected a staggering 21% of PMWS cases in of a group of colostrum-fed, PCV2-infected pigs vaccinated with commercial *M. hyo* and *Actinobacillus pleuropneumoniae* vaccines, a stark

contrast to 0% in an unvaccinated group. Kyriakis *et al.* (2002) reported that 43% of pigs vaccinated with a commercial *M. hyo* vaccine developed PMWS, compared to a mere 11% of the sham-inoculated group.

Fostera® (Zoetis) is designed as an inactivated PCV1–2 chimera, using the non-pathogenic PCV1 as a genomic backbone to carry the immunogenic ORF2 capsid gene of PCV2. Though the study did not use the vaccine in question, Fenaux *et al.* (2003) demonstrated that chimeric PCV1-2 inoculated pigs showed sufficient seroconversion to antibodies against PCV2 ORF2 capsid protein albeit with shorter length of viremia, which could be due to lower level of replication of the chimeras. The briefer viremia could also be attributed to the limited infection with milder pathological lesions of the lymphoid structures observed in the chimeric-inoculated group within the same study. Paphavasit *et al.* (2009) reached a similar conclusion when conducting a field trial using Fostera® (Suvaxyn®), reaffirming that chimeric PCV1-2 advantageously retains the non-pathogenic phenotype of PCV1 but elicits specific immune responses against pathogenic PCV2. Fostera® Gold PCV (Zoetis) then takes one step further, combining PCV1–2a chimera with PCV1–2b chimera. PCV2a genotype has been gradually phased out by PCV2b (Xiao *et al.*, 2006) and subsequently PCV2d (Xiao *et al.*, 2016). The genotypic distribution of PCV2 still continues to evolve, even in the largely vaccinated population, the industry now has a new problem to tackle – concurrent infection of different PCV2 genotypes potentiating PCVADs (Karuppannan *et al.*, 2017). Distinctive motifs on the PCV2 *cap* differentiate the genotypes, for instance PCV2d codes an additional lysine residue at the C-terminal end (Opriessnig *et*

al., 2013). A seemingly small variation – a single amino acid (aa) substitution between PCV2a and b – was enough to cause a failure of conformational neutralizing epitope recognition by monoclonal antibodies (Huang *et al.*, 2011; Saha *et al.*, 2012). Cases of apparent vaccine failures (Franzo 2016 *et al.*; Jeong 2015 *et al.*; Salgado 2014 *et al.*; Seo 2014 *et al.*; Xiao 2012 *et al.*,) were linked to isolation of PCV2d which differs from PCV2a by 23aa, thus the issue of 'leaky vaccine' arises (Karuppannan *et al.*, 2017). Vaccines derived from PCV2a are feared to be inadequate in conferring protection under conditions of repeated exposure of PCV2 and influence of PCV2 genotypic shifts. Given the specificity of conformational neutralizing epitope recognition by antibodies, Fostera® Gold PCV incorporates PCV1-2b chimeric with PCV1-2a chimeric in an effort to broaden the coverage to PCV2d, given that PCV2b and 2d share more similar epitopes (Lekcharoensuk *et al.*, 2004). There is currently no published study to validate the efficacy of this vaccine.

The global swine industry is emphasizing on vaccination as one of the most crucial health management tools in modern pig farming, as seen with the global vaccine market valued at US$ 578 million in 2008. From the total market size, PCV2 vaccines have taken up a value of close to US$ 198 million, with 50% of market shared dominated by Boehringer Ingelheim's Ingelvac CircoFLEX®. Since PCV2 vaccines were introduced to various market including U.S., Canada and Mexico in 2006 – 2008, the vaccination rate has been recorded to be up to 95% (The Pig Site, 2009; Pig Progress, 2010). Various trials in European and Asian countries have shown successful application of vaccines against PCV2, as summarized by Siebel (2010). In U.S., there were reports of

average daily weight gain improving from 650g to 675 g per day, financial benefit to cost ratio of 5.7:1, mortality rate decreasing from 9.5% to 2.4%. In Europe, reports reflected feed conversion ratio improving from 2.53 to 2.44, reduction on 22% veterinary fees and complete disappearance of PDNS related mortality. In Asia, 12.4% decrease in average post-weaning mortality rate and 5.7 days earlier of reaching marketable weight were reported. Apart from the published good field results of Boehringer Ingelheim's Ingelvac CircoFLEX®, the factor as to why CircoFLEX® captured the market fast and wide could be attributed to the ease of its one-shot application which can be conveniently given simultaneously with the company's *Mycoplasma hyopneumoniae* vaccine, Ingelvac MycoFLEX®.

In Malaysian commercial pig farms, post-weaning multisystemic wasting syndrome (PMWS) is the most prevalent form of porcine circovirus-associated disease (PCVAD), typically peaking two to three weeks post-weaning. This condition is frequently exacerbated by co-infection with porcine reproductive and respiratory syndrome (PRRS) virus. Strategies to manage PCV2 challenges include routine vaccination, serological monitoring of herds, and isolating animals showing PMWS symptoms. PCV2 vaccination is widely practiced, with varying approaches across farms: sow vaccination, piglet vaccination, or combined sow and piglet vaccination. Vaccination schedules are often tailored based on herd serology profiles and anticipated PCV2 challenges.

Common vaccination practices in Malaysian commercial pig farms include:

Boars	:	Generally not vaccinated to avoid pyrexia, which could affect semen quality; if vaccinated, this is limited to twice annually
Sows	:	Two doses are administered before the first mating, followed by blanket vaccination every 4–5 months. Some farms administer a single dose before each mating
Porkers	:	A single dose is given at 2–3 weeks of age

Currently available commercial vaccines include Circovac® (Ceva), Ingelvac CircoFLEX® (Boehringer Ingelheim), Fostera® PCV (Zoetis) and Porcilis® (MSD). Positive outcomes, such as improved growth rates, reduced mortality, and better health metrics, have been reported following vaccination (Lim *et al.*, 2007; Kam *et al.*, 2013; Shen *et al.*, 2014; Wen *et al.*, 2015; Ng *et al.*, 2022). Diagnosis of PCVAD in the field typically relies on clinical observations such as stunted growth, pallor, respiratory distress, and increased mortality, coupled with post-mortem findings like generalized lymphadenopathy and interstitial pneumonia. However, diagnostic practices are evolving, with serological antibody titers and qPCR detection of PCV2 viremia titers increasingly used for more accurate and early identification of PCV2 infections.

