FULL PAPER *Virology*

Genotypic Change and Phylogenetic Analysis of Porcine Circovirus Type 2 in Taiwanese Pig Herds

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(Received 2 February 2012/Accepted 18 May 2012/Published online in J-STAGE 1 June 2012)

ABSTRACT. Porcine circovirus type 2 (PCV2) is the primary causative agent of postweaning multisystemic wasting syndrome. Two major PCV2 genotypes, PCV2a and PCV2b, have been identified. To explore the prevalence of different subgroups of PCV2 in Taiwan, 37 PCV2 isolates collected during 2002–2011 were analyzed. The genotypes of the PCV2 isolates collected before 2007 belonged to either PCV2a or PCV2b. However, all of the isolates collected after 2008 were PCV2b. Most of the isolates obtained since 2008 have been classified into a novel genotype within a subgroup of PCV2b based on complete ORF2 sequence analysis. Moreover, analysis of the PCV2 isolates from the same pig farm but from different years revealed that the viruses shifted from a PCV2b genotype to a novel subgroup of the PCV2b genotype. Collectively, PCV2b was the dominant PCV2 genotype in Taiwan currently, and the viruses have shifted into a new emerging subgroup of the PCV2b genotype.

KEY WORDS: genotype, open reading frame 2, porcine circovirus type 2, sequence analysis.

doi: 10.1292/jvms.12-0044; *J. Vet. Med. Sci.* 74(10): 1303–1310, 2012

Porcine circovirus type 2 (PCV2) is the primary causative agent of porcine circovirus-associated diseases (PCVADs) in pigs and causes significant economic losses in most pig-producing countries [27]. PCVADs can be subclinical or can include 1 or more clinical manifestations such as multisystemic disease with weight loss, respiratory signs, enteric signs, porcine dermatitis and nephropathy syndrome, and reproductive problems individually or in combination in pigs raised on farms or in other groups of pigs [27]. PCVADs are multifactorial syndromes, and factors that are currently thought to influence the outcome of PCV2 infection include the viral strain, the host, coinfections, and immune modulation [27].

PCV2 is a small, non-enveloped, circular, single-stranded DNA virus containing a circular genome of 1766–1768 nucleotides [17, 34]. PCV2 is a member of the genus *Circovirus* of the family *Circoviridae* [27]. Another member of this genus includes PCV1, which does not produce disease in pigs [35]. The PCV2 genome contains three open reading frames (ORFs). ORF1 encodes two replicase proteins, Rep and Rep' [4]. The viral capsid protein is encoded by ORF2 [25], which is involved in the host immune response [24]. The third ORF, ORF3, is in a different reading frame embedded within ORF1 and encodes a protein associated with apoptosis [22].

PCV2 viruses are further classified into three genotypes (2a, 2b, and 2c) that differ with respect to the ORF2 sequence [9, 26]. The signature motif (amino acid positions 86–89) of ORF2 can be used to distinguish PCV2a and 2b, which contain the TNKI and SNPR (L) motifs, respectively [6]. The PCV2c genotype has only been found in Denmark to date [31]. In recent years, several reports have suggested that PCV2b is the predominant genotype in many countries worldwide, including Brazil [8, 11], Canada [14, 15], China [17, 21, 38], Cuba [29], Denmark [12], Germany [30], Japan [32], Korea [1, 2, 19, 20], Slovakia [37], Spain [10], Sweden [33], Switzerland [39], Thailand [18] and the U.S.A. [6, 23]. PCV2b might be more pathogenic than PCV2a [16, 32]. In Taiwan, the first PCV2 outbreak was confirmed in 2001 [3], and a high prevalence of PCV2 infection was noted thereafter. Up to 92% of pig herds and 68.8% of weaned pigs were infected by PCV2 in Taiwan [7]; however, the genotype prevalence of PCV2 in Taiwan is not currently known. The aim of the present study was to investigate the prevalence of the PCV2 genotype in Taiwan in the last decade.

