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Retrospective Study of Porcine Circovirus Type 2a and 2b Between 1999 and 2016 in Taiwan

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Abstract: Porcine circovirus type 2 (PCV2) is one of the major swine viral diseases and caused significant economic loss to pig producers worldwide, including Taiwan. PCV2 has been considered as the causative agent of postweaning multisystemic wasting syndrome (PMWS) as well as other clinical diseases. All these associated syndromes have been categorized as PCV2 associated diseases (PCVAD). The purpose of this study was to investigate the positive rate and genetic shift of two distinct genotypes of PCV2, which include PCV2a and PCV2b, in Taiwanese pig farms. A total of 1094 specimens originating from pigs between years 1999 and 2016 were analysed. The PCV2a and PCV2b sequences were amplified and distinguished using 1oop-mediated isothermal amplification (LAMP). Results showed that 24.8% (272/1094) pigs were PCV2a positive, 67.3% (737/1094) were PCV2b positive. These results also indicated that PCV2a was the predominant virus between 1999 and 2001, and that PCV2b became the most prevalent virus since 2003.

Keywords: Genetic Shift, LAMP, Porcine Circovirus Type 2, Predominant

1. Introduction

Porcine circovirus type 2 (PCV2) is a member of the genus *Circovirus* within the family *Circoviridae* [1, 2]. PCV2 have non-enveloped icosahedral virion with a size of 17 nm and containing single–stranded circular deoxyribonucleic acid (DNA) genome of 1,767–1,768 nucleotides (nt) [1, 2]. PCV2 have three major open reading frames (ORF), ORF1-3 [1, 2, 3]. The ORF1 encodes two major replication associated proteins: the complete Rep, and the spliced form Rep, which has a frame shift Rep′ [1]. ORF2 encodes the capsid protein (Cap), which contains the immunodominant antigenic epitopes, and has been used as a target for vaccine development [2, 4]. Interestingly, some variable amino acid residues located in the putative epitopes of ORF2 are exposed outside the virion, and these residues are suggested to be constantly under positive or negative selection forces due to constant exposure to high immunologic pressure [2, 4]. The ORF3 encodes a protein that has been implicated with cellular apoptosis in the porcine kidney PK-15 cell line, as well as in peripheral blood mononuclear cells [3].

PCV2 is a widespread swine viral pathogen that has caused economic loss to pig farms worldwide [5, 6, 7]. It has been considered to be the major aetiological agent of postweaning multisystemic wasting syndrome (PMWS), as well as several clinical diseases, including porcine respiratory disease complex (PRDC), porcine dermatitis and nephropathy syndrome (PDNS), and congenital tremors (CT) [5, 6, 7]. Collectively, these PCV2-associated diseases have been termed porcine circovirus disease (PCVD) or porcine circovirus associated disease (PCVAD) [7]. In addition, previous studies have suggested that PCV2 infections may occur in the absence of the typical clinical signs of PMWS and PCVAD [7]. PCV2 infection could be limited to a few lymph nodes in infected pigs, and not display clinical disease [7]. Previous studies have confirmed the combined infection of PCV2 with other viral

pathogens, including porcine parvovirus (PPV), porcine reproductive and respiratory syndrome virus (PRRSV), pseudorabies virus (PrV), classical swine fever virus (CSFV), porcine enterovirus type 8 (PEV8), porcine teschovirus (PTV), torque teno sus virus (TTsuV) and other viral pathogens [6, 8, 9]. Co-infection of PCV2 with other viral pathogens increases the severity of PMWS and PCVAD [6, 10].

Previous studies indicated that PCV2 has diverged into two separate genotypes, which include PCV2a and PCV2b, based on phylogenetic studies of the complete genome and the ORF2 region [4]. Prior to 2003, both PCV2a and PCV2b were prevalent in European and Asian countries, whereas PCV2a was the only prevalent strain in North American [11]. Since 2003, the global genetic shift from PCV2a to PCV2b has been discovered in swine populations, with increased severity of PCVAD [4, 5, 11, 12]. However, the detailed mechanism of this sudden shift from PCV2a to PCV2b predominant prevalence remains unclear [13]. Under experimental conditions, there may be no difference in the pathogenesis between PCV2a and PCV2b, but PCV2a contains 1,768 nt and PCV2b contains 1,767 nt [4, 5, 11, 14]. Co-infection of PCV2a and PCV2b in clinical samples obtained from pigs affected by PMWS and other PCVAD has been suggested [15].

