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A proposal on porcine circovirus type 2 (PCV2) genotype definition and their relation with postweaning multisystemic wasting syndrome (PMWS) occurrence

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Abstract

Porcine circovirus type 2 (PCV2) is the essential infectious agent of postweaning multisystemic wasting syndrome (PMWS). Despite first sequencing studies did not find any association between PCV2 sequences and PMWS occurrence, recent works have suggested the opposite. In the present study, 87 open reading frame 2 (ORF2) sequences obtained from pigs with different clinical conditions and coming from farms with different PMWS status were analyzed. Results further confirmed the existence of two genogroups and the definition of two PCV2 genotypes (1 and 2) is proposed. All sequences included in genotype 1 came from pigs from PMWS affected farms, while all sequences obtained from non-PMWS affected farms corresponded to genotype 2.

Moreover, infection of single pigs from PMWS affected farms harbouring both genotypes is described. Present results suggest that PCV2 genotype 1 may potentially be more pathogenic than PCV2 genotype 2.

Keywords: porcine circovirus type 2 (PCV2), postweaning multisystemic wasting syndrome (PMWS), genotype, phylogeny, epidemiology, pathogenicity

Introduction

Porcine circovirus type 2 (PCV2) is recognized as the essential infectious agent of postweaning multisystemic wasting syndrome (PMWS), which is considered to have a severe economic impact on swine production (Segalés et al., 2005). The major clinical sign of PMWS is wasting and growth retardation, but can also include pallor of the skin, icterus, respiratory distress and diarrhoea (Harding and Clark, 1998).

PCV2 belongs to the family *circoviridae*, genus *cirvovirus*, and is a small, non-enveloped, single-stranded DNA virus containing a circular genome of 1767-1768 bp (Hamel et al., 1998; Meehan et al., 1998; Mankertz et al., 2000). The genome contains three open reading frames (ORF): *ORF1* encodes the replicase (*rep* and *rep'*) proteins involved in virus replication (Mankertz et al., 1998), *ORF2* encodes the *capsid* (*cap*) protein (Nawagitgul et al., 2000) and *ORF3* encodes a protein that is not essential for PCV2 replication with potential apoptotic activities (Liu et al., 2005, 2006). It was proposed that, since *cap* protein is the most variable PCV2 protein, a link between capsid protein variation and pathogenicity of PCV2 could exist (Larochelle et al., 2002; Todd et al., 2002). In addition, *ORF2* has been shown as a good phylogenetic and

epidemiologic marker for PCV2, since it was able to reconstruct the same phylogenetic tree as using the whole viral PCV2 genome (Olvera et al., 2007).

The first description of PMWS was in Canada in 1991 (Harding and Clark, 1998) and, since then, it has been described in many parts around the world (Segalés et al., 2005). Retrospective analysis in pig samples demonstrated that PCV2 infection in the livestock occurred many years before the epizootic outbreaks described by mid and late 90s (Magar et al., 2000; Rodriguez-Arrioja et al., 2003). This fact together with presence of PCV2 in both PMWS and non-PMWS affected pigs and farms also suggested the possible existence of differences in pathogenicity between different PCV2 strains. Despite most sequencing studies did not find any relation between PCV2 sequences and the occurrence of the disease (Larochelle et al., 2002; Pogranichniy et al., 2002; Larochelle et al., 2003; de Boisseson et al., 2004; Grierson et al., 2004; Wen et al., 2005; Martins Gomes de Castro et al., 2007), some recent studies and field observations (Timmusk et al., 2005; Opriessnig et al., 2006; Cheung et al., 2007a, 2007b; Stevenson et al., 2007; Woodbine et al., 2007) have suggested the opposite. Moreover, epidemiological Danish and British studies strongly suggested that the spread of porcine circovirus diseases (PCVDs) is consistent with the introduction of a "new infectious agent" or a "new strain" of a known agent into a naïve population (Vigre et al., 2005; Woodbine et al., 2007).

Based on the controversy of potential different pathogenicity among PCV2 strains, the main objective of this study was to elucidate if *ORF2* PCV2 sequences could be correlated with different health or disease status of farms and/or pigs. Concomitantly, a potential definition of genotypes in PCV2 was explored. Moreover, we intended to

75 determine if multiple sequences can be present in the same animal at the same time, as
76 previously suggested (de Boisseson et al., 2004; Opriessnig et al., 2006; Cheung et al.,
77 2007a).
78
79 **Materials and methods**
80 *Sample collection*
81 Six farms (No. 1 to 6, table 1) located in North-Eastern Spain with historical records of
82 PMWS were included in a longitudinal case-control study performed during years 2005
83 and 2006. Actually, farms 4 and 5 corresponded to two batches of pigs from the same
84 farm. The diagnosis of PMWS at farm level was confirmed before the start the study.
85 Diagnostic procedures included a high percentage (>10%) of pigs with wasting and
86 mortality in postweaning (nurseries, fattening and finishing) areas and the individual
87 diagnosis of PMWS (Segalés et al., 2005) in at least 1 out of 5 necropsied pigs.
88
89 One hundred piglets per farm from 12 to 14 randomly selected sows were ear-tagged at
90 birth and followed up until PMWS outbreak occurrence. Sows were bled at farrowing.
91 When PMWS compatible clinical picture (Segalés and Domingo, 2002) appeared, those
92 animals showing clinical signs were bled, euthanized and necropsied (n=8 to 12 per
93 farm). Moreover, one healthy age matched pig per every two diseased pigs was also
94 euthanized and sampled in the same manner with a maximum of 5 per farm (n=4 to 5
95 per farm). Sections of lymph nodes (tracheobronchial, mesenteric, superficial inguinal
96 and submandibular) and tonsil were collected and fixed by immersion in neutral-
97 buffered 10% formalin.
98