9.9.4 PCV3 Vaccine Development

Current Research and Development

The development of vaccines for Porcine Circovirus Type 3 (PCV3) has become increasingly important due to its potential economic impact and

association with clinical syndromes such as reproductive failure and multisystemic inflammation in swine populations. Unlike PCV2, which has several commercial vaccines available, efforts to create PCV3 vaccines are still in their infancy and face unique challenges. Various innovative approaches, including autogenous vaccines and recombinant technologies, are being explored. One prominent example is the use of RNA particle technology under the MSD SEQUIVITY® platform, which allows the production of customized autogenous vaccines tailored to specific farm needs (Zabriskie, 2022). This process begins with sequencing the gene of interest, which is electronically transferred to Sequivity® analysts for synthesis. The RNA is then inserted into a production platform where it undergoes incubation, particle release, and purification before being formulated into a vaccine. However, this platform requires approximately 8–12 weeks to complete a custom vaccine, potentially limiting its use in urgent disease outbreaks (Zabriskie, 2022). Another significant advancement is the development of virus-like particle (VLP) vaccines, which mimic the structure of PCV3 to induce a robust immune response without requiring live virus cultivation. In 2020, Boehringer Ingelheim filed a patent for a PCV3 VLP vaccine that utilizes the capsid protein (ORF2) expressed via a recombinant baculovirus vector. The vaccine is adjuvanted with Montanide ISA27VG or Carbopol to enhance immune responses. Importantly, this vaccine can potentially be combined with antigens for other significant swine pathogens, such as PCV2, *Mycoplasma hyopneumoniae*, and swine influenza, highlighting its versatility (Iyer *et al.*, 2020).

Challenges in PCV3 Vaccine Development

The development of PCV3 vaccines is hindered by several key challenges. Firstly, the unculturable nature of PCV3 in standard cell lines complicates in vitro efficacy testing and hinders the study of its immunogenic properties. Efforts to culture PCV3 in PK-15 and ST cell lines have largely been unsuccessful, necessitating alternative approaches such as the use of infectious clones or innovative cell culture techniques (Pranoto *et al.*, 2023). Secondly, limited understanding of PCV3 pathogenesis and immune responses poses a barrier. Unlike PCV2, whose lymphoid tissue tropism and immunosuppressive effects are well-characterized, the immune evasion mechanisms and tissue tropism of PCV3 remain poorly understood. This lack of information complicates the identification of optimal vaccine targets (Maity *et al.*, 2023). Additionally, co-infections with pathogens such as PCV2 and PRRSV further obscure the clinical significance of PCV3, as its role in disease onset is often difficult to isolate (Pranoto *et al.*, 2023).

Promising Vaccine Candidates and Innovations

Recent studies have explored a range of vaccine platforms for PCV3. For example, chimeric vaccines combining PCV3 and PCV2 antigens have shown promise. A chimeric subunit vaccine by fusing truncated capsid proteins from PCV2d and PCV3 with a flexible GS linker has been developed (Peswani *et al.*, 2023) developed Expressed in E. coli, this vaccine provides a cost-effective production method and was shown to elicit neutralizing antibodies against PCV2d in animal models. However, its immunogenicity against PCV3 remains to be evaluated due to the absence of specific assay systems. The use of

infectious clones represents another significant innovation. By inserting the entire genome of PCV3 into plasmid vectors, researchers have successfully replicated the virus in controlled conditions, enabling the study of its pathogenicity and the development of prototype vaccines. The generation of infectious PCV3 clones would be capable of inducing PDNS-like symptoms in pigs, providing a useful tool for vaccine testing (Jiang *et al.*, 2023; Pranoto *et al.*, 2023).

Lessons from PCV2 Vaccine Development and Future Directions

The extensive experience with PCV2 vaccine development offers valuable insights for addressing challenges in PCV3 vaccine research. For example, targeting highly conserved epitopes on the capsid protein was crucial in creating effective PCV2 vaccines. Similar strategies could be applied to PCV3, provided conserved immunogenic regions are identified (Maity *et al.*, 2023). Additionally, the optimization of adjuvant formulations played a key role in enhancing the efficacy of PCV2 vaccines and could similarly improve PCV3 vaccine responses (Pranoto *et al.*, 2023). Field trials have also demonstrated the importance of multivalent vaccines that address co-infections. Lessons from these trials could guide the development of combination vaccines incorporating PCV3 antigens alongside those for PCV2 and other swine pathogens. Moving forward, improved understanding of pathogenesis and tissue tropism of PCV3, immune evasion strategies and interactions with the host immune system ought to be the focus of PCV3 vaccine development. Developing standardized assays for evaluating PCV3 immunogenicity and vaccine efficacy is also critical for advancing clinical trials.

9.9.5 Outlook on PCV Vaccination for Comprehensive Control

The integration of PCV2 and potential PCV3 vaccines into herd health management represents a critical step toward comprehensive control of Porcine Circovirus diseases. Future vaccine development should focus on creating universal or combined formulations capable of addressing multiple genotypes and species, such as PCV2a, PCV2b, PCV2d, and PCV3, to enhance cross-protection and streamline vaccination protocols. These innovations could significantly benefit industry stakeholders by improving productivity through better disease control, reducing economic losses associated with morbidity and mortality, and fostering improved animal welfare through more robust and adaptive disease prevention strategies. Comprehensive vaccination programs, complemented by stringent biosecurity measures, offer a pathway to sustainable swine production in the face of evolving viral challenges.

9.10 Viral Load, Co-Infection and Quasispecies in PCV Epidemiology

Viral Load in PCV Epidemiology

In this study, conventional PCR was utilized to detect PCV2, PCV3 and PCV4, focusing on the primary objective of molecular identification rather than quantifying viral load. Real-time PCR (qPCR) which is more effective for assessing viral load was not employed. In studies of PCV2, qPCR has been extensively used to quantify viral loads, revealing a clear distinction between clinical and subclinical infections. Pigs with clinical signs of postweaning multisystemic wasting syndrome (PMWS) typically exhibit viral loads

exceeding 10^7 copies/mL of serum or 500 ng tissue sample, whereas subclinical cases often present with significantly lower viral loads, around 10^3 – 10^5 copies/mL (Brunborg *et al.*, 2004; Segalés *et al.*, 2012).

