Emergence of a new type of porcine circovirus in swine (PCV): a type 1 and type 2 PCV recombinant.

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ABSTRACT

In late September 2008, tissue samples from piglets experiencing an acute outbreak of porcine reproductive and respiratory syndrome (PRRS) were submitted to the Veterinary diagnostic service of the University of Montreal. Several diagnostic assays were performed including a multiplex real-time quantitative PCR assay (mrtqPCR) for the detection and differentiation of porcine circovirus (PCV) type 2a and 2b genotypes in the lung and lymph nodes. The pig samples were found to be positive for PCV2a using the mrtqPCR but odd results were obtained. The Ct values obtained with mrtqPCR probes targeting the ORF1 and ORF2 of PCV2 were not as expected which suggested the presence of genomic variations in the PCV2 viral genome. Ultimately, a total of three diagnostic cases with mrtqPCR unusual results were investigated. After virus isolation and sequence analyses, a new type of PCV was identified in those three cases. Based on sequence analyses, this new PCV genome contains the ORF1 of PCV1 and the ORF2 of PCV2a and its entire viral genome nucleotide identity compared to PCV1, PCV2a and 2b is 86.4%, 88.7% and 86.5%, respectively. It is proposed to name this new PCV by taking into account the nomenclature of Segales et al. (2008) and by indicating the origin of the ORF1 at first and the origin of the ORF2 in second. Consequently, the name proposed for this new PCV is PCV1/2a. The prevalence of PCV1/2a seems to be very low in Quebec, Canada (2.5% of PCV positive cases), and its origin is now in debate.

Keywords: Porcine circovirus, PCV1/2a, PCV recombinant, new PCV genotype.

INTRODUCTION

Porcine circovirus (PCV) was first recognized as a contaminant of the continuous porcine kidney cell line (PK15) (ATCC-CCL31) in 1974 in Germany and described as picornavirus-like virus (Tischer et al., 1974). Under experimental conditions, the PCV-PK15-isolate did not induce disease in pigs (Allan et al., 1995). In the late 1990's, PCV was associated with a newly-emerged disease syndrome in pigs described as "postweaning multisystemic wasting syndrome" (PMWS) (Allan et al., 1998). Sequence analysis of the PMWS-associated PCV revealed differences in comparison to the earlier described PCV (Hamel et al., 1998; Meehan et al., 1998). In order to distinguish the pathogenic PMWS-associated PCV from the non-pathogenic PCV, the pathogenic type was designated porcine circovirus type 2 (PCV2) and the non-pathogenic type as porcine circovirus type 1 (PCV1). Following the death rate increase related to PMWS in late 2004 in Canada, viral sequence analyses confirmed the emergence of a new PCV2 genotype in swine, named PCV2b(Gagnon et al., 2007). . Recently, a nomenclature has been proposed which takes into account the classification of Gagnon et al (2007) and identifies two major genotypes (PCV2a and PCV2b) circulating worldwide and a third one (PCV2c) that has been reported sporadically in the 80's (Segales et al., 2008).

Following the appearance of the PCV2b genotype, a new multiplex real-time quantitative polymerase chain reaction diagnostic assay (mrtqPCR) that could differentiate PCV2a and 2b was developed (Gagnon et al., 2008).

In late September 2008, tissue samples from piglets experiencing an acute outbreak of porcine reproductive and respiratory syndrome (PRRS) were submitted to the Veterinary diagnostic service of the Faculté de médecine vétérinaire (FMV) of the Université de Montréal (UdeMtl). Several diagnostic assays were performed including the mrtqPCR for the detection of PCV2 from lung and lymph nodes. Interestingly, the samples were positive for PCV2a but odd results were obtained. The Ct values obtained with the PCV2 and PCV2a specific mrtqPCR DNA probes were not similar, on the opposite to what has been previously reported (Gagnon et al., 2008), suggesting the presence of genomic variations in the PCV2a viral genome. Consequently, a new investigation was conducted to establish the nature and extent of those genomic variations. Herein, this manuscript reports the emergence of a new type of PCV in swine (named: PCV1/2a) whose genome is composed of the ORF1 of PCV1 and the ORF2 of PCV2a.

