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Complete Genome Characteristics of Porcine circovirus Type 2 (PCV2) Isolates from Papuan Pigs, Indonesia

Widi Nugroho[1], Farhid Hemmatzadeh[1], Sidna Artanto[2] and Michael P. Reichel[1]

[1]School of Animal and Veterinary Sciences, Roseworthy Campus, University of Adelaide, Roseworthy, Australia
[2]Faculty of Veterinary Medicine, Gadjah Mada University, Jl. Olahraga, Karangmalang, Yogyakarta, Indonesia

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Abstract Porcine circovirus type 2 (PCV2) has been recognised as an important pathogen in the pig industry world-wide. The virus was recently identified in Papuan pigs, yet information about the characteristics of Papuan PCV2 isolates is limited. The aim of the present study was to characterise the complete genome of Papuan PCV2 isolates. Viral DNA was isolated from 13 blood samples of village pigs from the Jayawijaya region. Four of the PCR positive samples were selected for full genome sequencing. Neighbour Joining phylogeny with P-distance model showed that the four Papuan PCV2 isolates belong to genotype PCV2-IM3. Sequence identity analysis of the Papuan PCV2 genomes further showed 99.5% to 99.6% similarity with a Chinese PCV2-IM3 reference strain. The current study revealed that genotyping based on the ORF2 sequence resulted in a substantially different characterisation of PCV2 genotypes compared to classification based on the complete genome sequences. Furthermore, the study showed that the topology of the PCV2 phylogeny based on the ORF2 sequence was different from the topology based on capsid proteins. Genotyping using the complete genome, ORF2 region or capsid protein sequences resulted in substantial variance in the classification of the PCV2 isolates. The clinical consequence of these different genotyping methods needs further study.
Keywords *Papua; Pigs; ORF2; Neighbour Joining; PCV2-IM3 Genotype*

1. Introduction

Porcine circovirus type 2 is an emerging pathogen causing economic loss in the pig industries worldwide. Infection with PCV2 manifests in various clinical outcomes, termed porcine circovirus associated diseases (PCVD) and characterized by a respiratory disease complex, dermatitis and nephropathy syndrome or post weaning multisystemic wasting syndrome (PMWS). In order for PCVD to be produced, co-infection with PCV2 and other factors is required (Baekbo et al., 2012). Reported PCVD co-factors included porcine reproductive and respiratory syndrome (PRRS), porcine parvovirus (PPV), Swine Hepatitis E virus (HEV), *Mycoplasma hyopneumoniae, Salmonella spp.* Or *Metastrongylus elongatus* (Allan et al., 2000; Ha et al., 2005; Kennedy et al., 2000; Marruchella et al.,

2012; Opriessnig T. et al., 2004; Yang et al., 2015) as well as environmental stressors, such as changes in temperature and high stocking density (Patterson et al., 2015).

Genotype variations of PCV2 virus have been observed; one widely recognised method for the classification of PCV2 genotypes is the Neighbour Joining (NJ) phylogenetic tree approach with pairwise proportional difference of nucleotides (P-distance) model, using the ORF2 gene region or the complete genome as target sequences. The cut off value of proportional nucleotide diversity for genotype demarcation is 0.035 in ORF2 based analysis or 0.02 when the complete genome is used (Segales et al., 2008).

Using the NJ method with P-distance model, four major genotypes of PCV2 have been established, namely PCV2a, PCV2b, PCV2c and PCV2d. Further, a few intermediate (IM) groups; IM1-IM4 have also been reported (Xiao et al., 2015). The most prevalent circulating genotype in farmed pigs has been PCV2b, followed by PCV2a. Genotype PCV2d may be a new emerging genotype in farmed pigs and recently has been identified amongst herds with vaccination failure in USA, Korea and Brazil (Segales, 2015; Xiao et al., 2015). Genotype PCV2c consists so far of only four strains (Franzo et al., 2015). Intermediate clades have consisted of less than fifty strains (Xiao et al., 2015).