This distinction has been proposed as a diagnostic criterion to differentiate between PCV2-associated diseases and asymptomatic infections, thus informing diagnostic thresholds and management strategies of PCV2 in farms. Similarly, emerging research on PCV3 has begun investigating viral load levels in both subclinical and clinical cases, although the findings remain preliminary. While PCV3 has been associated with various clinical presentations, a significantly higher PCV3-positive rate was reported in diarrheal weaned pigs compared to non-diarrheal weaned pigs. Lower Ct values were observed more frequently in samples from fattening or weaned pigs exhibiting respiratory disease and diarrhea, compared to asymptomatic pigs (Zhai *et al.*, 2017). Viral loads from oral fluids, serum and faecal samples collected from farms previously confirmed to be PCV3-positive ranged from $10^{2.5}$ to $10^{7.2}$ genome equivalent copies/mL (Wozniak *et al.*, 2020). While higher viral loads tend to correlate with clinical symptoms, a consistent and reliable pattern has not yet been established, complicating efforts to define a diagnostic threshold based on viral load alone. Further research is needed to clarify PCV3 disease progression and develop more precise diagnostic criteria.

Genetic Diversity and Quasispecies

PCV2 exhibits a remarkably high mutation rate, with a nucleotide substitution rate estimated at 1.2×10^{-3} substitutions/site/year, the highest known for

single-stranded DNA viruses. This genomic plasticity is attributed to the relaxed replication requirements of the rolling circle replication mechanism (Firth *et al.*, 2009; Olvera *et al.*, 2007; Perez *et al.*, 2011). Both mutation and genetic recombination play crucial roles in PCV2 evolution. Selective pressure is evident across the entire PCV2 genome, particularly influencing the ORF2 gene, which encodes the capsid protein, the major immunogenic protein and the primary component of PCV2 vaccines. Recombination within ORF2, especially at antigenic epitope regions, significantly affects the phenotypic and antigenic diversity of PCV2, potentially impacting vaccine efficacy. Both inter- and intra-genotype recombination events have been reported, further demonstrating the adaptability of PCV2 under immune and environmental pressures (Ssemadaali, Ilha & Ramamoorthy, 2015). Mutations within the rep gene, responsible for replication-associated proteins, also reflect host-driven evolution. These genetic adaptations underline the virus's capacity to evade host immunity (Olvera, Cortey & Segales, 2007).

As a consequence of its high mutation rate, PCV2 exists as a dynamic "cloud" of genetic variants, a concept referred to as ‘quasispecies’ encompassing the full spectrum of possible variations within a single viral population (Eigen, 1993; Drew, 2011). Within a viremic animal, these viral populations, also known as a ‘mutant spectrum’, emerge due to replication errors, resulting in subtle genetic variability. Such diversity may provide adaptive advantages, such as enhancing the virus's capacity for replication, immune evasion and dissemination. Environmental and management factors significantly shape the composition of these viral populations. Variables such as host immunity,

nutritional status, pollutants, vaccination practices, stress and co-infections with other pathogens exert selective pressures that influence the emergence of viral variants. These pressures enable the virus to adapt to new environmental conditions or host defenses, potentially altering disease progression and transmission (Drew, 2011).

This study identified significant within-host diversity, with two genetically distinct PCV2 strains detected in a single pig's lung and lymph nodes. Specifically, sequences PCV2/SW51L/UPM/MY009/OM524606 and PCV2/SW51I/UPM/MY010/OM524607, derived from the lung and inguinal lymph nodes of the same animal, were found to be both genetically and phylogenetically distinct (Tan *et al.* 2022; Chapter 4). These findings underscore the ability of PCV2 to establish diverse viral populations within a single host, contributing to its evolutionary resilience. This intrahost genetic variability is a hallmark of quasispecies as discussed earlier. Evidences of co-infection and recombination involving multiple PCV2 genotypes have been documented, particularly in cases where pigs from diverse sources were mixed. Correa-Fiz *et al.* (2018) reported recombinant strains arising after the mixing of animals from different farms. These recombination events can generate viral variants with enhanced fitness or altered antigenic properties. Furthermore, selective pressures induced by the host immune response drive the evolution of PCV2, particularly within the antigenic epitope regions of the capsid protein encoded by ORF2. Furthermore, in this study, a notable co-infection rate of 28.77% was observed between PCV2 and PCV3, with 21 out of 73 samples testing positive for both viruses (Tan *et al.*, 2022; Chapter 4).

Such co-infections can exacerbate disease severity and alter immune responses, as interactions between PCV strains have been shown to influence viral replication and pathogenicity (Klaumann *et al.*, 2018).

The Role of Vaccination

Vaccination against PCV2 has been shown to significantly reduce the clinical signs of the disease. Field data consistently demonstrate the success of PCV2 vaccines, with observable productivity gains in vaccinated animals, including increased average daily weight gain and reduced wean-to-finish mortality (Coll *et al.*, 2010; Kristensen *et al.*, 2011; da Silva *et al.*, 2014). Studies indicate that vaccination has contributed to a reduction in the total number of positive PCV2 cases reported to veterinary diagnostic laboratories, with peak case numbers observed in 2007 sharply declining by 2009, following the introduction of vaccines in 2006. However, a slight resurgence of positive cases occurred between 2012 and 2016, coinciding with the emergence of PCV2d (Afghah *et al.*, 2016). This suggests that while vaccination alleviates disease symptoms, the virus continues to circulate within production systems. In countries where PCV2 vaccination has been aggressively implemented, a sharp decline in PCV2 prevalence has been observed in herds practicing robust vaccination strategies (Dvorak *et al.*, 2016; Eddicks *et al.*, 2016). Nevertheless, it remains concerning that several new variants have emerged during this period (Franzo *et al.*, 2016).