MATERIALS AND METHODS

Specimen collection and histopathological examination: Clinical samples (lungs, lymph nodes, spleen, brain and serum) were collected from 785 pigs from 186 herds in central and southern Taiwan during 2002–2011. These samples were mainly acquired from weaning and growing pigs with

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Table 1. The genotypes of 37 porcine circovirus type 2 isolates collected from Taiwanese pig herds during the last decade

Isolation time	Name[a)]	Age[b)]	Genotype	ORF2 length (nt)	Accession number	Diagnosis[c)]
2002/Feb.	A/2002	10 wk	2a	702	JF683387	BP
2002/Aug.	B/2002	10 wk	2b	702	JF683390	AP, Sal, Poly
2002/Jul.	P/2002	6 wk	2b	702	JF927976	PRRS
2002/Feb.	Q/2002	9 wk	2b	702	JF927977	BP, Sal, Myco
2002/Mar.	R/2002	13 wk	2a	702	JF927978	AP, BP
2002/Apr.	S/2002	9 wk	2a	702	JF927979	Sal, Myco
2003/Feb.	A/2003	8 wk	2a	702	JF683388	BP, PRRS, Poly
2003/Apr.	C/2003	8 wk	2a	702	JF683391	BP, Poly, Myco
2003/Mar.	T/2003	12 wk	2b	702	JF927980	AP, BP
2003/Apr.	U/2003	13 wk	2b	702	JF927981	Myco
2003/Sep.	V/2003	8 wk	2b	702	JF927982	Sal, BP
2005/Nov.	D/2005	10 wk	2b	702	JF683396	Poly
2006/Dec.	E/2006	8 wk	2b	702	JF683400	BP, AP
2007/Mar.	D/2007-2	8 wk	2a	702	JF683398	Sal, BP
2007/Jul.	D/2007-1	8 wk	2b	702	JF683397	BP, Poly
2007/Oct.	F/2007	10 wk	2a	702	JF683403	AP, Sal
2007/Dec.	H/2007	6 wk	2b	702	JF927983	Sal
2008/Dec.	E/2008	1 wk	2b[d)]	705	JF683401	Enteritis
2009/Jun.	F/2009	10 wk	2b[d)]	705	JF683404	BP
2009/Aug.	G/2009-1	6 wk	2b	702	JF683407	BP
2009/Sep.	G/2009-2	8 wk	2b[d)]	705	JF683408	BP, Poly
2010/Dec.	C/2010-1	sow	2b[d)]	705	JF683392	RF
2010/Dec.	C/2010-2	1 d	2b/2b[d, e)]	702/705	JF683393/JF683394	Abortion
2010/Dec.	C/2010-3	9 wk	2b	702	JF683395	Poly, Myco
2010/Jan.	D/2010	11 wk	2b[d)]	705	JF683399	Sal
2010/Apr.	E/2010	6 wk	2b[d)]	705	JF683402	BP
2010/Jul.	F/2010-2	12 wk	2b	702	JF683406	BP, Poly
2010/Sep.	F/2010-1	12 wk	2b[d)]	705	JF683405	BP, Poly
2010/Apr.	I/2010	6 wk	2b[d)]	705	JF927984	BP
2010/Apr.	J/2010	7 wk	2b[d)]	705	JF927985	BP, Sal
2010/Apr.	K/2010	8 wk	2b[d)]	705	JF927986	BP, Sal
2010/Apr.	L/2010	12 wk	2b	702	JF927987	BP
2010/Apr.	M/2010	8 wk	2b[d)]	705	JF927988	BP
2010/Apr.	N/2010	8 wk	2b[d)]	705	JF927989	Sal
2010/Jul.	O/2010	13 wk	2b	702	JF927990	BP
2011/Mar.	A/2011	8 wk	2b[d]	705	JF683389	BP, Poly

