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1 **Genomic analysis of PCV2 isolates from Danish archives and a current PMWS**
2 **case-control study supports a shift in genotypes with time**

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9
10 *Keywords:* PMWS; PCV2; Case-control study; Phylogeny, genotypes

11
12 **Abstract**

13
14 Porcine circovirus type 2 (PCV2) is the primary cause of Postweaning Multisystemic Wasting
15 Syndrome (PMWS) in pigs. PCV2, however, is found in both PMWS-affected herds and non-
16 affected herds. The objective of this study was to clarify if PCV2 genome nucleotide sequences
17 isolated from pigs from PMWS-affected herds and non-affected herds cluster phylogenetically in
18 two separate groups. All isolates (45) belonged to PCV2 group 1 and shared a nucleotide sequence
19 identity of 99.4-100% indicating a very homogeneous PCV2 population in Denmark. Phylogenetic
20 analysis of the PCV2 isolates revealed no distinctive clustering of case- and control-herds
21 suggesting that there is no link between PCV2 sequences and herd disease status. The appearance of
22 only PCV2 group 1 isolates in this study (isolates from 2003/2004) led us to determine if PCV2
23 nucleotide sequences had changed in Denmark over time. Interestingly, all PCV2 isolates from
24 before the first outbreak of PMWS (2001) belonged either to a new PCV2 group identified for the

1 first time in this study and named group 3 (isolates from 1980, 1987 and 1990) or PCV2 group 2
2 (isolates from 1993 and 1996). The shift from PCV2 group 2 to 1 was confirmed on a more global
3 scale by placing all full genome PCV2 sequences submitted to GenBank from 1997-2006 in either
4 of the groups by phylogenetic analysis. The analysis showed that the shift happened in 2003 or even
5 earlier. This may indicate that PCV2 group 1 is a more adapted form of PCV2 and possibly could
6 be more pathogenic.

7

8 **1. Introduction**

9

10 Infection with PCV2 is associated with PMWS in pigs aged 2 to 4 months. PMWS was first
11 observed in Canada in 1991 (Harding and Clark, 1997) and is now considered as one of the most
12 important swine diseases worldwide. Affected pigs show wasting or unthriftiness, enlarged lymph
13 nodes, and occasionally respiratory distress, jaundice and diarrhea (Segales et al., 2005). The most
14 distinct microscopic lesions in lymphoid organs are lymphoid cell depletion, and formation of
15 multinucleated giant cells as well as basophilic intracytoplasmic inclusion bodies (Segales and
16 Domingo, 2002). Infection with PCV2 is needed for full expression of PMWS clinically as well as
17 histopathologically. However, far from all pigs infected with the virus develop the full range of
18 symptoms associated with PMWS (Segales et al., 2005).

19 PCV2 is member of the virus family circoviridae. The virion is icosahedral, nonenveloped and 17
20 nm in diameter. A single capsid protein of approx. 30 kDa has been identified for PCV2
21 (Nawagitgul et al., 2000). The genome of PCV2 is circular, covalently closed, single-stranded DNA
22 (ssDNA) of about 1.76 Kb (Chae, 2004). Upon infection, the ssDNA genome is converted into a
23 double-stranded intermediate, which serves as template for rolling-circle synthesis of the viral
24 ssDNA strand (Steinfeldt et al., 2006). The genome contains two major reading frames: ORF1

1 encodes the replicase and ORF2 is encoding the capsid protein. The PCV2 genome is ambisense so
2 that the encapsidated viral DNA strand serves as a template for transcription of the capsid protein
3 gene (ORF2), while the complementary DNA strand of the replicative intermediate functions as a
4 template for transcription of the replicase gene (ORF1) (Mankertz et al., 2004).

5 The similarity of PCV2 genomes is at least 93% and, phylogenetically, PCV2 can be divided into
6 two major groups (1 and 2) (Olvera et al., 2007). The most distinct nucleotide differences between
7 the two groups are found in the capsid protein gene with 33 differences. In contrast, only 13
8 nucleotide differences are found in the replicase gene (Olvera et al., 2007). There is apparently no
9 link between PCV2 group and disease status or geographical area (Olvera et al., 2007). However,
10 some countries have observed a shift from PCV2 group 2 to 1. This shift has been reported in
11 Switzerland in 2003 (Wiederkehr et al., 2007), in Canada in 2005 (Ellis et al., 2006) and in USA in
12 2005 (Cheung et al., 2007).

