



RESEARCH ARTICLE

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

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ABSTRACT

Porcine circovirus type 4 (PCV4) is the newest member in the porcine circovirus family, first reported in 2020. To date, the presence of PCV4 has only been reported in China, South Korea and most recently in Thailand. Detection of PCV4 have been reported in various production stages of pigs from piglets, finishers to sows; associated with a myriad of clinical manifestations including porcine dermatitis and nephropathy syndrome (PDNS), postweaning multisystemic wasting syndrome (PMWS), respiratory, enteric and neurological diseases. While successful virus isolation and culture has yet to be reported, pathogenicity of PCV4 has been demonstrated through infectious clone studies. The objective of this study is to investigate the presence of PCV4 in Malaysian porcine population to update the epidemiology of porcine circoviruses in Malaysia. A total of 49 samples from commercial intensive pig farms, abattoir and wild boar population were subjected to conventional polymerase chain reaction assay to detect PCV4 capsid (*cap*) genome. Resulting *cap* nucleotide sequences were analyzed for maximum likelihood phylogeny relationship. Results revealed that PCV4 is present in Peninsular Malaysia at a molecular prevalence of 4.08% (2 / 49 samples). Both 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. With this latest update of the novel PCV4 in Malaysia, it is clear that more attention needs to be given to the investigation of novel porcine circoviruses (PCV) and management of PCV diseases.

Keywords: Malaysia pig farming; porcine circoviruses; porcine circovirus type 4 (PCV4); porcine circovirus associated diseases (PCVAD); molecular detection.

INTRODUCTION

Circoviruses have been of veterinary importance especially in the swine and poultry 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-D az *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.*, 2020; 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.

MATERIALS AND METHODS

Sample Collection

A sample subset comprising 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, 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 of 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 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 year 2016 to 2021.

Molecular Detection & Molecular Characterization

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 1.

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 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 *et al.*, 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).

RESULTS & DISCUSSION

PCV4 was confirmed to be present in Malaysia at a detection rate of 4.08% (2 / 49 samples) (Table 2). 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, 2022).

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 1).

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 2). 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 3).

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 year 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 & Opriessnig, 2011; Segal's, 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

Table 1. Primers and PCR cycling conditions used in this study to generate complete nucleotide sequence of PCV4 capsid

Primer Pair	Nucleotide Sequence (5'–3')	Product Length (bp)	PCR Cycling Condition (Temperature / Time)					
			Initial Denaturation	Number of Cycle	Denaturation	Annealing	Extension	Final Extension
Zhang-F	TGAGGGAGGATGGGCAGTTGTATG	842	95°C / 5 min	35	95°C / 20 s	58°C / 30 s	72°C / 1 min	72°C / 10 min
Zhang-R	CACCACCCACAGATGCCAATCA							

Table 2. 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
Negative	Positive	Wild boar	10	11	0	10
		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%)