On the other hand, in 2006, eighty pigs from 2 different farms (No. 7 and 8) located also in North-Eastern of Spain and without history of PMWS were bled at 3 months, which corresponds to an age where PCV2 viremia is usually present (Larochelle et al., 2003; Shibata et al., 2003).

All treatments, housing, husbandry and slaughtering conditions were conformed to the European Union Guidelines and Good Clinical Practices.

Histopathology

Formalin-fixed paraffin-embedded blocks containing the abovementioned lymphoid tissues were prepared. Two consecutive 4 µm thick sections corresponding to each pig were cut from each block. One section was processed for histopathology, while the other was processed for PCV2 nucleic acid detection by *in situ* hybridization (ISH) (Rosell et al., 1999). PMWS was diagnosed when pigs fulfilled international accepted criteria (Segalés et al., 2005). Pigs were finally classified into three different categories: **i) PMWS cases**: pigs showing clinical wasting, moderate to severe PMWS characteristic histopathological lymphoid lesions and moderate to high amount of virus within the lesions; **ii)Wasted non-PMWS cases**: pigs showing clinical wasting but without or slight PMWS characteristic histopathological lymphoid lesions and none or low amount of PCV2 genome within lymphoid tissues; and **iii) Healthy pigs**: pigs showing good clinical condition, which presented none or slight PMWS characteristic histopathological lymphoid lesions and none or low amount of PCV2 genome within lymphoid tissues.

Screening of serum samples by polymerase chain reaction (PCR) and sequencing

DNA from serum was extracted using a commercial kit (Nucleospin® Blood, Macherey Nagel) and tested using a previously described PCV2 PCR (Quintana et al., 2001). From samples positive by PCV2 PCR, whole ORF2 was amplified using specific primers (capFw 5'-CTTTTTTATCACTTCG TAATG-3' and capRw 5'-CGCACTTCTTTCGTTTTC-3') under previously reported conditions (Fort et al., 2007). Amplicon products from ORF2 PCV2 PCR positive samples were purified (MiniElute®, Qiagen) and both strands were sequenced at least twice, using the same above mentioned specific primers. Cycle sequencing was carried out with BigDye® Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) and an ABI 3730 DNA sequencer (Applied Biosystems, Foster City, CA, USA) following the manufacturers' instructions.

When sequences with multiple peaks at the same position were observed in the chromatogram, PCR products were cloned and sequenced in order to elucidate possible multiple sequences. The ORF2 PCV2 gene was amplified as already mentioned (Fort et al., 2007) with a final extension of 72ºC for 20 min and was cloned into the pGEMT® vector system (Promega), transformed in *Escherichia coli* TOP10 competent cells and screened following manufacturers' instructions. Positive colonies were detected with the mentioned ORF2 PCR with the unique variation of the first denaturation, being at 96ºC for 10 min. Plasmid DNA was extracted by QIAprep spin Kit according the manufacturers' instructions and sequenced using universal M13 primers.

Phylogenetic analysis of ORF2 PCV2 sequences

Sequence data was compiled and analyzed using Sequence Analysis (Applied Biosystems, Foster City, USA) and Fingerprinting II software (Informatix^{tm} Software,

2000). Sequences were aligned using Crustal W method. Phylogenetic relationships among sequences were analyzed as described in Olvera et al. (2007) using parsimony and nucleotide distance methods. Firstly, the heuristic search option of PAUP 4.0.b (Swofford, 1998), considering a single stepwise addition procedure, and a tree bisection-reconnection (TBR=100) branch swapping algorithm, was used for unweighted Maximum Parsimony analysis (MP). A majority rule consensus tree was then generated from the 100 most parsimonious trees found in each of the 1000 bootstrap replicates of the analysis. Secondly, we computed a nucleotide distance matrix between sequences to infer phylogenies by a Neighbor-Joining (NJ) and a Maximum Likelihood (ML) trees using respectively MEGA 3.1 (Kumar et al., 2001) and TreePuzzle 5.0 (Schmidt et al., 2002). Confidence in the NJ tree was estimated by 1000 bootstrap replicates. The tree search quartet puzzling algorithm directly assigned estimations of support to each internal branch of the ML tree. Trees were rooted with two ORF2 PCV1 sequences (accession numbers AY660574 and AY193712).