a) The letters in each name indicate the herd's name. The number indicates the year of PCV2 isolation. b) Animal age at the time of sample collection. wk: weeks. d: days. c) Diagnosis was based on histopathological diagnosis, bacterial and viral isolation and PCR detection. All of the cases were PCV2 infections. AP: *Actinobacillus pleuropneumoniae* infection. BP: bronchopneumonia. PRRS: porcine reproductive and respiratory syndrome. Poly: polyserositis. Sal: Salmonellosis. Myco: Mycoplasmal pneumonia. RF: reproductive failure. d) These PCV2 isolates were classified as a novel genotype belonging to PCV2b. e) Two PCV2 isolates were collected from this pig simultaneously.

clinical signs of growth retardation, dyspnea or jaundice. Some samples were obtained from sows and neonatal piglets with reproductive disorders. Tissues were fixed in 10% non-buffered formalin for 24 hr and routinely embedded in paraffin. Sections (4–5 μm) were cut and stained with hematoxylin-eosin for histopathological examination. The year of sampling, age, clinical history, genotype of PCV2, and final diagnosis are summarized in Table 1.

Sample preparation and PCV2 screening and amplification: All viral DNA was extracted from clinical samples (either serum or homogenized tissues) using a Genomic DNA Mini Kit (Geneaid Biotech, Ltd., Taipei, Taiwan) according to the manufacturer's protocol. All of clinical specimens were screened for PCV2 by polymerase chain reaction as described by Takahagi *et al.* [32].

Sequencing and sequence analysis: The DNA fragments were purified (Geneaid Biotech, Ltd., Taipei, Taiwan), and the target nucleotide sequences were determined from both orientations using an auto sequencer (ABI 3730XL, Foster City, CA, U.S.A.). The DNA sequences of ORF2 were then compared with those of reference PCV2a (AF055392), PCV2b-1A (AF055394, DQ141322), PCV2b-1B (AY682995), PCV2b-1C (AY713470, DQ151643), PCV2d (HM038017) [17], PCV2c (EU148503), and PCV1 (AY184287). Multiple alignments of the nucleic acid and amino acid sequences were performed by the ClustalW