MATERIAL AND METHODS

Diagnostic cases description

Three swine samples (containing lung and lymph nodes) were submitted to the Molecular diagnostic service of the FMV of the UdeMtl for the realization of several PCR diagnostic assays. Those three cases were submitted for various reasons such as PRRS virus (PRRSV) outbreak or only for surveillance program. Tissues of the three samples were taking from 3 to 10 weeks old piglets. None of the three submitted cases were related to PMWS and no animal in barns where the cases originated were experiencing outbreak of PMWS. The three cases, FMV08-1114252, FMV08-1133505 and FMV09-1134568, were submitted to the diagnostic laboratory in September 2008, November 2008 and January 2009, respectively.

PCV1 ORF1 and PCV2 ORF2 specific PCR assays

Three specific PCR assays were performed to detect the ORF1 of PCV1 and the ORF2 of PCV2 genes. For the first PCR assay, a set of primers PCV1.ORF1.S1 (5'-GCCAAG CAAGAAAAGC-3') and PCV1.ORF1.AS1 (5'-CGTTACAGGGAACTGCTC-3') was used to target the ORF1 gene of the PCV1 viral genome with an expected fragment of 415 nucleotide base pair (bp) in length. For the second PCR assay, PCV2.ORF2-F (5'-CTCTGAATTGTACATACATGGTTACACGGA-3') and PCV2.ORF2-R (ATGACG TATCCAAGGAGGCGTTA) primers were used to target the ORF2 gene of the PCV2 viral genome with an expected fragment of 667 bp in length. For the third PCR assay, the following set of primers, PCV1.ORF1.S1 and Circo-DR (5'-

CGCACCTTCGGATATACTATCA-3') was selected to amplify both ORF1 of PCV1 and ORF2 of PCV2 genes simultaneously with an expected fragment of 1533 bp in length. The PCR reactions were performed with 5 µL of previously tissue isolated DNA templates (see the mrtqPCR assay protocol in Gagnon et al., 2008) added to 45 µL of reaction mixture, containing final concentrations of 1.25 mM MgCl 2, 1X PCR buffer, 0.2 mM of each dNTP, 0.5 µM of each primer, and 2.5 U of *Taq* DNA polymerase (New England Biolabs). The DNA was amplified by 40 cycles of denaturing at 95°C for 40 sec, annealing at 55°C for 40 sec, and extension at 72°C for 1 min.

PCV sequencing and phylogenetic analyses.

The entire PCV2 genome was amplified by PCR, using 2 sets of oligonucleotides (PCV1.ORF1.S1 and Circo-DR: 5'-CGCACCTTCGGATATACTATCA-3'; Circo-DF: 5'-GGGCCAGAATTCAACCTTAA-3' and PCV1.ORF1.AS1) that produced 2 overlapping PCR products at both ends of 1533 nucleotides (nt) and 820 nt, respectively. The PCR products were purified by using a commercial kit (QIAquick PCR purification kit; Qiagen) according to the manufacturer's instruction. Both strands of the purified DNA PCR products were sequenced by using the same primer sets with standard automated sequencing methods as previously described (Gagnon et al., 2007). Resulting sequences were compared with references strains such as PCV1 (PK-15 infected cells; Genbank: U49186), PCV2a (1010 Stoon; Genbank: AF055392) and PCV2b (FMV05-6302; Genbank: DQ220739) reference strains. Software (BioEdit Sequence Alignment Editor version 7.0.5.2, Ibis Therapeutics; Carlsbad, CA, USA) using the CLUSTAL W alignment method was used. An unrooted phylogenic tree was

constructed by using the distance-based neighbor-joining method as previously described (Gagnon et al., 2007).

Virus isolation

The PCV isolation experiment was conducted using the PK15A cell line as previously described (Gagnon et al., 2007). Virus isolation was conducted during 9 consecutive passages with about 1-2 cm^3 pools of piglet samples (which consist of homogenates of lung and lymph nodes that were prepared as previously described by Gagnon et al. (2007)) .To confirm the presence of viruses in PK15A infected cells, an immunofluorescence assay (IFA) was performed at 96 hrs pi using an anti-PCV2 pig serum as described previously (Racine et al., 2004). Cells were visualized using a DMI 4000B reverse fluorescence microscope. Photographs of the cells were taken with a DFC 490 digital camera and the images were analyzed using the Leica Application Suite Software, version 2.4.0 (Leica Microsystems).