PCV2 vaccination does not completely eradicate the virus, allowing it to persist at low levels and potentially promote immune-evading mutations (Kekarainen

et al., 2014). Despite the effectiveness of PCV2 vaccines in reducing clinical signs, the persistence of subclinical infections remains a significant challenge. These subclinical infections can exacerbate co-infections and weaken the overall immune response, potentially compromising herd health. PCV2 remains detectable in both clinically healthy and diseased pigs, even with widespread vaccination, highlighting the vaccine's inability to prevent transmission entirely (Read *et al.*, 2015). This persistent circulation of PCV2 raises concerns about "leaky vaccines" that prevent clinical symptoms but do not fully inhibit viral shedding, which may inadvertently drive the evolution of more virulent variants. Studies indicate that vaccination alone is insufficient for eradication; while repeated vaccinations may temporarily reduce PCV2 to undetectable levels, the virus re-emerges shortly after vaccination ceases (Feng *et al.*, 2014). The rapid evolution of PCV2 following the introduction of commercial vaccines has prompted speculation that vaccine-induced selection pressure may be driving viral evolution (Ssemadaali, Ilha & Ramamoorthy, 2015). For instance, PCV2d, now the predominant strain in many regions, is believed to have emerged under immune pressure from PCV2a and PCV2b vaccines (Franzo *et al.*, 2016). A study on synonymous codon usage in PCV1 and PCV2 indicates that while mutational bias influenced PCV1 evolution, both mutational bias and natural selection have shaped PCV2 evolution. This suggests that the widespread use of PCV2 vaccines, along with potential selective pressure, has driven the current evolution of PCV2 (Chen *et al.*, 2014). Comparisons of PCV2 genetic sequences from farms vaccinated with the PCV2a vaccine to those from unvaccinated farms showed higher non-synonymous mutations in the vaccinated group. Several of these mutations in

the capsid protein localized to immuno-dominant epitopes, suggesting that vaccination may have altered the immunogenicity or conformation of the virus (Kekarainen *et al.*, 2014). These findings support the idea that the introduction of PCV2 vaccines has influenced the molecular epidemiology of the virus.

In conclusion, the dynamics of viral load, co-infection, and quasispecies significantly influence the epidemiology of PCV infections. The presence of multiple strains, recombination events, and the impact of immune pressure from vaccination underscore the complexity of managing these infections. To address these challenges, integrated strategies involving genomic surveillance, adaptive vaccination, and robust biosecurity measures are essential.

9.11 Wild Boars in PCV2 Dynamics: Spillover and Spillback Risks

Global Context and Increasing Wild Boar Populations

The global rise in wild boar populations (Saez-Royuela & Telleria, 1986; Acevedo *et al.*, 2006) has raised concerns about their potential role in disease transmission. Increased population densities result in higher host-to-host contact rates, amplifying the risk of pathogen spread (Acevedo *et al.*, 2007). Reports of postweaning multisystemic wasting syndrome (PMWS) in wild boars are rare but have been documented in North America and Europe, primarily affecting animals aged 4 to 10 months (Ellis *et al.*, 2003; Schulze *et al.*, 2003; Vicente *et al.*, 2004). These cases revealed clinical and pathological findings similar to those in domestic pigs, including increased piglet mortality within herds and hunting estates (Vicente *et al.*, 2004). While wild boars may

harbour PCV2, the extent to which they act as reservoirs for domestic pigs remains unclear. PCV2 isolates from wild boars are often genetically identical to those found in domestic pigs, even across distant regions (Knell *et al.*, 2005; Cságola *et al.*, 2006). This similarity suggests that PCV2 infection in wild boars likely originates from domestic pigs, particularly given the high prevalence of PCV2 in swine herds. However, there is limited knowledge regarding the directionality of transmission on whether wild boars are passive carriers through spillover or actively contribute to pathogen spread through spillback.

Key Findings from Malaysian Wild Boars

This study confirmed 100% prevalence of PCV2 in wild boar lung samples (28/28), marking the first evidence of PCV2 circulation in Malaysian wild boars (Tan *et al.*, 2022; Chapter 4). Genetic analysis revealed at least 80.7% nucleotide identity between wild boar and domestic pig PCV2 sequences. While most wild boar strains clustered into distinct clades, some were interspersed within domestic pig clades, with bootstrap values ranging from 58% to 97%. Among wild boar sequences, 92.86% (26/28) were identified as PCV2d, consistent with the 98.04% (50/51) PCV2d prevalence in domestic pigs. Two wild boar strains were classified as PCV2a, while one was identified as PCV2b. The PCV2b strain shared a clade with a 2007 strain from the northern region of Malaysia, suggesting the persistence of older genotypes in wild populations. These findings suggest that while PCV2d dominates both populations, older genotypes may still circulate within isolated wild boar populations.

Spillover, Spillback, or Both?

Spillover refers to the transmission of a pathogen from its primary host to a novel, susceptible host species, as seen with Nipah virus transmission from bats to pigs (Sparrer *et al.*, 2023). In the context of this study, spillover likely occurred when PCV2 moved from domestic pigs to wild boars due to shared environments near farms. In Malaysia, pig farms are often situated near forests and oil palm plantations, increasing the likelihood of interactions between wild boars and domestic pigs. These interactions may occur directly or indirectly through fomites such as leftover feed. The migratory behaviour of wild boars adds complexity to these dynamics. Additionally, migratory behaviour among wild boars, particularly through corridors linking Malaysia to neighbouring countries like Thailand and Vietnam, may facilitate regional pathogen spread. Conversely, spillback describes the reintroduction of a pathogen from a novel host back into its original host, akin to SARS-CoV-2 transmission from humans to wild-caught bats (Sparrer *et al.*, 2023). The detection of PCV2a and PCV2b in wild boars suggests either spillback, or independent maintenance of these older genotypes within wild populations.

Implications for Disease Control

The proximity of wild boars to pig farms in Malaysia, often near forests or oil palm plantations, heightens the risk of PCV2 transmission. Direct contact between domestic pigs and wild boars or indirect exposure through fomites such as feed waste are possible transmission pathways. The dominance of PCV2d in both populations suggests recent spillover from domestic pigs to wild boars, but the presence of older genotypes indicates the potential for historical

spillback events. Although no PCV3 or PCV4 was detected in this study, the small sample size of 28 wild boar lungs limits definitive conclusions. Larger, geographically diverse studies are needed to explore the full scope of PCV3 and PCV4 presence in wildlife and their potential to spill over into domestic pigs. Enhanced surveillance and phylogenetic analyses will be critical in identifying emerging viral threats and understanding their transmission pathways.