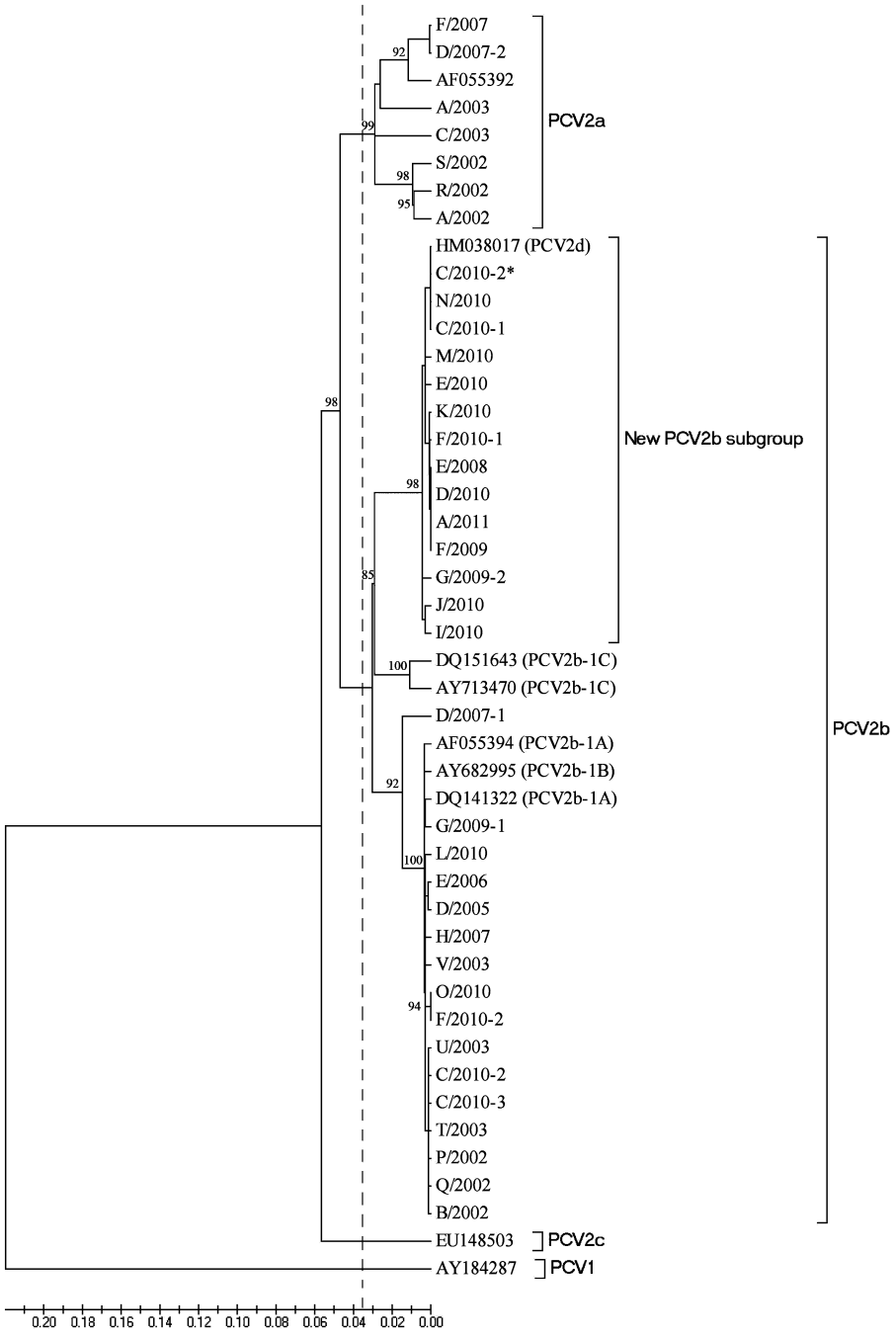

Fig. 1. Phylogenetic relationship determined by the ORF2 sequences of the local PCV2 and reference PCV1 and PCV2 strains. The phylogenetic trees were generated using MEGA 4. The number of branches was calculated using bootstrapped values from 1,000 replicates. Bootstrap support values >85 are shown. Letter/number represents the pig farm/year of sampling. The dashed line indicates the *p*-distance threshold of 0.035 proposed for the genotype definition in PCV2. *Two PCV2 isolates were collected from the same pig simultaneously.

method using the MegAlign program (DNASTAR, Madison, WI, U.S.A.). The phylogenetic relationships were generated by the neighbor-joining method based on the *p*-distance using MEGA 4.

RESULTS

Thirty-seven PCV2 isolates from 36 pigs from 22 herds were selected in this study based on the farm geographic

location and longitudinal study. The genotypes of the ORF2 nucleotide sequences of the 37 isolates were designated according the genotype definition and nomenclature proposed by the EU consortium on PCVAD [9, 31]. All of the PCV2 isolates were clearly separated into two genotypes with high bootstrap values. According to phylogenetic relationship, the new subgroup PCV2b viruses were clustered together without PCV2b-1A, 1B and 1C (Fig. 1). Among 15 pig farms, 6 (40%) and 9 (60%) pig farms were found to harbor PCV2a and PCV2b, respectively, during the years 2002–2007 (Fig. 2). PCV2a viruses disappeared, whereas all of the pig farms harbored the PCV2b genotype beginning in 2008. Surprisingly, a new genotype subgroup within PCV2b was found in most pigs and pig farms, and this was supported by high bootstrap values (Figs. 1 and 2). Most isolates were found in nursery pigs, whereas two novel PCV2b viruses were found in a gilt with reproductive failure and a weak newborn piglet (Table 1). Moreover, analysis of the PCV2 isolates from the same pig farm but from different years revealed that all isolates shifted from the PCV2a or 2b genotype to a new subgroup of the PCV2b genotype. All of the novel PCV2b isolates were found late in the year (Table 1). We also found more than 2 genotypes coexisting at pig farm C, even in the same pig (Table 1). Taken together, our results show that PCV2b was the dominant PCV2 genotype in recent years in Taiwan and that the viruses shifted into a new emerging subgroup within the PCV2b genotype.