13 The aim of this study was to compare the genome nucleotide sequences of 45 PCV2 isolates from
14 Danish pigs originating from PMWS-affected herds (case) and non-affected herds (control),
15 respectively. In Denmark we experienced the first cases of PMWS in 2001 (Vigre et al., 2005).
16 Therefore, PCV2 genome nucleotide sequences from this case-control study (2003/2004) were
17 compared with PCV2 genome nucleotide sequences obtained from Danish pig serum collected in
18 1980, 1987, 1990, 1993 and 1996. The development in the Danish PCV2 sequences from 1980-
19 2004 were placed in a broader context by comparison with the development in PCV2 sequence
20 submissions from other countries to GenBank in the period 1997-2006.

21

22 **2. Materials and methods**

23

24 *2.1. Sample collection*

1

2 The case-control study was carried out in 2003/2004 on pigs from 32 PMWS-affected herds and 13
3 non-affected herds distributed throughout Denmark. PMWS-affected herds were selected according
4 to the definition by Sorden et al. (2000) where the occurrence of PMWS requires that a pig/group of
5 pigs have all the following characteristics: 1) clinical signs characterized by wasting/failure to
6 thrive; 2) histologic lesions characterized by depletion of lymphoid tissues and/or lymphohistiocytic
7 to granulomatous inflammation in any organ, typically lungs and/or lymphoid tissues; and 3) PCV2
8 within characteristic lesions as determined by immunohistochemistry or in situ hybridization.

9 Non-affected herds were selected by the absence of clinical signs of PMWS and low mortality.

10 Non-affected herds developing PMWS within 3 months after termination of the study were
11 excluded.

12 Pig serum samples from Danish archives (1980, 1987, 1990, 1993 and 1996) collected before the
13 first cases of PMWS in Denmark (2001) were included in the study.

14

15 *2.2. DNA extraction and PCV2 sequencing*

16

17 DNA was purified from lung tissue and serum with the QIAamp DNA Mini kit (Qiagen, Ballerup,
18 Denmark). The PCV2 genome was PCR amplified as three overlapping PCR products with the
19 primer pairs PCV2-1for (5'-CTTCCGAAGACGAGCGC-3') and PCV2-1rev (5'-
20 TAGCATTCTTCCAAAATACCAAG-3') for PCR product 1, PCV2-2for (5'-
21 GTTTACATAGGGGTCATAGG-3') and PCV2-2rev (5'- TGCTTCTTCACAAAATTAGCG-3')
22 for PCR product 2, and PCV2-3for (5'-TAGAGACTAAAGGTGGAAGTGTGTA-3') and PCV2-3rev
23 (5'- TCCTGGGCGGTGGACATG-3') for PCR product 3. All primers used in this study were
24 delivered by MWG-Biotech AG (Ebersberg, Germany).

1 The PCR reactions (50 μ l) contained 5 μ l *pfu* DNA polymerase reaction buffer (Stratagene, La
2 Jolla, CA, USA), 0.1 mM of each deoxynucleoside triphosphate (Amersham Biosciences GMBH,
3 Hillerød, Denmark), 0.4 μ M of each primer, 1 μ M MgCl₂, 1 μ l *PfuTurbo* DNA Polymerase
4 (Stratagene) and 200 ng DNA purified from tissue. PCR was performed on a T3 Thermocycler
5 (Biometra GmbH, Göttingen, Germany) under the following conditions: 2 min at 95 °C followed by
6 38 cycles of 30 sec at 95 °C, 30 sec at 51 °C, 1 min at 72 °C and a final step of 10 min at 72 °C. The
7 PCR products were purified with a QIAquick PCR purification kit (Qiagen) and sequenced on both
8 strands with the sequencing primers SeqPCV2-1for (5'-GGATCTTCCAATATCCCTATTTG-3')
9 and SeqPCV2-1rev (5'-AGAGCTTCTACAGCTGGG-3') for PCR product 1, SeqPCV2-2for (5'-
10 CAGCACTGGAGCCCCTC-3') and SeqPCV2-2rev (5'- GTGAGGTGTTTCGTCCTTCC-3') for
11 PCR product 2, and SeqPCV2-3for (5'-GTATTCTGATTACCAGCAATC-3') and SeqPCV2-3rev
12 (5'-TACAGAATAAGAAAGGTTAAGG-3') for PCR product 3.