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APPENDICES

Appendix A

Microscopic Images of *In situ* Hybridisation (ISH) Results

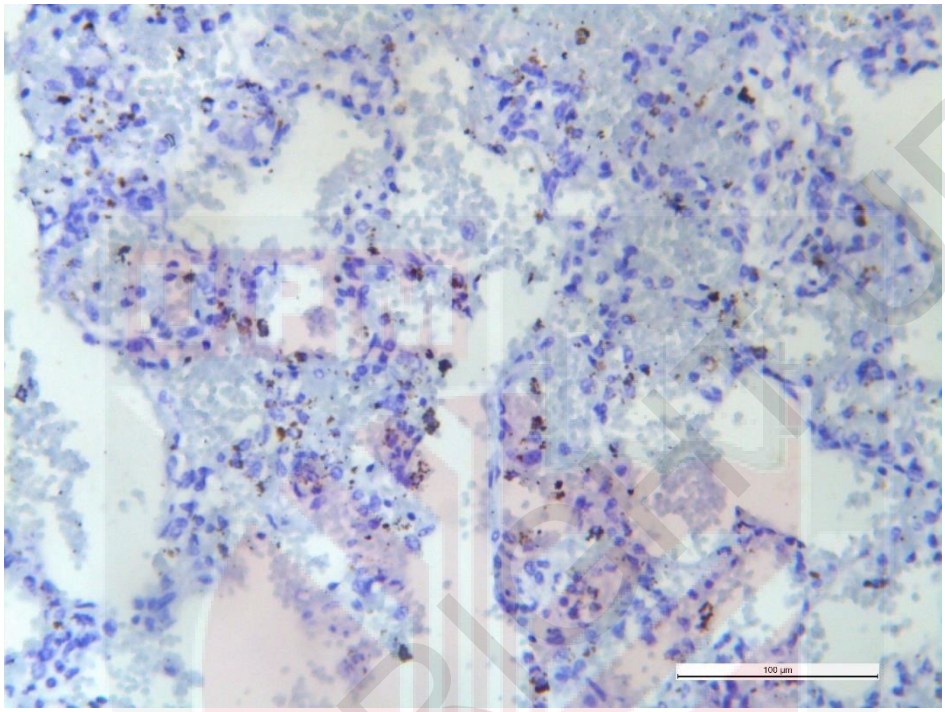


Figure 1A: Positive in situ hybridisation (ISH) results from lung tissues. Multifocal ISH positive signals were observed as brown grains of chromogenic staining within cytoplasm of pneumocytes. 200× magnification with scale bar equivalent to 100 µm

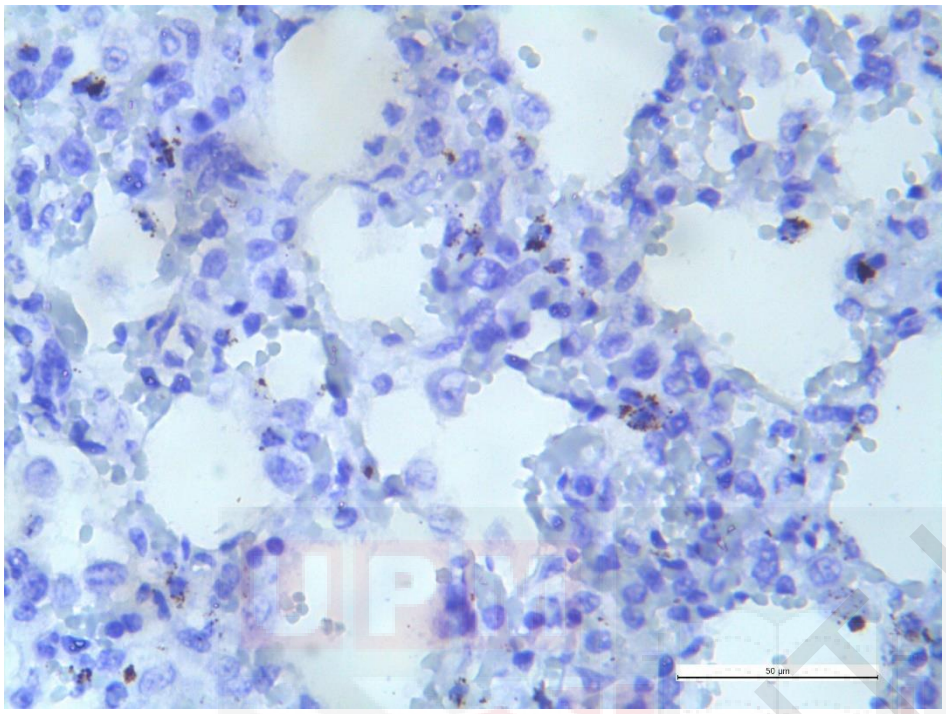


Figure 1B: Positive ISH results from lung tissues. Multifocal ISH positive signals were observed as brown grains of chromogenic staining within cytoplasm of pneumocytes. 400× magnification with scale bar equivalent to 50 µm