The complete ORF2 sequences were 702 or 705 base pairs (bp) in length. The strains were categorized into three types according to the length of the ORF2 genomes: PCV2a had a genome of 702 bp in length, PCV2b had a genome of 702 bp in length, and the novel PCV2b had a genome of 705 bp in length (Table 1). The nucleotide sequences were further analyzed using the DNASTAR software, revealing that the novel PCV2b genotype had a single base mutation at position 700, resulting in an ORF2 of 705 bp in length (data not shown). Comparisons of the complete ORF2 nucleotide sequence revealed 92.0–99.9 and 90.0–94.4% homology within the analyzed local PCV2a isolates and between local PCV2a and 2b isolates, respectively (Table 2). In comparison to the low nucleotide sequence similarity between PCV2b and novel PCV2b (92.4–94.7%), the homology among all analyzed novel PCV2b isolates (97.7–100%) appeared to be much higher (Table 2).

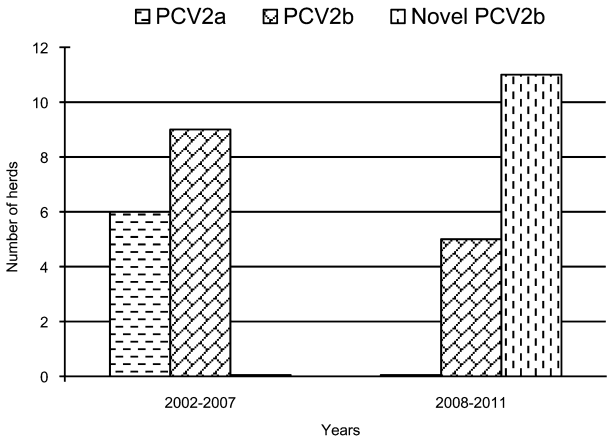

Fig. 2. The number of herds with detected differences in the PCV2 genotypes at two time points.

Amino acid comparisons among the 37 isolates and the 7 reference strains revealed that three major regions with greater diversity were found at amino acid positions 53–91, 121–151 and 190–215 (Fig. 3). Seven amino acid substitutions were observed in PCV2b isolates [V80L, T86S, K88P, I89R(L/H), I(L)91V, S190A(T) and K(H)232N]. Seven unique amino acid substitutions were also found in the novel PCV2b (53I, 59K, 68N, 90T, 134N, 215I and 234K). In addition, novel PCV2b shared ten amino acid substitutions (77N, 80L, 86S, 88P, 89L, 91V, 151T, 191G, 206I and 232N) with PCV2b (Fig. 3 and Table 3). We also examined the 4 amino acid sites (positions 86–89) in the ORF2 protein identified by Cheung *et al.* [6]. These 4 amino acids were proposed to distinguish between PCV2a and PCV2b. Our alignment results revealed that the PCV2a and 2b isolates contained the TNKI and SNPR(L/H) motifs, respectively (Fig. 3 and Table 3). Interestingly, all novel PCV2b genotype isolates had the signature motif of PCV2b. Taking the phylogenetic analyses

Table 2. Sequence homology among ORF2 of local PCV2 viruses and reference PCV2 viruses