RESULTS

Identification of a new genomic PCV variant

All the obtained results converge to demonstrate the emergence of a new PCV (Table 1; Figure 1 and 2). At first, the Ct values obtained with the three DNA probes of the mrtqPCR PCV diagnostic assay (Gagnon et al., 2008) suggested the presence of genomic variation within the viral genome of PCV2a (data not shown). Then, specific sets of primers were used in a conventional PCR assay to determine the nature of the ORF1 and ORF2 genes of the PCV2a genomic variant. As illustrated in Figure 1a, the set of primers specific for the ORF1 of PCV1 gave a DNA band at the expected molecular weight of 415 nucleotides (nt) for PCV1 positive control and the FMV08-1114252, FMV08-1133505 and FMV09-1134568 diagnostic cases. As illustrated in Figure 1b, the set of primers specific for the ORF2 of PCV2 gave a DNA band at the expected molecular weight of 667 nt for PCV2 positive control and for the FMV08-1114252, FMV08-1133505 and FMV09-1134568 cases. To establish that the PCV2a mutant possesses the ORF1 of PCV1 and the ORF2 of PCV2a, a set of primers were selected where the forward primer targets the ORF1 of PCV1 and the reverse primer target the ORF2 of PCV2. The results obtained with the FMV08-1114252, FMV08-1133505 and FMV09-1134568 cases indicate the presence of both PCV1 ORF1 and PCV2 ORF2 genes in the PCR products (Figure 1c).

Finally, to confirm the genomic nature of the new PCV, its viral genome was sequenced directly from pig tissue homogenates and from their corresponding PK15A isolated viruses. The FMV08-1114252 and FMV08-1133505 were found to be 100% identical

(Table 1, Figure 2). The FMV09-1134568 was found to be 99.9% identical to both FMV08-1114252 and FMV08-1133505 since only one nucleotide (nt) located in ORF1 was different (data not shown). As indicated in Table 1, the ORF1 of FMV08-1114252, FMV08-1133505 and FMV09-1134568 was more genomically related to the ORF1 of PCV1 with a nt identity of 99.7% compared to ORF1 of PCV2a and 2b with a nt identities of 82.5% and 82.8%, respectively (Table 1). On the other hand, the ORF2 of FMV08-1114252, FMV08-1133505 and FMV09-1134568 was more genomically related to the ORF2 of PCV2a with a nt identity of 98.5% compared to ORF2 of PCV1 and PCV2b with a nt identities of 66.7% and 92.5%, respectively (Table 1). The nt identity of the entire viral genome of the new PCV was 86.4%, 88.7% and 86.5% compared to PCV1, PCV2a and PCV2b respectively (Table 2). The Genbank accession numbers assigned to these new viral sequences were FJ655418, FJ655419 and FJ790425 for FMV08-1114252, FMV08-1133505 and FMV09-1134568, respectively. Overall, these latest results confirm that the viral genome of the new type of PCV that emerged in swine in Canada is composed of ORF1 of PCV1 and ORF2 of PCV2a.

Virus isolation.

Attempt to isolate the virus from swine lung and lymph nodes tissue homogenates have been realized in PK15A cells. Viruses were isolated from FMV08-1133505 and FMV09-1134568 samples. No live virus was isolated from FMV08-1114252 possibly because there might be no live virus existing in the tissue samples or not enough tissues were submitted to the diagnostic laboratory. As illustrated in Figure 3, an immunofluorescence assay (IFA) was conducted. IFA positive staining cells were observed using anti-PCV2

pig serum at 3 and 9consecutive passages from cases FMV09-1134568 and FMV08-1133505, respectively. After two additional passages the expression of the viral Cap protein of PCV2 encoded by the ORF2 gene was confirmed in PCV infected cells (Figure 3).