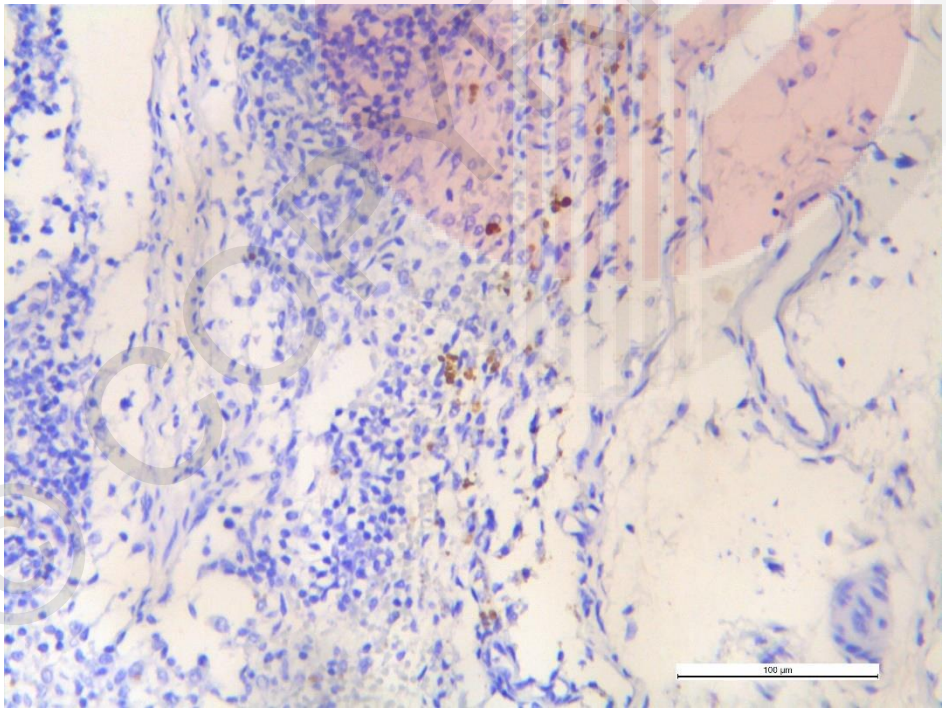


Figure 1C: Positive ISH results from inguinal lymph node tissue. Multifocal ISH positive signals were observed as brown grains of chromogenic staining within cytoplasm of lymphocytes. 200× magnification with scale bar equivalent to 100 µm

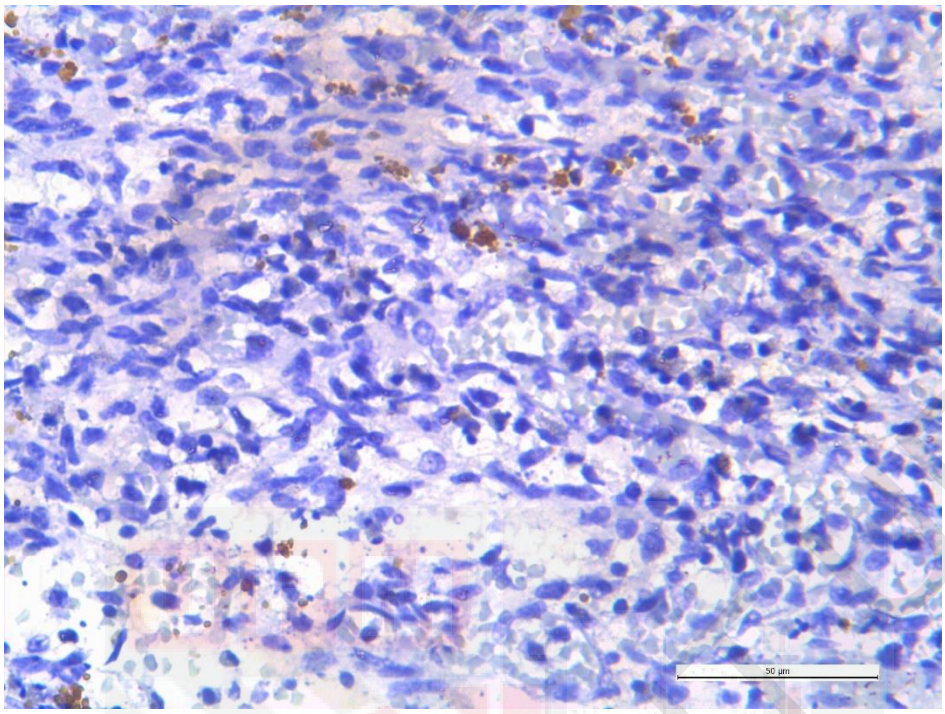


Figure 1D: Positive ISH results from mesenteric lymph node tissues. Multifocal ISH positive signals were observed as brown grains of chromogenic staining within cytoplasm of lymphocytes. 400× magnification with scale bar equivalent to 50 µm

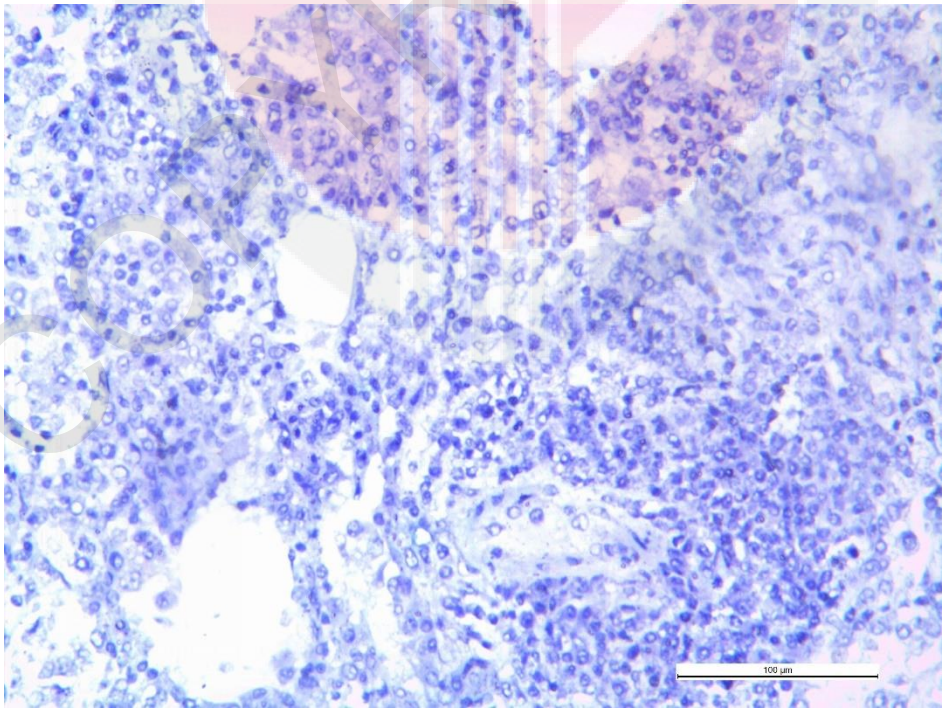


Figure 2A: Negative in situ hybridisation (ISH) results from lung tissues. In situ hybridisation positive signals were not observed. 200× magnification with scale bar equivalent to 100 µm