Recently, there are several excellent methods that can be used to identify the PCV2a and PCV2b; these include polymerase chain reaction (PCR) assay, nested PCR, real-time PCR and restriction fragment length polymorphism (RFLP) [5, 14, 15, 16]. These methods are convenient and allow the testing of large number of clinical samples. However, these methods require relatively well-equipped laboratories with a well-trained staff and involve multiple steps to amplify the nucleic acid, and the sensitivity of the method can be affected by limited template nucleic acid. For this reason, the authors have developed a loop-mediated isothermal amplification (LAMP) method that is based on the ORF2 of PCV2, which allows for the simultaneous detection and classification of PCV2a and PCV2b in clinical samples [17]. Importantly, LAMP is a simple, rapid and cost-effective tool that can provide differential diagnosis of PCV2a and PCV2b [17, 18, 19, 20]. Consequently, investigations on the different genotypes of PCV2 are particularly important for Taiwan. The aim of this study was to use LAMP to investigate the genotypes of PCV2a and PCV2b in Taiwan between 1999 and 2016.

2. Materials and Methods

2.1. Samples and DNA Extraction

In this study, various tissue samples (including tonsil, lymph nodes, spleen, liver, lung and brain) were collected at the Division of Hog Cholera, Animal Health Research Institute, Council of Agriculture, Executive Yuan, from 1094 pigs at 4 to 16 weeks of age with PMWS or non-PMWS from 184 pig farms between 1999 and 2016. The PMWS and non-PMWS were identified using a set of PMWS diagnostic criteria [7]. All specimens were prepared as a 10% (w/v) emulsion by homogenizing tissue samples in Eagle's minimum essential medium (EMEM) and then froze at -80°C until used. Viral DNA was extracted directly from 200 µl of the 10% (w/v) emulsion of tissue samples using the QIAamp DNA Extraction Kit (Qiagen, USA), according to the manufacturer's instructions. For evaluating the analytical specificity of LAMP, 36 virus isolates were used, comprising eight PCV2a, 11 PCV2b, four PCV type 1 (PCV1), two PPV, three PrV and eight PRRSV, and all of the isolates were identified by determining their partial nucleotide sequences as previously studies [9, 21]. The DNA of PCV2a, PCV2b, PCV1, PPV, and PRV and the complementary DNA (cDNA) of PRRSV isolates were produced as previously studies [9, 21], and stored at –20°C until use or used immediately for this study.

2.2. LAMP Reaction

Specific regions within the ORF2 of PCV2a and PCV2b were amplified and distinguished with LAMP by using specific oligonucleotide primers (Table 1) as previously described [17]. A 25 µl reaction mixture consisted of 12.5 µl of 2 × LAMP reaction buffer, 1 µl of Bst DNA polymerase (Eiken, Japan), 2 µl of DNA extracted from lymph node samples, 40 pmol each of FIP and BIP primers, and 5 pmol each of the F3 and B3 primers for PCV2a and PCV2b. LAMP was performed by incubating the reaction mixture at 65°C for 50 min and then inactivating the Bst DNA polymerase by heating the mixture to 80°C for 2 min. The reaction was monitored in real time at 6 sec intervals by measuring the turbidity at A_{650} using a LA-320 real-time turbidimeter (Eiken, Japan). The results were determined within 50 min, and turbidity at A_{650} above 0.1 was interpreted as positive using the LA-320 software package (Eiken, Japan). LAMP products were also analysed by electrophoresis through a 2% agarose gel containing 0.5 mg/mL SYBR Safe DNA gel stain (Invitrogen, USA) in Tris-acetate-EDTA (ethylenediamine tetra-acetic acid) solution.

Table 1. *Oligonucleotide primer sets used for the LAMP of PCV2a and PCV2b.*

Primer	Position	Type	Sequence (5'-3')
PCV2a-F3	1379-1396	Forward outer	GCC CAC TCC CCT ATC ACC
PCV2a-B3	1589-1572	Backward outer	CTC CCG CAC CTT CGG ATA
PCV2a-FIP	1474-1451 1408-1423	Forward inner F1c-TTTT-F2	AAA ATC TCT ATA CCC TTT GAA TAC-TTTT[a]-GGG AGC AGG GCC AGA A
PCV2a-BIP	1487-1506 1561-1543	Backward inner B1c-TTTT-B2	TCC CGG GGG AAC AAA GTC GT -TTTT[a]-CGT ACC ACA GTC ACA ACG C
PCV2b-F3	1378-1393	Forward outer	GCC CAC TCC CCT GTC A
PCV2b-B3	1606-1588	Backward outer	CAT CTT CAA CAC CCG CCT C

Primer	Position	Type	Sequence (5'-3')
PCV2b-FIP	1474-1457 1410-1429	Forward inner F1c-TTTT-F2	CCC CG/TC TCT GTG CCC TTT- TTTT[a]-AGC AGG GCC AGA ATT CAA CC
PCV2b-BIP	1516-1537 1587-1572	Backward inner B1c-TTTT-B2	TCT CAT CAT GTC CAC CGC CCA G-TTTT[a]-TCC CGC ACC TTC GGA T

[a]The inner primers FIP and BIP contained two binding regions connected by the TTTT bridge.