13

14 2.3. Sequence assembling and analysis

15

16 Complete PCV2 genomes were assembled using the ChromasPro (version 1.33) program
17 (<http://www.technelysium.com.au/ChromasPro.html>) and deposited in the GenBank database under
18 the accession numbers shown in Table 1. The PCV2 genomes were aligned using the ClustalW
19 method available in the MEGA (version 3.1) program (<http://www.megasoftware.net>) (Kumar et al.,
20 2004). Phylogenetic trees were constructed by the neighbor-joining method using 1000 bootstrap
21 replicates in the MEGA 3.1 program.

22

23 3. Results and discussion

24

1 *3.1. A Danish case-control- study performed in 2003/2004*

2

3 Among 45 PCV2 isolates sequenced in the current case-control study, 27 different genome
4 sequences were identified (Table 1). The sequence with accession number EF565344 occurred in
5 15 isolates while the other sequences occurred only once or twice (Table 1). All 45 PCV2
6 sequences shared a nucleotide identity of 99.4-100% indicating a very homogeneous PCV2
7 population in Denmark. A PCV2 isolate (sequence accession number EF565366) from a non-
8 affected herd had an 11 nucleotide (nt) insertion at position 42, nine bases upstream from the
9 initiation codon of ORF1. This 11 nt base insertion constituted a third repeat at the site. A similar
10 insertion was previously reported by Boisseson et al. (2004) for a PCV2 strain (AY321993) from a
11 non-affected herd in France. It seems unlikely that this 11 nt insertion enhances the pathogenicity of
12 PCV2 as it is an infrequent event and occurs only in strains from non-affected herds.

13 To determine whether characteristic PCV2 sequences were associated with isolates from PMWS-
14 affected herds and non-affected herds, respectively, a phylogenetic tree was constructed from the
15 current 27 PCV2 sequences (Fig. 1). The analysis revealed no distinctive clustering of case and
16 control sequences suggesting that there is no link between PCV2 sequences and herd disease status.
17 This is supported by the finding of two PCV2 sequences (EF565344 and EF565365) present in both
18 case and control herds (Table 1). Furthermore, the result is consistent with case-control studies from
19 France (Boisseson et al., 2004), the Netherlands (Grierson et al., 2004), USA (Pogranichniy et al.,
20 2002) and Canada (Larochelle et al., 2003). PCV2 full genome sequences from the French and
21 Dutch studies were included in the phylogenetic tree (Fig. 1), confirming that there is no distinctive
22 clustering of case and control sequences even in this larger material. All the isolates from our
23 Danish case-control study belonged to PCV2 group 1. Similarly, all the PCV2 isolates from the

1 French and Dutch studies included in Fig. 1 belonged to this PCV2 group except for the French
2 strain (AY322004) from a control herd, which belonged to PCV2 group 2.

3

4 *3.2. Development of PCV2 genome sequences over time*

5

6 The finding of PCV2 group 1 sequences, exclusively, in this Danish case-control study lead us to
7 determine if PCV2 sequences obtained from pigs in Denmark had changed over time. Interestingly,
8 PCV2 sequences from serum samples collected in 1980 (GenBank accession nr. EU148503), 1987
9 (EU148504) and 1990 (EU148505) all belonged to a new group (group 3) of PCV2 identified for
10 the first time in this study (Fig. 2). PCV2 sequences isolated from serum samples collected in 1993
11 (EU148506) and 1996 (EU148507) belonged to PCV2 group 2 (Fig. 2). Therefore, on a time scale
12 PCV2 group 3 is the earliest identified PCV2 group in Denmark followed by PCV2 group 2 and
13 finally, PCV2 group 1 after the first outbreaks of PMWS in 2001. This observation suggests a link
14 between PCV2 group 1 and disease in Denmark. PCV2 group 3 showed higher nucleotide sequence
15 identity with group 1 (approx. 95%) than with group 2 (91-93.6%). The reason for this is unknown
16 but could indicate introduction of new strains to the Danish herds during time rather than mutational
17 development of existent strains.