DISCUSSION

Conventional PCR assays, and the sequence analyses clearly demonstrate the presence of a new type of PCV which is composed of the ORF1 of PCV1 and the ORF2 of PCV2 (Table 1, Figure 1 and 2). Furthermore, the isolation of an infectious virus (Figure 3) and its viral genome sequence results confirm the existence of this new PCV (Table 1, Figure 2).

It is now important to update the nomenclature of PCV to take into account the origin of the viral genes. It is proposed to identify the type of this new PCV by indicating the origin of the ORF1 at first and the origin of the ORF2 in second. To harmonize this proposition with the nomenclature proposed by (Segales et al., 2008), if only one indication is made such as PCV1 or PCV2a, then both ORF1 and ORF2 are from the same origin. Consequently, we propose to name this new PCV, PCV1/2a or to add a third type to the present nomenclature and consequently the new PCV could be named PCV type 3 (PCV3). Eventually, the selection of an appropriate nomenclature to describe this new PCV will have to be addressed by a PCV international expert committee.

Two hypotheses could explain the emergence of the PCV1/2a in swine: 1) the natural genetic recombination between PCV1 and PCV2a in infected animals; or 2) a human made virus originating from a chimeric killed vaccine strain. The genetic recombination between various strains of viruses is a phenomenon well known and it has already been demonstrated to occur between PCV2a and 2b viruses (Cheung, 2009; Hesse et al., 2008; Ma et al., 2007; Olvera et al., 2007). The genetic recombination phenomenon between

PCV1 and PCV2a could have occurred, however, the prevalence of both viruses is very low in the province of Quebec, Canada, where the three PCV1/2a positive cases have been found. Since 2007, the Diagnostic service of the FMV does not test anymore for the presence of PCV1 because it is a non-pathogenic virus and because its prevalence at that time was very low (0.1% of the PCV positive cases) (data not shown). In addition, PCV2a is the second less frequent PCV found at the diagnostic laboratories with a prevalence of 8.2% of the PCV positive cases (including PCV2a alone and PCV2a + 2b mix infection cases) compared to PCV2b alone with a prevalence of 89.3% (Table 2) which was corroborated in a previous report (Gagnon et al., 2008). Still, even if PCV1 and PCV2a are found in low amount in Quebec swine, the genetic recombination between PCV1 and PCV2a is a possible explanation. Following sequence analyses, the recombination sites between PCV1 and PCV2a were estimated to be at both nt interval located between ORF1 and ORF2 genes (1731-36 and 1004-1020 nt) (data not shown).

In early 2008, a new vaccine was commercially available in Canada (Suvaxyn® PCV2). This vaccine uses a chimeric killed or inactivated PCV (Gillespie et al., 2008; Meng et al., 2007). This chimeric PCV genome is composed of the ORF1 of PCV1 and the ORF2 of PCV2a. The nt sequences of the PCV1/2a strains were found to possess 5 to 6 nt differences compared to chimeric PCV vaccine strain (Figure 2) with a nt identity of 99.6 to 99.7 %. Noteworthy, all the nt differences were located in the ORF1 gene (data not shown). It will be an unexpected conclusion if it is proven that the new PCV1/2a virus originates from the use of a killed virus vaccine but further investigation will have to be conducted to ascertain this hypothesis. Noteworthy, we have not been able to isolate infectious viruses from one vaccine bottle sold in Canada (Suvaxyn® PCV2; batch

#1861164A which was distributed during the time period the three PCV1/2a positive cases were identified) using PK15A cells (data not shown). The virus isolation negative result from the commercial vaccine leads us to suggest another hypothesis. Is it possible that small size residual DNA, such as the entire genome of PCV, could be included in a killed vaccine formulation and function as a DNA vaccine, thus leading to the production of infectious viruses in vaccinated animals? In that regard, experimental inoculation of pigs with PCV infectious DNA clones have already been shown to induce the production of infectious virus (Fenaux et al., 2003).