Strains	Strains				
	AF055392 (PCV2a)	Taiwanese PCV2a[a)]	AF055394 (PCV2b)	Taiwanese PCV2b[b)]	Taiwanese PCV2b[c, d)]
AF055392 (PCV2a)	100.0	93.3–97.7	93.3	92.6–94.2	89.9–91.0
Taiwanese PCV2a		92.0–99.9	90.7–94.0	90.0–94.4	88.3–92.3
AF055394 (PCV2b)			100	97.6–99.9	93.6–95.0
Taiwanese PCV2b				96.9–100.0	92.7–94.7
Taiwanese PCV2b[d)]					97.7–100.0

a) Accession numbers: JF683387, JF 683388, JF683391, JF683398, JF683403, JF927978, JF927979. b) Accession numbers: JF683390, JF683393, JF683395, JF683396, JF683397, JF683400, JF683406, JF683407, JF927976, JF927977, JF927980, JF927981, JF927982, JF927983, JF927987, JF927990. c) Accession numbers: JF683389, JF683392, JF683394, JF683399, JF683401, JF683402, JF683404, JF683405, JF683408, JF927984, JF927985, JF927986, JF927988, JF927989. d) This PCV2 isolate was classified as a novel genotype belonging to PCV2b.

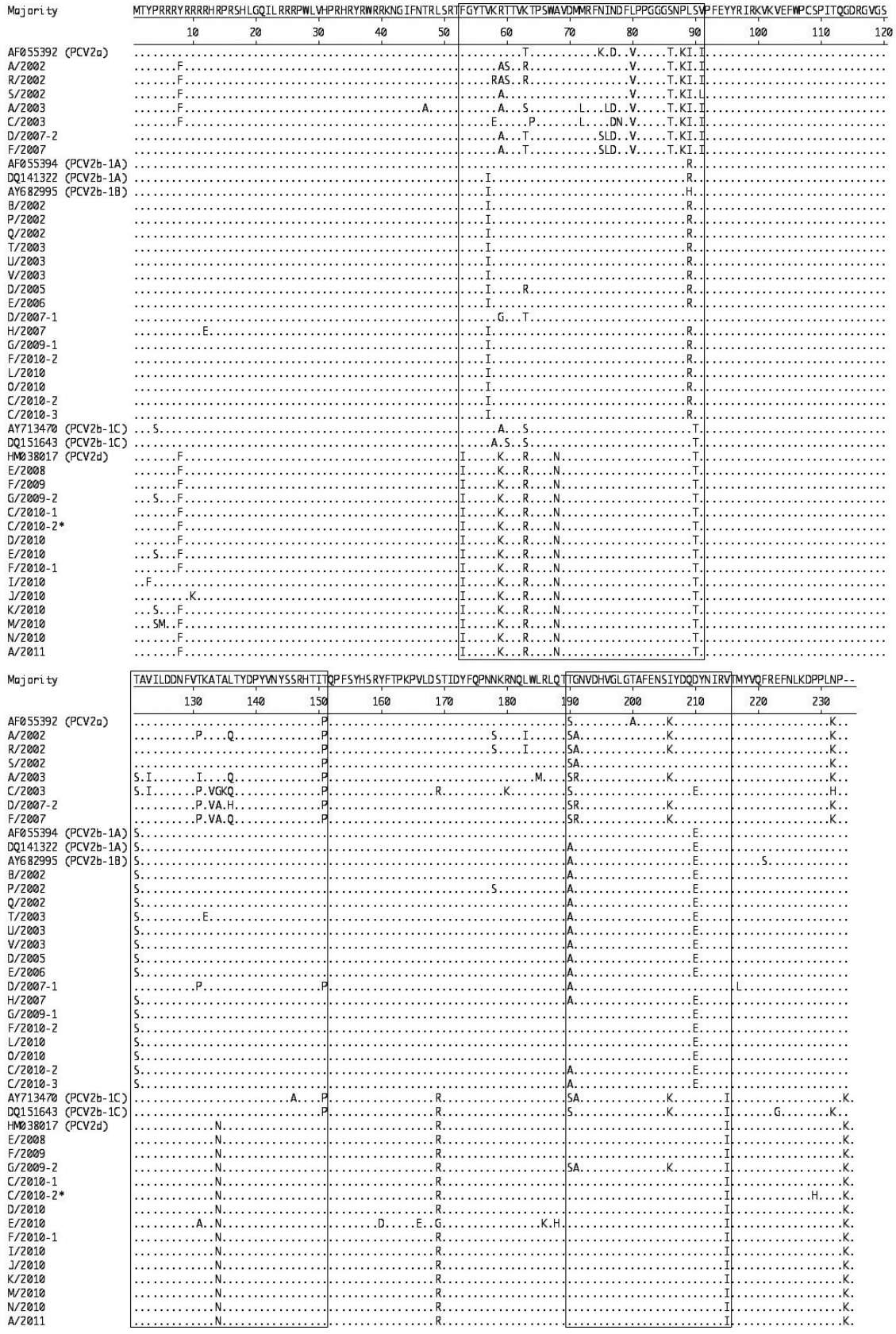

Fig. 3. Multiple amino acid sequence alignments of ORF2. Only those amino acid sequences differing from the overall consensus sequence are shown. Heterogenic regions are boxed.

Table 3. Amino acid substitutions in ORF2 of PCV2a, PCV2b and novel PCV2b

Positions	Strains		
	PCV2a	PCV2b	New PCV2b
8	F(Y)	Y	F(Y)
53	F	F	I
57	V	I(V)	V
59	A(R)	R(G/A)	K
63	T(R/K/S)	K(R/T/S)	R
68	A	A	N
77	D(N)	N	N
80	V	L	L
86–89[a)]	TNKI	SNPR(L/H)	SNPL
90–91	SI(L)	SV	TV
121	T(S)	S(T)	T
134	T(A/G)	T	N
151	P	T(P)	T
169	S(R)	S	R(G)
190–191, 206, 210[b)]	SA(R/G)K(I)D(E)	A(T/S)G(A)I(K)E(D)	T(S)G(A)I(K)D
215	V	V	I
232	K(H)	N(K)	N
234	-	-(K)	K

a) Positions 86–89 were proposed to distinguish between PCV2a and 2b. b) Positions 190–191, 206 and 210 are important for viral replication *in vitro*.

together, we believe that the new emerging genotype isolates clustered with PCV2b.