2.3. Statistical Analysis

Duncan's new multiple range test was applied to compare the differences in the means amongst the different genotypes. Comparisons of the positive rates in different genotypes of PCV2 were made using Chi-square analysis. A P value less than 0.05 was considered to indicate statistical significance.

3. Results

The specificity of LAMP was evaluated using DNA extracted from PCV2a, PCV2b, PCV1, PPV, and PRV and cDNA from PRRSV. The PCV2a (Figure 1A) and PCV2b (Figure 1B) LAMP procedures successfully detected specific PCV2a (Figure 1A, lane 1) and PCV2b (Figure 1B, lane 2) target DNA without cross-reaction and generated ladder-like product, respectively.

Among the 1094 pigs, 88.1% (964 (227+692+45) /1094) were PCV2-positive, 24.8% (272 (227+45)/1094) were PCV2a positive, 67.3% (737 (692+45)/1094) were PCV2b positive, and 4.1% (45/1094) were positive for both PCV2a and PCV2b (Table 2). The positive rate of PCV2b infection was significantly higher than that of PCV2a infection (P < 0.05) in Taiwan. Notably, no PCV2a-positive sample was found in 2006, 2015 and 2016 (Table 2). However, the positive rate of PCV2a infection (76.4%, 149 (31+56+62)/195 (43+66+86)) was significantly higher than that of PCV2b (5.6%, 11/195 (43+66+86)) (P < 0.05) between 1999 and 2001 (Table 2). Interestingly, sometime shortly after 2001 a shift occurred, and in 2003 the positive rate of PCV2b infection (77.2%, 61 (58+3)/79) became significantly higher than that of PCV2a (17.7%, 14 (11+3)/79) (P < 0.05) (Table 2). This trend continued for the remainder of the period we surveyed, with the positive rate of PCV2b infection being significantly higher than that of PCV2a infection (P < 0.05) between 2004 and 2016 (Table 2).

Table 2. *Detection of PCV2 in lymph node tissue samples between 1999 and 2016.*

Year	No. of samples	PCV2a only	PCV2b only	PCV2a and 2b[a]	Negative
1999	43	31	0	0	12
2000	66	56	0	0	10
2001	86	62	11	0	13
2002	81	32	28	17	4
2003	79	11	58	3	7
2004	75	4	63	1	7
2005	57	5	50	0	2
2006	43	0	41	1	1
2007	46	1	35	2	8
2008	68	7	55	3	3
2009	74	6	59	4	5
2010	61	7	47	5	2
2011	63	3	54	3	3
2012	78	2	72	2	2
2013	46	0	37	1	8
2014	52	0	33	3	16
2015	37	0	28	0	9
2016	39	0	21	0	18
Total	1094	227	692	45	130

[a]Positive for both PCV2a and PCV2b in this study.

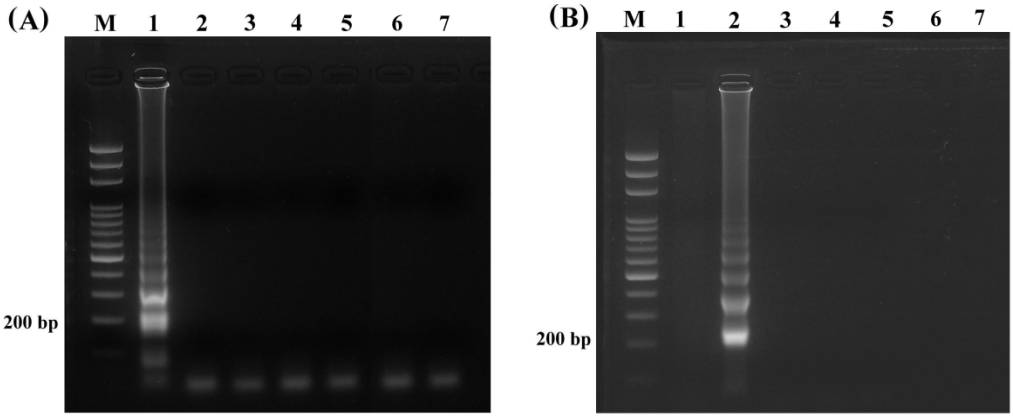

Figure 1. *Specificity of the PCV2a (A) and PCV2b (B) LAMP were observed using agarose gel electrophoresis. Lane M, 3000-100 bp ladder marker (50 U/μL; Portec, Taiwan); lanes 1 to 6 represent PCV2a, PCV2b, PCV1, PPV, PRV and PRRSV, respectively; lane 7, negative control.*