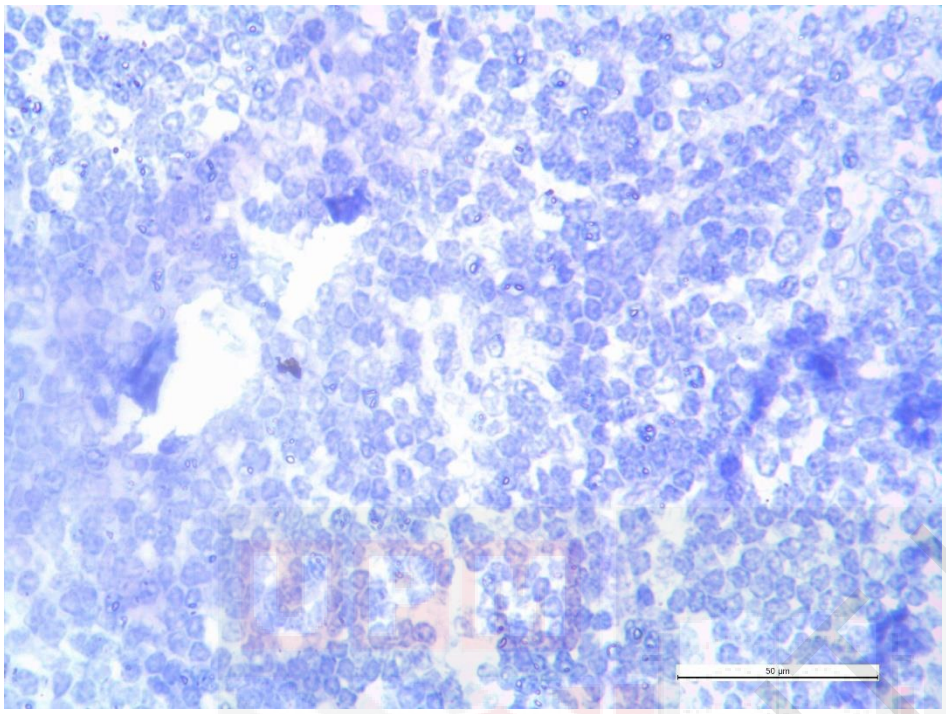
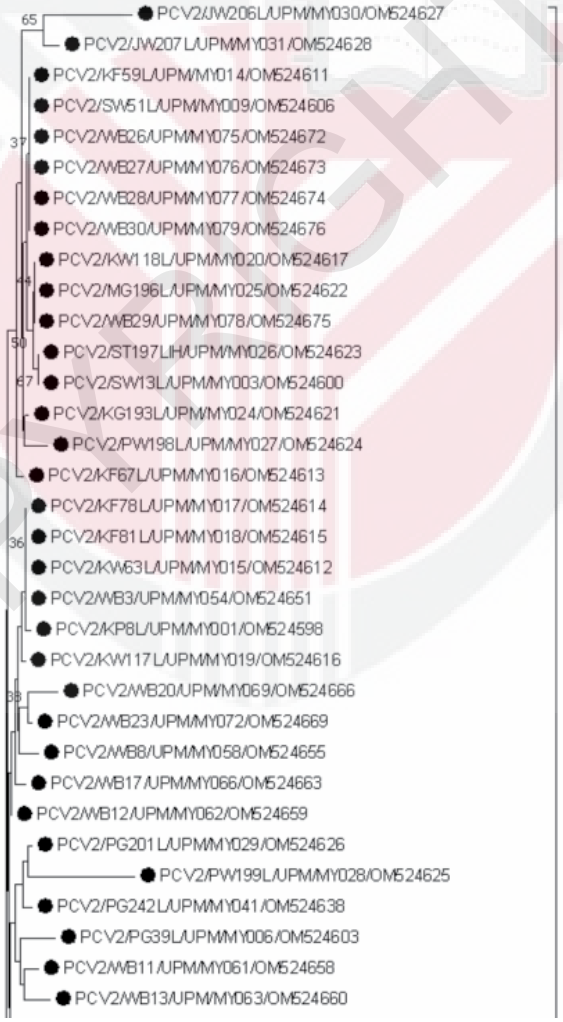
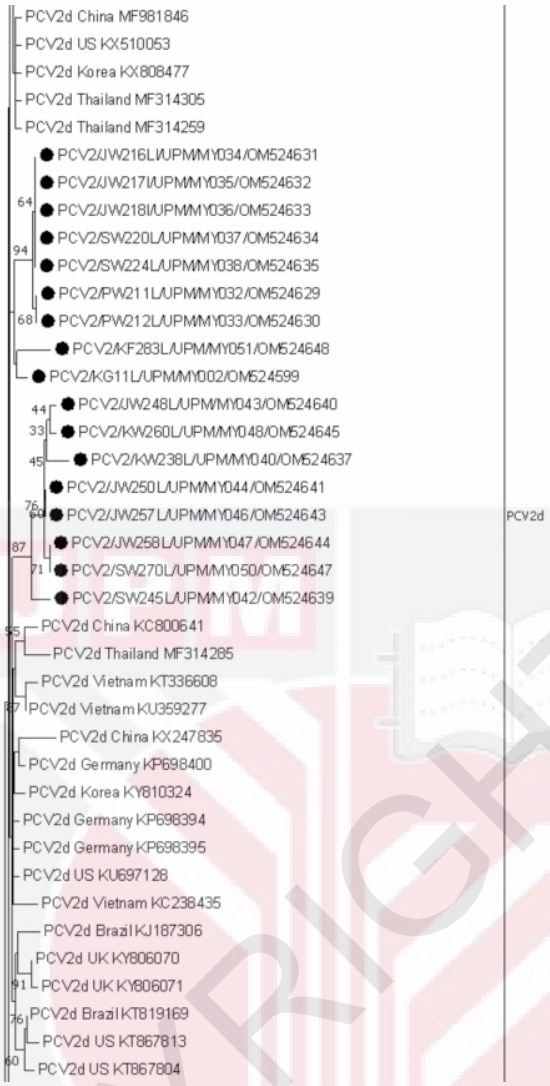