Commercial vaccines against PCV2 have been developed based on the capsid protein of ORF2 genes of the PCV2a genotype and known to be protective against infections with PCV2a and PCV2b (Segales, 2015). However, the observation that PCV2d can be retrieved from vaccinated pigs invites a discussion on the cross protection provided by commercial vaccines for PCV2d (Segales, 2015; Xiao et al., 2015). Furthermore, the level of cross protection of available vaccines against PCV2c and intermediate groups is unknown.

To date, only two papers have been published on the topic of PCV2 from Indonesia, one from Western Indonesia (Manokaran et al., 2008) and the other one was from Papua (Nugroho et al., 2015). Papua province has the fifth highest pig population in Indonesia (Siagiaan, 2014) and village pigs are important livelihood assets for the Papuan community (Nugroho et al., 2015). The aim of the present study was to characterise the Papuan PCV2 isolates based on either the complete genome sequences, ORF2 region and capsid protein using NJ method. This information will contribute to a better knowledge of the epidemiology of PCV2 infections in Papua, Indonesia.

2. Materials and Methods

2.1. PCR and Complete Genomes Assembly

Total DNA from thirteen serologically PCV2 positive blood samples was used in present study. The samples were retrieved from village pigs from Jayawijaya Region, Papua, Indonesia. The two pairs of primers used in the current full genome sequencing study were 20 base pair (bp) length respectively, reported previously (Fenaux et al., 2000). The first pair of primers, CV1 and CV2, amplified a 989bp fragment. The CV1 primer is 5'-AGGGCTGTGGCCTTTGTTAC-3', situated at position 1336-1355 in PCV2 genome and CV2 is 5'-TCTTCCAATCACGCTTCTGC-3' located at position 536-556 of PCV2 genome. Second set of primers, CV3 and CV4, amplified a 1,092-bp fragment. The sequence of CV3 and CV4 were 5'-TGGTGACCGTTGCAGAGCAG-3' and 5'-TGGGCGGTGGACATGATGAG-3' respectively, positioned at 452-471 and 1525-1544 in PCV2 genome. The PCR products of PCV2 DNA resulting from amplification using these two pairs of primers overlap at positions 452-536 and 1355-1544 in PCV2 genome.

Amplification of PCV2 genomes was conducted using conventional PCR. Each of the PCR mix contained 2.5 µL of 10X buffer, 0.5 µL of 10 mM dNTP, 1 µL of 50 mM $MgSO_4$, 1 µL of each of primers, 0.2 µL Platinum *Taq*DNA Polymerase High Fidelity (Invitrogen, USA) and 3 µL DNA. The

PCR cycle was done using Biorad T-100 thermocycler (Bio-Rad, USA) and programs for both primer pairs consisted of an initial denaturation of 94^{o}C for 2 min, followed by 35 cycles of consecutive denaturation at 94^{o}C for 30 sec, annealing at 55^{o}C for 30 sec and extension at 68^{o}C for 1 min. The PCR was completed at a final extension of 68^{o}C for 5 min. Only PCR products that showed clear target bands with minimal additional lower bands were selected for further sequencing.

The selected sets of PCR products were sequenced using the Sanger method at the Australian Genome Research Facility (AGRF, Adelaide, Australia). The sequences were assembled based on the overlapping sequences for each sample compared to a few selected sequences available at NCBI (http://www.ncbi.nlm.nih.gov/).

2.2 Genotyping and Capsid Proteins Analyses

In order to infer the genotype of the Papuan PCV2 isolates, the selected Papuan complete genomes, as well as their ORF2 genes were aligned with 1390 PCV2 sequences obtained from GenBank, using ClustalX 2.1 (Larkin et al., 2007). Phylogenetic trees were subsequently constructed based on the Neighbour Joining method and P-distance model with 1000 bootstraps pseudo replication, in *MEGA* 5 software (Tamura et al., 2011). The phylogenetic tree was generated using FigTree (http://tree.bio.ed.ac.uk/software/figtree/) and genotyping using the full genome sequence was compared with genotyping using ORF2 sequence. For the analysis of the capsid protein, amino acids coded by the ORF2 genes of the Papuan isolates and 1390 PCV2 sequences obtained from GenBank were translated into Bioedit (Hall, 1999) and aligned in ClustalX 2.1 (Larkin et al., 2007). NJ P-distance phylogenetic trees were then constructed to compare the groups of capsid proteins amongst PCV2 strains.