18 The shift of PCV2 groups in Denmark with time further lead us to determine on a more global scale
19 if the proportion of PCV2 groups submitted to GenBank had changed since the first PCV2 sequence
20 (group 2) was submitted in 1997. A phylogenetic tree was constructed from all the 218 full-length
21 PCV2 genome sequences available from GenBank in February 2007 (data not shown).
22 Subsequently, all PCV2 sequences were assigned to PCV2 group 1 or 2 and year of submission to
23 GenBank (Fig. 3A). The analysis demonstrated that PCV2 group 1 has become dominating with
24 time and that a major shift from PCV2 group 2 to PCV2 group 1 occurred in year 2003. However,

1 the expected delay between the actual PCV2 isolation and the submission of PCV2 sequences to
2 GenBank suggest that the shift could have happened earlier.

3 To clarify if this shift was connected to certain regions, PCV2 sequences submitted before (Fig. 3B)
4 and after (Fig. 3C) the shift in 2003 were assigned to geographical regions. Before 2003, Canada,
5 USA and Korea/Japan only submitted sequences belonging to PCV2 group 2, whereas submissions
6 from the EU and China/Taiwan/Singapore were mixed group 1 and 2 but dominated by group 2.
7 After the shift in 2003, all the countries submitted primarily group 1 sequences. This indicates that
8 the shift from group 2 to 1 is global except for Korea/Japan and Australia, which have only
9 submitted PCV2 sequences belonging to group 2 in the period from 1997 to 2006. As Australia is
10 free from PMWS (Muhling et al., 2006) it will be interesting to follow if outbreaks of PMWS in
11 this country, like in Denmark, will coincide with a shift from PCV2 group 2 to 1.

12 Only recently, USA and Canada experienced this shift from PCV2 group 2 to group 1 resulting in
13 new outbreaks of PMWS with a significant increase in mortality (Cheung et al., 2007; Ellis et al.,
14 2006). This observation can be explained by low cross protection in the pigs between PCV2 group 1
15 and 2 as most nucleotide differences between PCV2 group 1 and 2 are found in the capsid protein
16 gene (Olvera et al., 2007). In addition, we speculate that PCV2 belonging to group 1 more
17 effectively attach to and enter the cells, and thereby more easily spread between cells. This could
18 explain the observation that PCV2 group 1 viruses with time out-compete PCV2 group 2. This,
19 however, needs to be further investigated.

20

21 **4. Conclusion**

22

23 Historically, Denmark had PCV2 group 3 followed by group 2 in the herds before the first
24 outbreaks of PMWS in 2001. Hereafter, in 2003/2004 only PCV2 group 1 sequences were found.

1 This suggest that PCV2 group 1 is more powerful in inducing PMWS despite that also PCV2 group
2 2 is capable of inducing disease (Hamel et al. 1998). In the current case-control study all PCV2
3 isolates belonged to PCV2 group 1 and were highly similar revealing no clustering connected with
4 PMWS-affected herds and non-affected herds. It is therefore likely that all PCV2 group 1 isolates in
5 this study are potentially capable of inducing PMWS; hence, other factors, such as co-infection with
6 other porcine viruses or pathogenic bacteria, immunostimulation, husbandry forms, and/or genetics
7 of the pigs, are probably necessary for the full development of the disease. This is supported by
8 reproduction of PMWS in Swedish and Danish pigs using a PCV2 isolate from a herd in Sweden
9 collected at the time the country was free from PMWS (Haslung et al., 2005).