4. Discussion

This study indicated that the PCV2a genotype was the predominate form expressed in the analysed population between 1999 and 2001 in Taiwan (Table 2). PCV2b genotype was firstly founded in 2001, and since 2003 the PCV2b genotype has been predominant in the analysed population (Table 2). Therefore, these results indicate that a shift occurred

in the virus genotypes from the PCV2a to the PCV2b in Taiwanese pig farms, and PCV2b has now become the predominant PCV2 genotype. However, the origins of the different genotypes or clusters of PCV2 isolates in Taiwan were not clearly identified in the present study, because no Taiwanese PCV2 samples were collected before 1999. Several reports have observed the same situation in endemic areas where the PCV2a persisted for early 2000s, and was replaced by the PCV2b genotype [4, 5, 11, 14, 22]. PCV2 currently circulating in the field might have undergone constant genetic changes over the years. This phenomenon remains unclear what caused the shift between the different genotypes. The possible explanations include: first, PCV2b may possess higher replication rate than the PCV2a in pigs; second, PCV2b may contain higher affinity to compete the cellular receptors. These preliminary findings have proven useful in directing retrospective studies on PCV2 in Taiwan. Similarly, future studies are needed to investigate the presence and role of PCV2b in Taiwanese pig population.

In addition, this study also indicated that 4.7% (45/946) of the pigs were infected with both PCV2a and PCV2b (Table 2). Previous study has indicated that simultaneous heterologous inoculation of PCV2a and PCV2b (PCV2a/2b) in germ-free pigs induces more severe illness and wasting behaviour typical of PMWS than similar titters of homologous inoculation with PCV2a or PCV2b alone [23]. However, the homologous inoculation with PCV2a (PCV2a/2a) or PCV2b (PCV2b/2b) resulted in significantly less severe clinical disease, with lower gross and histological lesions [23]. Consequently, it will be important for future studies to identify the different genetic and infectious factors associated with the pathogenesis of dual infection of PCV2a and PCV2b in clinical cases.

The reasons for the switch of different virus populations of PCV2 in the field are not clear. However, the recent virus populations have become more variable and some specific positions in the putative epitopes of ORF2 vary among different groups, which may represent viral survival strategy in the host for better adaptive abilities to the selection forces [24]. Diversity in the putative epitopes of residue variation may facilitate recent virus population a better recognization of host cell receptor binding domains, or a better attachment to and entry into host cell [24]. Mutations in ORF2 of PCV2 may also alter viral antigenicity which relates to ‘immune escape’, and may contribute to the switch of virus populations [24]. Therefore, genetic comparison of the ORF2 sequences of different groups may give insight into the diversity of the ORF2 which are closely associated with the potential of immune escape, vaccines development and disease control [2, 24].

Recently, the commercial PCV2 vaccines based on PCV2a genotype have been used to protect pigs since the 2006 [25]. These commercial PCV2 vaccines were immediately successful in preventing the severe mortality associated with PMWS and PCVAD [25]. However, some previous studies indicates that vaccines based on the PCV2a genotype do not protect pigs perfectly against possible infection from the PCV2b [22, 25]. Whether the PCV2b contain higher ability to escape from antibody neutralization than the PCV2a will require additional investigation [22]. PCV2b isolates have become more variable and may reflect their better adaptive abilities to the selection forces than PCV2a isolates, which contains a greater number of residue variations in the putative epitopes of the ORF2 [22, 25]. As a result of the mutations in ORF2 of PCV2b, the virus may possess altered viral antigenicity, and may have increased adaptive abilities to the selection forces for immune escape with PCVAD in cases of suspected vaccine failures [22, 25]. Thus, in order to improve vaccine efficacy, novel vaccine development should take these factors into consideration.

5. Conclusion

By analyzing the specimens from 1094 pigs in Taiwan during 1999 to 2016, PCV2a genotype was the predominate population between 1999 and 2001, and then PCV2b isolates instead of PCV2a became predominate population begin in 2003. These results reveal that there has been a switch in the virus populations from the PCV2a to the PCV2b circulating in Taiwanese pig farms. PCV2 currently circulating in the field might have undergone constant genetic changes over the years. These results also agreed with the results reported by Olvera et al [4]. It is important to collect Taiwanese PCV2 isolates continuously to further study the epidemiological changes in the future.

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