Furthermore, the P-distance matrix of the Papuan isolates and selected reference strains of different genotypes were calculated in MEGA 5 (Tamura et al., 2011). P-distance analyses were performed at the level of the complete genome, ORF2 genes as well as at the predicted amino acid sequences of the capsid proteins. Additionally, a sequence identity matrix (SIM) and classical dendograms of complete genomes was constructed for the Papuan PCV2 isolates and a few selected reference strains of PCV2-IM3, PCV2a, PCV2b, PCV2c and PCV2d genotypes.

3. Results

3.1. Complete Genomes Description and Genotyping

Four out of positive 13 samples that showed clear PCR products on agarose gel were selected for complete genome sequencing. The full genome of the four PCV2 isolates comprised of 1,767 bp length. The nucleotide divergence of the Papuan isolates ranged from 0.002-0.004. The size of the ORF1 sequence was 945 bp, situated at nucleotide position 51-995, encoding a protein of 314 amino acids (aa) size. The size of the ORF2 sequence was 705 bp, at position 1734-1030, encoding a protein of a size of 234 aa.

The complete genomic phylogenetic tree suggested that the Papuan PCV2 isolates are grouped together with strains belong to the PCV2-IM3 genotype. The members of this genotype, apart from the Papuan isolates are Brazilian isolates (KJ094602, KJ094605, KJ094606), the Croatian isolate (HQ591381), the Indian isolate (LC004753) and a Chinese isolate (HM776452) (Figure 1a), with a mean pairwise genetic distance of 0.018 (SE: 0.002, range: 0.001-0.035). Genotyping based on the ORF2 gene however, excluded the Brazilian strains (KJ094602, KJ094605, KJ094606) from the genotype, but included a further 39 Chinese isolates and retained the Croatian (HQ591381), Indian (LC004753) and Chinese (HM776452) isolates in the IM3 genotype. This comparison resulted in a larger sized group that comprised 46 strains (Figure 1b) with a genetic distance of 0.033 (SE: 0.003,

range: 0.001-0.088). The P-distance of the Papuan strains and IM3 genotypes, based on the complete genome and ORF2 genes, was 0.004 and 0.007 respectively, lower than the threshold of 0.02 and 0.035, respectively supporting the position of Papuan PCV2 isolates in the IM3 genotype (Table 1). SIM (Table 2) and classical dendogram (Figure 2) further confirmed that the Papuan isolates belong to the PCV2-IM3 genotype. Additionally, variation within the Papuan isolates occurs in the intergenic region at position 42, in the ORF1 gene at positions 131, 389, 405, 604, 910, and in the ORF2 gene zat positions 1558, 1561, 1591 and 1619.