10 The global shift from PCV2 group 2 to PCV2 group 1 over time confirms the Danish observations
11 and indicates that group 1 could be a more adapted or even a more pathogen form of PCV2. This is
12 supported by the predominance of PCV2 group 2 in healthy herds in Sweden and Spain, whereas
13 PCV2 group 1 is more related to PMWS affected herds (Timmusk et al., 2005; Grau-Roma et al.,
14 2007).

15 At present the nomenclature of PCV2 genotypes is confusing. Hence, PCV2 group 1 proposed by
16 Olvera et al. (2007) matches genotype 2 (Timmusk et al., 2005) and “new type 321” (Ellis et al.,
17 2006). Further, the PCV2 group 2 of Olvera et al. (2007) matches genotype 1 (Timmusk et al.,
18 2005), and “old type 321” and “type 422” (Ellis et al., 2006). With this article the nomenclature
19 becomes even more complicated by introduction of a third genotype (group 3). A common
20 nomenclature of PCV2 genotypes should therefore be implemented in the near future.

21

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23

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4

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11

1 Table 1. Identification of pigs used in this case-control study and their origin from PMWS-affected
2 or non-affected herds. Genbank accession numbers are shown for PCV2 strains isolated from the
3 pigs.

4
5 Fig. 1. Phylogenetic analysis of full-length PCV2 genomes from this study, France (Boisseson et
6 al., 2004) and the Netherlands (Grierson et al., 2004). The PCV2 genomes were assigned to PCV2
7 group 1 and 2 according to Olvera et al. (2007). The rooted phylogenetic tree was constructed by
8 the neighbor-joining method. Significant bootstrap values are indicated as a percentage for 1000
9 replicates.

10
11 Fig. 2. Phylogenetic analysis of full-length PCV2 genomes from GenBank and Danish archive pig
12 serum. In the phylogenetic tree, year of submission is given for PCV2 genomes obtained from
13 GenBank, whereas year of serum collection is given for the archive samples. The PCV2 genomes
14 were assigned to PCV2 group 1 and 2 (Olvera et al., 2007), and group 3 (this study). The rooted
15 phylogenetic tree was constructed by the neighbor-joining method. Significant bootstrap values are
16 indicated as a percentage for 1000 replicates. The following abbreviations have been used: CAN:
17 Canada; USA: United States of America; BRA: Brazil; CHI: China; TW: Taiwan; JAP: Japan;
18 AUS: Australia; The Netherlands: NL; Denmark: DK; Austria: AUT; France: FRA; Hungary:
19 HUN; United Kingdom: UK; Germany: GER; Spain: SPA.

20
21 Fig. 3. Submission of full genome PCV2 sequences to GenBank assigned to PCV2 group 2 (White
22 bars) and PCV2 group 1 (Black bars). (A) All submissions from 1997 to 2006, (B) submissions
23 from geographical regions 1997-2002, and (C) submissions from geographical regions 2003-2006.
24 The following abbreviations have been used: CAN: Canada; USA: United States of America; BRA:

- 1 Brazil; EU: European Union; CHI: China; TW: Taiwan; SING: Singapore; KOR: Korea; JAP:
- 2 Japan; AUS: Australia.
- 3

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Pig identification	Herd PMWS (+/-)	PCV2 accession no.
408	+	EF565344
418	+	EF565344
422	+	EF565344
424	+	EF565344
427	+	EF565344
452	+	EF565344
462	+	EF565344
463	+	EF565344
466	+	EF565344
468	+	EF565344
473	+	EF565344
573	-	EF565344
537	-	EF565344
562	-	EF565344
597	-	EF565344
411	+	EF565345
405	+	EF565342
538	-	EF565363
529	-	EF565362
429	+	EF565350
451	+	EF565350
444	+	EF565355
575	-	EF565366
544	-	EF565364
475	+	EF565360
455	+	EF565357
524	-	EF565361
542	-	EF565361
459	+	EF565358
419	+	EF565346
442	+	EF565354
421	+	EF565347
592	-	EF565368
450	+	EF565356
581	-	EF565367
428	+	EF565349
470	+	EF565349
456	+	EF565365
558	-	EF565365
433	+	EF565351
406	+	EF565343
440	+	EF565353
423	+	EF565348
438	+	EF565352
471	+	EF565359