The signature residues at positions 173–175 and 179 are important for antibody recognition [36]. Among our samples, we observed amino acid conservation at these 4 positions (Fig. 3). In addition, amino acid positions 190–191, 206 and 210 of ORF2 are important for viral replication [5]. In this study, our amino acid alignment revealed that the motifs SA(R/G)K(I)D(E), A(T/S)G(A)I(K)E(D) and T(S)G(A)I(K)D were present in the genomes of PCV2a, PCV2b and novel PCV2b, respectively (Table 3). Our results show that novel PCV2b contained a new amino acid substitution within this motif. Interestingly, one isolate (strain G/2009-2) that grouped with novel PCV2b had the SAKD motif of PCV2a.

DISCUSSION

PCV2 is distributed worldwide, and it causes porcine circovirus-associated diseases and serious economic problems in swine production [27]. Genetic variation among PCV2 isolates could be used to further classify viruses into two genotypes (PCV2a and PCV2b) that differ in their amino acid sequence and phylogenetic relationships [6, 9, 26]. This is the first study to investigate the prevalence of PCV2a and 2b in Taiwan. A previous study showed that PCV2b was the dominant PCV2 genotype from 2001 to 2008 in Spain [10]. Our results indicate that the majority of PCV2 infections in Taiwan were due to PCV2b viruses during the study period (2002–2011). Surprisingly, a new emerging PCV2 virus was noted within the PCV2b subgroup, which has been a dominant genotype subgroup in Taiwan since 2008. This PCV2 genotype subgroup not only causes respiratory signs in nursery piglets but was also found in a gilt with reproductive failure and a weak newborn piglet. Thus, the pathogenesis of this new virus requires further investigation.