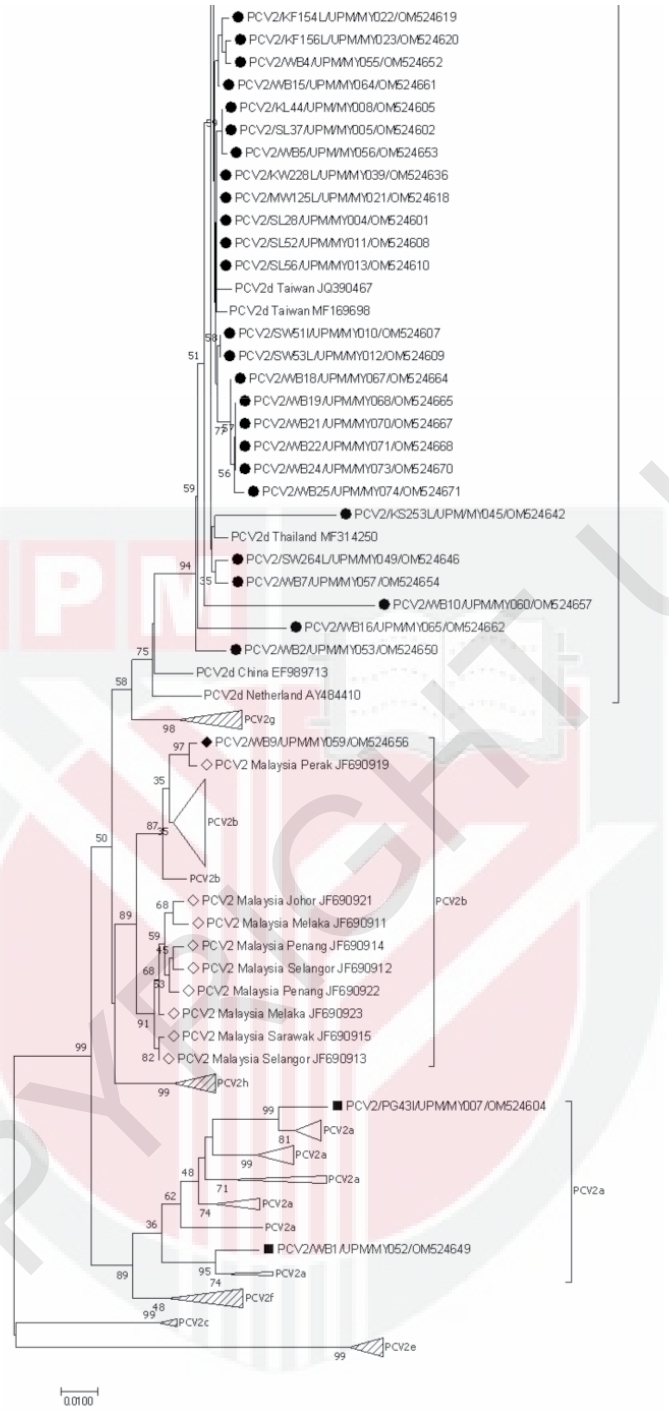
Figure 2B: Negative ISH results from inguinal lymph node tissues. In situ hybridisation positive signals were not observed. 400× magnification with scale bar equivalent to 50 µm

Appendix B

Figure 2: Condensed phylogenetic analysis of PCV2 cap gene nucleotide sequences. Seventy-nine Malaysian PCV2 cap gene nucleotide sequences (■ PCV2a, ◆ PCV2b, ● PCV2d) were compared with 162 other GenBank reference sequences from different countries and different genotype clades including previously published Malaysian sequences (denoted ◇). PCV2a and PCV2b clades were partially condensed, showing only Malaysian sequences. PCV2c, PCV2e, PCV2f, PCV2g and PCV2h clades were entirely condensed. GenBank accession numbers, origin country and genotype are as indicated. Malaysian PCV2 cap gene sequences were additionally labelled with sequence ID. The tree was constructed using the NJ method, p-distance nucleotide substitution model with 1000 bootstrap replicates. The scale bar indicates branch length measured in number of substitutions per site







BIODATA OF STUDENT

Chew Yee grew up in a small town on the east coast of Malaysia. She always loved the sea, and is very fond of animals big and small, hairy and furry. When her mother asked her to pick her choice of primary school, she requested for SJK (C) Kong Min citing 'I can see the beach from my classroom' and 'there are goats outside the school compound". It wasn't a surprise when she decided to pursue a Diploma in Animal Health and Production in Universiti Putra Malaysia Kampus Bintulu which sent her into cattle, goat, chicken, and fish farms. This sparked her interest in livestock, and she eventually decided to pursue a degree in Veterinary Medicine in Universiti Putra Malaysia. Luck and fate have it that her mentor is a kindly and wise lecturer named Dr. Ooi Peck Toung. He took her under his wing and opened her eyes to the world of pig farming. Tan immediately fell in love with the adorable pink creatures with their ever-curious snouts and intelligent eyes. Together with Dr. Ooi's research family, she explored the world of swine medicine from every aspect possible: from wallowing in the mud for hands-on husbandry to needling the pigs for herd health management. The endless discussion in the field on Porcine circovirus 2 begin to plant a seed for postgraduate research in Chew Yee's mind. The path to into the field of research was cemented when Prof. Dr. Roongroje became the external examiner of her DVM Comprehensive Examination. When she is not talking or thinking about pigs, Chew Yee read indiscriminately across genres. She is currently reading Hans Rosling's "Factfulness".

LIST OF PUBLICATIONS

Journal Article

Tan, C. Y., Opaskornkul, K., Thanawongnuwech, R., Arshad, S. S., Hassan, L., & Ooi, P. T. (2020). First molecular detection and complete sequence analysis of porcine circovirus type 3 (PCV3) in Peninsular Malaysia. *PLoS One*, *15*(7), e0235832.

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Ng, S. C., Tan, C. Y., Foong, Y.C., Berg, S., Lin, H. Y. (2022). Comparing the efficacy of porcine circovirus 2 vaccines in reducing viremia titer. In Proceedings of the International Pig Veterinary Society (IPVS) 2022 (p. 16).