At the moment, the prevalence of PCV1/2a is very low (2.5%) (Table 2) and a genomically similar virus, the chimeric PCV vaccine strain, has been demonstrated to be non-pathogenic (Fenaux et al., 2004; Fenaux et al., 2003). Furthermore, Fenaux et al (2004) have suggested that this chimeric PCV vaccine strain can be used in a modified live vaccine. Consequently, the health of infected pigs should not be at risk. Right now, the only important concern should be the accuracy of the diagnostic tests that are used for PCV diagnosis. In fact, several laboratories have developed PCV PCR diagnostic assays with their own specific design of gene targets (Gagnon et al., 2008; Horlen et al., 2007; McIntosh et al., 2009; Pal et al., 2008; Yang et al., 2007). Those laboratories may have to modify their PCR diagnostic assays if they want to identify the new PCV1/2a virus and if they wish to establish an accurate PCV identification that will cover all type of PCV genotypes.

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FIGURE LEGENDS

Figure 1. Specific PCR assay results of the detection of the ORF1 gene of PCV1 and the ORF2 gene of PCV2. Panel A: PCR products obtained with the set of primers specific for the ORF1 gene detection of PCV1. Panel B: PCR products obtained with the set of primers specific for the ORF2 gene detection of PCV2. Panel C: PCR products encompassing the ORF1 of PCV1 and ORF2 of PCV2a genes that were obtained with a specific set of primers targeting the ORF1 of PCV1 in the forward direction and ORF2 of PCV2a in the reverse direction. PCR products were visualized by electrophoresis on a 1% agarose gel and ethidium bromide staining. L: 100 bp DNA Ladder (Invitrogen Corporation); 1: FMV08-1114252 diagnostic case; 2: FMV08-1133505 diagnostic case; 3: FMV09-1134568 diagnostic case; 4: PCV-1 positive swine tissue control; 5: PCV2a positive swine tissue control; 6: PCV2b positive swine tissue control; 7: negative swine tissue control.

Figure 2. Phylogenetic tree of the entire viral genome of PCV1/2a strains. The GenBank accession numbers of each strain are indicated on the right, next to the name of the strains. The PCV chimeric vaccine strain DNA sequence was obtained from Meng et al. (2007). The strains have been classified in 5 genogroups (PCV1, PCV2a, PCV2b, PCV2c and PCV1/2a). Bootstrap values are indicated but some are non-indicated for clarity purpose. Horizontal scale bar indicates the distance between strains; a 0.1 distance means that the strains possess 90% nt identity.

Figure 3. PCV isolation in PK15A cells confirmed by immunofluorescence. The IFA was done as previously described (Racine et al., 2004). Two PCV (FMV08-1133505 and FMV09-1134568) were isolated from swine samples. The IFA was done at passage 11 for FMV08-1133505 and passage 5 for FMV09-1134568. Mock infected cells are illustrated as control.

Table 1. Nucleotide identities of the new PCV1/2a virus type compared to PCV1 and PCV2 reference strains

PCV1/2a virus genes	PCV1 (PK15 infected cells)[1]			PCV2a (1010-Stoon)[2]			PCV2b (FMV05-6302)[3]		
	Complete genome	ORF1	ORF2	Complete genome	ORF1	ORF2	Complete genome	ORF1	ORF2
FMV08-1133505 strain[4]									
Complete genome	86.4%			88.7%			86.5%		
ORF1		99.7%			82.5%			82.8%	
ORF2			66.7%			98.5%			92.5%
FMV09-1134568 strain[5]									
Complete genome	86.4%			88.6%			86.4%		
ORF1		99.6%			82.4%			82.7%	
ORF2			66.7%			98.5%			92.5%

[1]Genbank accession number: U49186

[2]Genbank accession number: AF055392

[3]Genbank accession number: DQ220739

[4]The nt sequences of FMV08-1114252 and FMV08-1133505 strains are 100% identical.

[5]The nt sequence of FMV09-1134568 strain is 99.9% identical to both FMV08-1133505 and FMV08-1114252 strains.

Table 2. PCV2 and PCV1/2a prevalence

Genotype classification	Total number of cases	Prevalence in %
PCV2a	5	4.1%
PCV2b	109	89.3%
PCV2a and PCV2b	5	4.1%
PCV1/2a	3	2.5%
Total	122	100%

Note 1: The prevalence was determined using the mrtqPCR assay as previously described (Gagnon et al., 2008).

Note 2: The mrtqPCR assay was done on Canadian swine tissue homogenates (lung and lymph nodes) submitted to the diagnostic laboratory from September 2008 until March 2009.