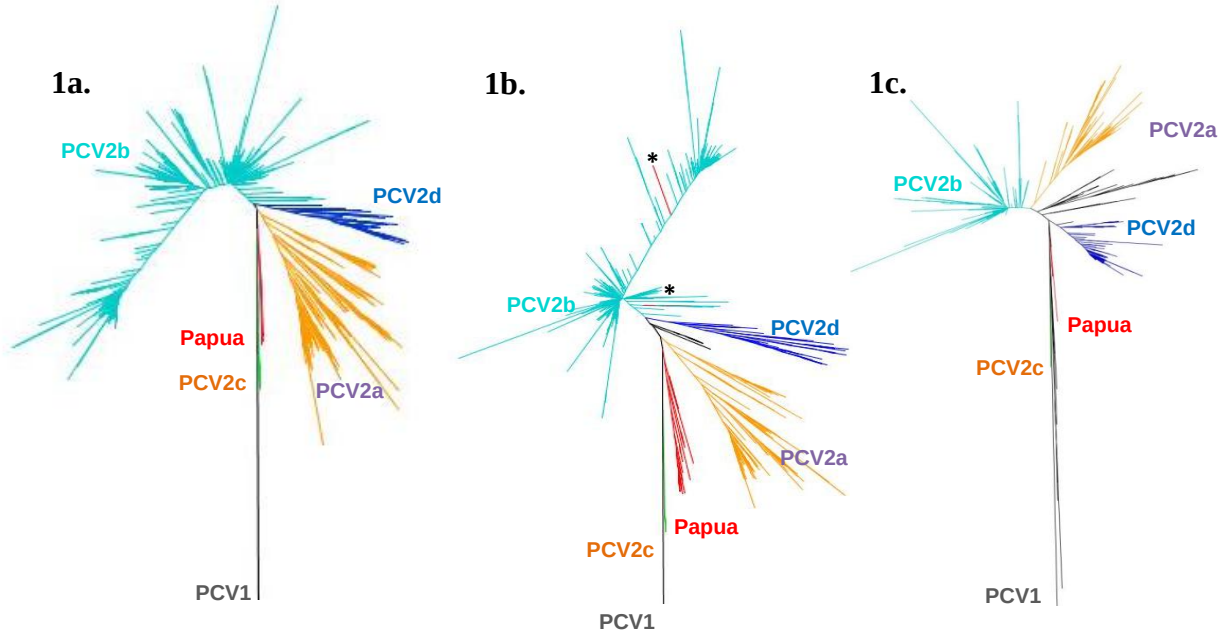

Figure 1: *Evolutionary relationships of the Papuan PCV2 isolates and other genotype strains. 1a. Phylogenetic tree based on complete genome (1682 nt), 1b. Phylogenetic tree based on ORF2 gene (664 nt) and 1c. Phylogenetic tree based on Capsid protein (222 aa). The analysis involved 1,395 nucleotide sequences. PCV1 was included as an outgroup. Evolutionary analyses were conducted in MEGA5. Color explanation: Orange, PCV2a; Light blue, PCV2b; light green, PCV2c; deep blue, PCV2d; red, Papuan and IM3 genotypes; black, PCV1.* (asterix) shows the position of Brazilian strains KJ094602, KJ094605 and KJ094606 which move from the Papuan group in Figure 1a. to PCV2 b genotype group in Figure 1b.*

Table 1: *Genetic divergence of Papuan isolates and different PCV2 genotypes, calculated using Pairwise distance method with 1,000 bootstrap pseudo replication. Analyses involved complete genomes, ORF1 genes, ORF2 genes and amino acid of capsid protein sequences. Papuan isolates show lowest genetic divergence from the PCV2-IM3 reference strains compared to its genetic divergence with other genotypes*

Region of DNA sequence	Within Papuan isolates (SE) n=4	Between Papuan isolates and other genotypes				
		PCV2-IM3	PCV2a	PCV2b	PCV2c	PCV2d
		HM776452	AF055392	AF055394	EF524532	AY181946
Complete genome (1735 nt)	0.003 (0.001)	0.004	0.04	0.036	0.047	0.041
ORF1 gene (933 nt)	0.004 (0.001)	0.002	0.014	0.022	0.02	0.028
ORF2 gene (691 nt)	0.002 (0.001)	0.007	0.081	0.058	0.086	0.063
Capsid protein (231 aa)	0.002 (0.002)	0.01	0.083	0.057	0.096	0.049