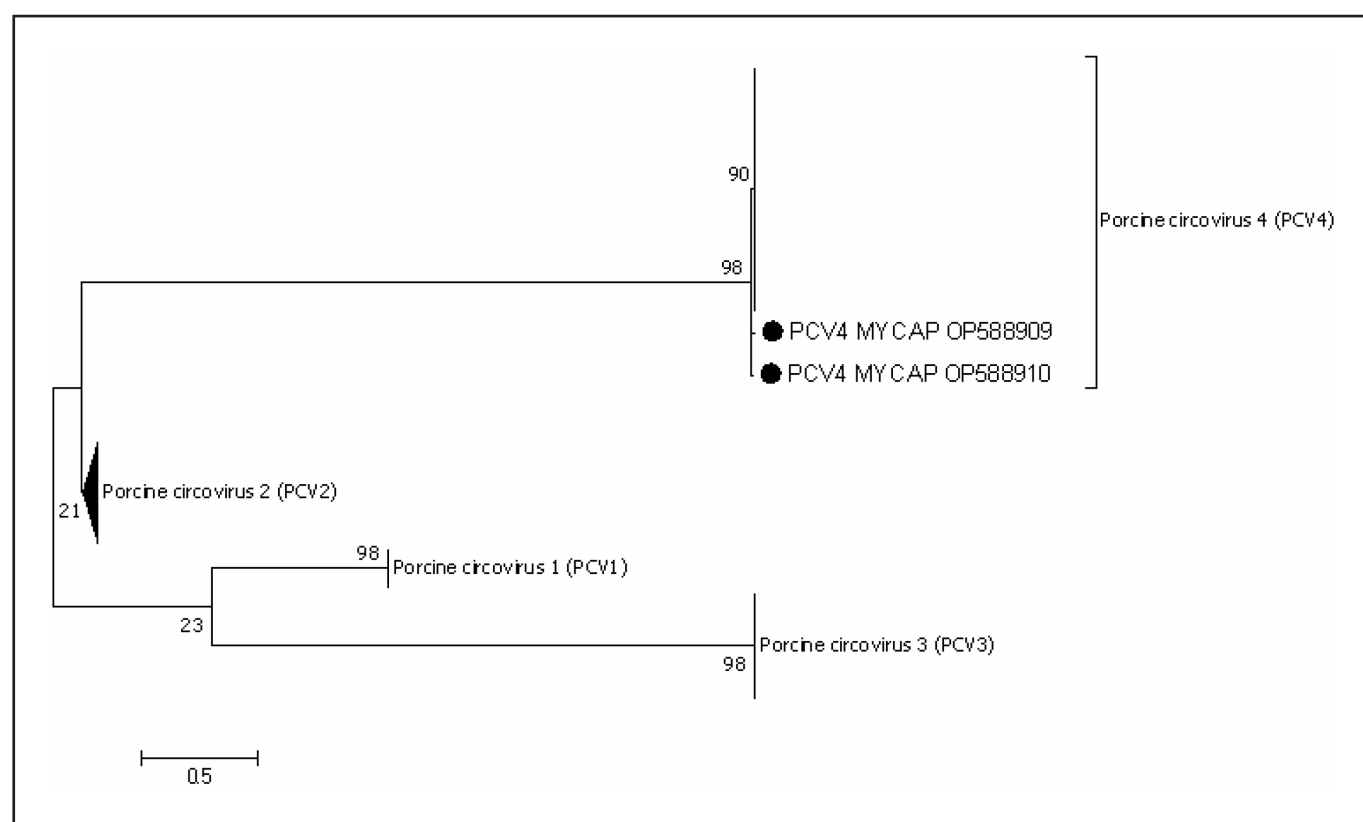


Figure 1. Condensed phylogenetic analysis of Malaysian PCV4 *cap* gene nucleotide sequences with reference to PCV1, PCV2, PCV3 and PCV4 *cap* gene nucleotide sequences. Two Malaysian PCV4 strains (●) were compared with 143 GenBank reference sequences which comprised of 11 PCV1 *cap* gene nucleotide sequences, 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.

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.

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 clone 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



Figure 2. 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 comprised 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 (○) 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.

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.

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Conflict of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

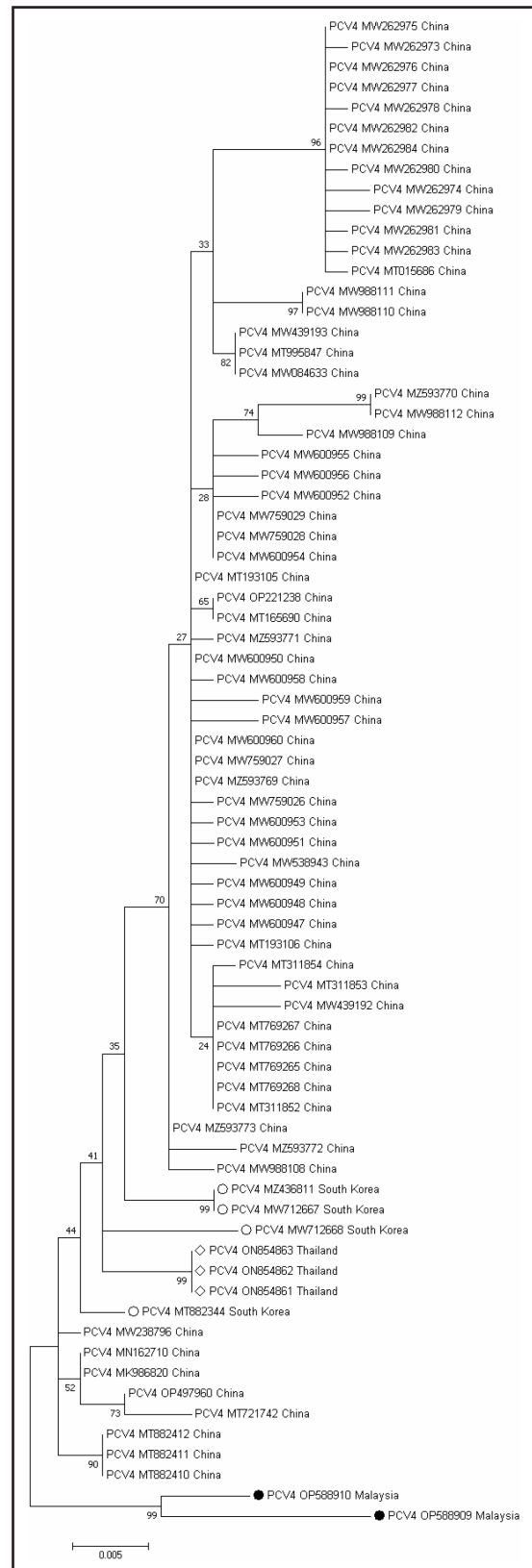


Figure 3. Phylogenetic analysis of Malaysian PCV4 cap gene nucleotide sequences with reference PCV4 cap gene nucleotide sequences. Two Malaysian PCV4 strains (●) were compared with 72 GenBank reference PCV4 cap gene nucleotide sequences. PCV4 strains from South Korea (○) and Thailand (◇) were additionally 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.

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