Based on our review of the GenBank database and sequence analysis, our novel PCV2b genotype subgroup was closely related to the PCV2 subgroup 2d according to Guo's report [17]. However, our new emergent genotype belonged to the PCV2b subgroup according to the amino acid sequence (positions 86–89) and phylogenetic analysis [6], with a *p*-distance threshold of 0.035 [9]. A similar genotype definition study was also conducted by Li *et al.* in eastern China [21]. Similar novel viruses were also found in China in 2007 (GenBank no. HM038017) and 2008 (GenBank no. FJ644929) by Guo *et al.* [17] and Li *et al.* [21], respectively. In contrast to these two studies, our data revealed that this novel genotype was predominant despite the short period of existence of approximately 3 years (2008–2011) in Taiwan. Our results also demonstrated that concurrent infection with two PCV2 strains is possible at the same pig farm, even in the same pig (farm C). Coinfection may provide a chance for viral recombination. In addition, one novel virus (G/2009-2) contained the SAKD motif of PCV2a. Taken together, our results suggest that the etiology of this new emerging strain may be due to i) importation from abroad or ii) evolution or recombination from existing PCV2 genotypes or unknown viruses. This is currently under investigation.

ORF2 encodes a viral capsid protein, which is the major structural protein of PCV2 and is involved in the host immune response and viral replication [24, 25]. Therefore, a small number of mutations may result in increased pathogenicity. The amino acid sequence positions 173–175 and 179 are important for antibody recognition [36]. Our results showed that all of the amino acids at these positions were

relatively conserved among PCV2a and 2b, and new emerging viruses (Fig. 3). In contrast, Guo *et al.* reported that a virus of the PCV2d (GenBank no. HM038031) group was not recognized by a monoclonal antibody that can neutralize PCV2 [17]. Hence, the effectiveness of commercial vaccines against this novel virus requires further evaluation. Cheung *et al.* previously reported that the amino acid motif 190-191-206-210 was associated with viral replication [5]. The results from that study suggested that viruses containing the motif AGIE (PCV2b) replicated better than those with motif SRKD (PCV2a) *in vitro*. Our findings revealed that the motifs SA(R/G)K(I)D(E), A(T/S)G(A)I(K)E(D), and T(S)G(A)I(K)D were present in the PCV2a and 2b, and novel genotype viruses, respectively. The results of the present study also showed that seven unique amino acid substitutions were noted in novel PCV2b. Further studies are needed to investigate the pathogenicity and viral replication of the different genotypes belonging to the PCV2b subgroup.

In our study, the number of PCV2a-positive pig farms declined during 2002–2007, and this genotype disappeared after 2008. Interestingly, analysis of the PCV2 isolates from the same pig farm but from different years revealed that the isolates shifted from genotypes PCV2a or 2b to an emerging subgroup of the PCV2b genotype. All of the novel PCV2b isolates were collected late in the year (Table 1). Taken together, we believe that a novel PCV2b emerged a few years ago. A comparison of viral replication between PCV2a and 2b revealed that the amount of infectious virus recovered from PCV2a-transfected cultures was at least 10 times less than the amount recovered from PCV2b-transfected cultures [5]. A previous study reported that cross-protection between PCV2a and PCV2b exists *in vivo* [28]. Vaccination with a commercial vaccine can prevent the development of viremia due to different viral genotypes and significantly decrease viral shedding via the nasal and fecal routes [13]. Taken together, these results might explain why PCV2b genotypes become predominant and why infection with the PCV2a genotype has declined around the world.

ACKNOWLEDGMENTS. We thank Dr. Zhi-Shian Ho and Dr. Paul Su for sample collection.

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