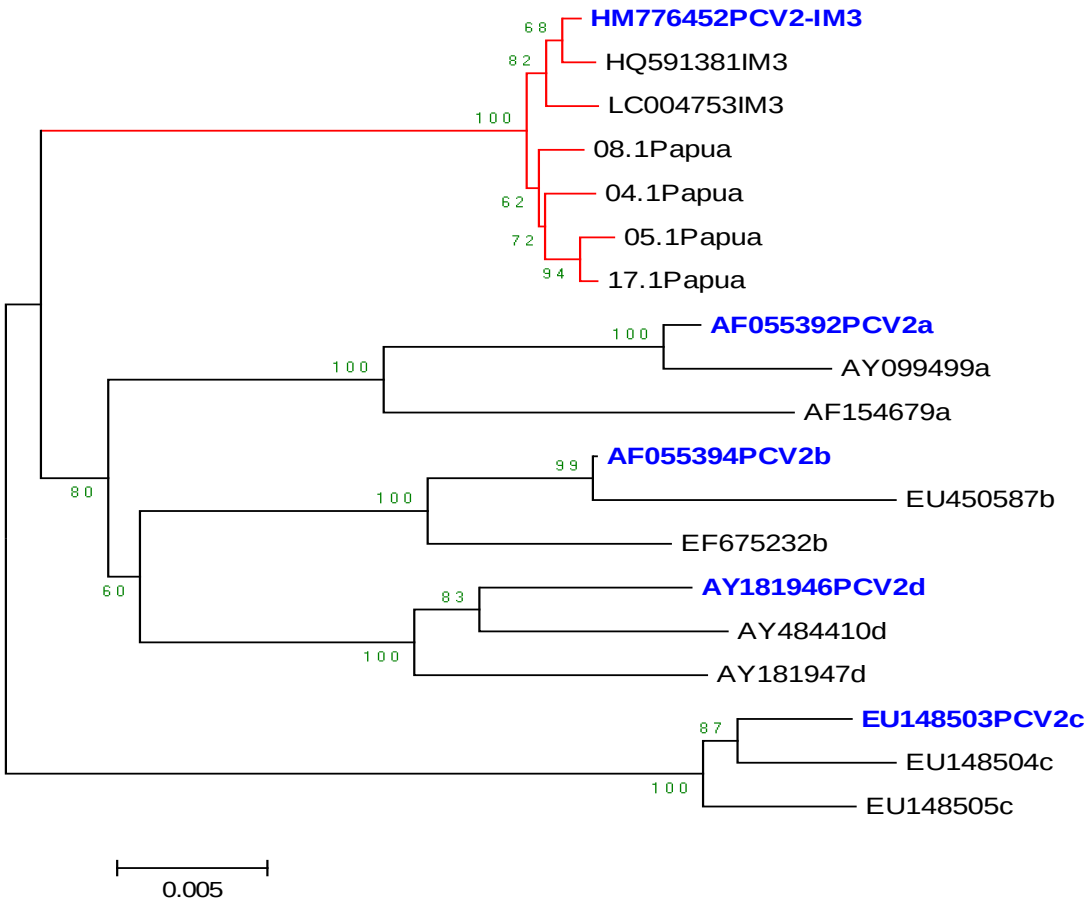

Figure 2: *Dendogram of Papuan PCV2 isolates and selected reference strains from different genotypes. The analysis involved 19 nucleotide sequences. There were a total of 1765 positions in the final dataset. Evolutionary analyses were conducted in MEGA5. Papuan PCV2 isolates are grouped in the clade of PCV2-IM3 genotype*

Table 2: *Sequence identity matrix (SIM) of complete genomes of Papuan PCV2 isolates and selected reference strains from different genotypes. Papuan isolates show high similarity of 99.5-99.6% with PCV2-IM3 genotype reference strain*

PCV2 strains	04.1Papua	05.1Papua	08.1Papua	17.1Papua	PCV2IM3	PCV2a	PCV2b	PCV2c	PCV2d
					HM776452	AF055392	AF055394	EU148503	AY181946
04.1Papua									
05.1Papua	99.6%								
08.1Papua	99.6%	99.6%							
17.1Papua	99.6%	99.8%	99.6%						
HM776452PCV2-IM3	99.6%	99.5%	99.6%	99.6%					
AF055392PCV2a	95.7%	95.7%	95.7%	95.7%	95.9%				
AF055394PCV2b	96.2%	96.2%	96.4%	96.2%	96.3%	96.2%			
EU148503PCV2c	95.0%	94.9%	95.1%	95.0%	95.0%	94.6%	95.3%		
AY181946PCV2d	95.8%	95.8%	95.8%	95.8%	95.9%	95.8%	96.8%	95.1%	
AY193712PCV1	76.6%	76.6%	76.6%	76.5%	76.4%	76.1%	76.5%	76.2%	75.7%

3.2. Predicted Capsid Protein Analyses

The topology of PCV2 phylogeny based on capsid proteins was slightly different from the topology of the PCV2 phylogeny based on the ORF2 gene. In the capsid proteins based phylogeny, PCV2b was located closer to PCV2a, while when analysis was based on the ORF2 gene, PCV2b is the sister clade of PCV2d. The capsid proteins of the Papuan isolates and PCV2-IM3, however remain in consistent topology as immediate descendants of PCV2c (Figure 1c). The amino acid divergence of the capsid protein of Papuan PCV2 isolates was lowest with PCV2d compared to their amino acid divergence with other major PCV2 genotypes. The amino acid sequences of the capsid proteins of the Papuan isolates differed only at position 39 (Arg39Lys).

3.3. GenBank Accession Number

The complete genomes of the Papuan PCV2 isolates used in this study can be retrieved from GenBank with the accession numbers KT369067, KT369068, KT369069 and KT369070.

4. Discussion

PCV2 is an important disease in the pig industry world-wide, causing significant economic loss (López-Soria et al., 2014). In Eastern Indonesia, where traditional pig husbandry practices are predominant, study of the infection with this pathogen is rare. In our current study, we characterized the complete genome of four Papuan PCV2 isolates obtained from village pigs. The Papuan PCV2 isolates in the current study can be grouped with the PCV2-IM3 genotype using NJ phylogeny with P-distance model.

Strains belonging to the PCV2-IM3 genotype have been isolated from pigs across Brazil, China, Croatia and India. In a Brazilian study (Franzo et al., 2015) and our current investigation strains were retrieved from feral pigs. There is no information available as to the host characteristics of the isolates from China and Croatia. PCV2-IM3 genotype might be actually more prevalent in feral pigs rather than in modernly farmed pigs.

The current study demonstrated that there were substantial differences between the results of genotyping based on the ORF2 region and the classification using complete genome sequences. Analysis using the complete genome showed that PCV2-IM3 has lower genetic diversity compared with genotyping using the ORF2 gene. Furthermore, in the current analysis, three Brazilian reference isolates, which belong to the PCV2-IM3 genotype in the complete genome, based analysis grouped with PCV2b when the ORF2 gene was used for genotyping. ORF2 was perceived as the region's representative of the complete genome variation and it has been suggested by others to use either the ORF2 region or full genome sequences in PCV2 genotyping (Segales et al., 2008). We suggest that complete genome sequences should always be used for genotyping of PCV2, as the methods to obtain complete genome of this small virus have largely been available.

A previous study reported that the three Brazilian reference isolates belong to the PCV2d genotype when the analysis involved only a small number of samples consisting of just 36 complete genome sequences (Franzo et al., 2015). Similarly, a study indicated that a phylogenetic analysis using just 48 strains resulted in a topology different from an analysis using a large (n=1, 680) set of PCV2 reference strains, noting the importance of large sample size when determining the classification of PCV2 (Xiao et al., 2015). Not only a different number of samples, but also a different set of sequences within the same number of samples used in the construction of phylogeny tree may produce a different topology. A guideline to genotyping PCV2 using a smaller number of samples may require further study.

Monovalent vaccines based on the capsid protein of either PCV2a or PCV2b genotype have been efficacious to prevent infections with either PCV2a, PCV2b or their mixed infections (Jeong et al., 2015; Opriessnig et al., 2013; Segales 2015). The PCV2d strains on other hand, were isolated from herds that had been vaccinated with PCV2a vaccines (Segales, 2015). Our current study showed that ORF2 of PCV2d was more similar to PCV2b than PCV2a, while ORF2 of PCV2a was highly similar to PCV2b compared to other genotypes. This might explain partly the cross reaction of PCV2a and PCV2b vaccines, and the failure of PCV2a vaccine to protect infection with PCV2d genotype. In the case of Papua PCV2 isolates, the similarity of their ORF2 genes with PCV2b is comparable to with PCV2a. However, the capsid protein of Papuan PCV2 isolates was more similar to PCV2d. The implication of these phenomena on the efficacy of commercial vaccines against Papuan PCV2 isolates requires further study.

5. Conclusion

In conclusion, the present study showed that Papuan PCV2 isolates belong to the PCV2-IM3 genotype. Distribution of this genotype currently encompasses China, Croatia, India and Indonesia. Genotyping using complete genome, ORF2 region or capsid protein sequences resulted in substantial difference in PCV2 strains classification. The clinical implication of these different genotyping methods requires further investigation.

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Conflicts of Interest

All authors declare no conflict of interest.

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