Genotype definition

Data from the pairwaise comparison of the obtained ORF2 PCV2 sequences together with 148 ORF2 PCV2 sequences present at the NCBI nucleotide database (http://www.ncbi.nlm.nih.gov) in September 2005 (Olvera et al., 2007) and two PCV1 ORF2 sequences (for rooting purposes) (accession numbers AY660574 and AY193712) were used to construct a matrix of p-distance values. P-distance is the proportion of nucleotide sites at which two sequences being compared are different. It is obtained by dividing the number of nucleotide differences by the total number of nucleotides compared (Kumar et al., 2000). Afterwards, p-distance/frequency histogram was

constructed in order to determine possible cut-off values to distinguish different PCV2 genotypes (Biagini et al., 1999; Rogers and Harpending, 1992).

Phylogenetic analysis among populations (farms)

Patterns of nucleotide diversity distribution among farms were estimated by a hierarchical nested analysis of molecular variance (AMOVA) (Excoffier et al., 1992) of the frequency distribution of sequences and their pairwise divergence at three hierarchical levels: within farms (φst), among farms within groups (φsc) and among groups (φct). Those analyses were performed using Arlequin software (version 3.1) and considered two groups of farms: i) PMWS affected farms (No. 1 to 6), and ii) non-PMWS affected farms (No. 7 and 8). Moreover, phylogenetic relationships among populations were assessed by computing a NJ population tree from the distance matrix of nucleotide divergence among farms.

Nucleotide sequence accession numbers

The ORF2 PCV2 sequences reported in this work have been deposited at GenBank (http://www.ncbi.nlm.nih.gov) under accession numbers EF647642- EF647728.

Results

Clinical Picture and PMWS diagnosis

Clinical picture compatible with PMWS appeared in farms No. 1 to 6 between 11 and 21 weeks of age, depending on the farm (Table 1). Acute clinical signs occurred during a period of about 3 weeks. Farms 1 to 5 suffered from PMWS based on clinical signs, histopathological lesions in lymphoid tissues and PCV2 detection within lesions. In farm 6, PMWS was suspected based on the occurrence of wasting in the growing phase,

198 but pathological studies could not finally confirm the diagnosis of the disease. No
199 clinical signs compatible to PMWS were observed in farms 7 and 8 and no pathological
200 studies were carried out. Mortality rates of the fattening period in each studied farm are
201 given in table 1.
202
203 *PCV2 PCR and ORF2 sequencing*
204 Prevalence of PCV2 by PCR in pig sera from PMWS affected farms calculated based on
205 necropsied animals ranged from 40 to 93.8%. The highest prevalence in those farms
206 was found in PMWS affected pigs (median 85.7%; max. 100%, min. 66.7%), followed
207 by wasted non-PMWS affected pigs (median 83.3%; 100- 33.3%), healthy pigs (median
208 60.0%; 75.0-20.0%) and sows (median 0%; 14.3%-0%). On the other hand, the
209 prevalence was 10% in both non-affected PMWS studied farms.
210
211 A total of 87 ORF2 PCV2 sequences coming from 60 animals (58 pigs and 2 sows)
212 were obtained (Table 1). Seven out of 60 (11.7%) serum samples tested corresponded to
213 pigs yielded more than one sequence; all of them from PMWS affected farms. Thirty-
214 four clones containing the ORF2 PCV2 sequence were obtained and sequenced from
215 those 7 serums containing multiple sequences. Results confirmed that those 7 animals
216 had more than one sequence at the same time. From these 34 sequences, 26 were
217 different in at least one nucleotide. Three out of the 7 animals harboured ORF2 PCV2
218 sequences showing low percentage of identity (91- 93%) (Table 2).
219
220 *Genotype definition*
221 The p-distance/frequency histogram obtained from the 235 ORF2 PCV2 sequences was
222 clearly bimodal; one mode corresponded to the number of differences between genotype

1 and genotype 2, and the other to differences among sequences within genotypes (Figure 1). Considering the possible different cut-off values tested for the definition of genotypes, only one gave results that matched phylogenetic results. That value was finally established at 3.5%, since it was located in a fairly equidistant position between both peaks and agreed with the distance observed between both groups 1 and 2 in the NJ phylogenetic tree. Thus, two ORF2 PCV2 sequences could be assigned to different genotypes (1 or 2) when the genetic distance between them was >3.5%. On the other hand, the branch length observed in the collapsed PCV1 rooted NJ tree (Figure 1) was higher in genotype 2 than in genotype 1, indicating major variability inside genotype 2.

According to this definition, sequences included in groups 1 and 2 (Olvera et al., 2007) would constitute genotypes 1 and 2, respectively. It is worthy to say that a similar p-distance/frequency histogram is obtained using the 148 whole PCV2 sequences present in the NCBI nucleotide database (http://www.ncbi.nlm.nih.gov) in September 2005 (Olvera et al., 2007). In that case, the cut-off value to differentiate PCV2 genotypes was established at 2.0% (data not shown).

Phylogenetic analysis of ORF2 sequences

All three algorithms for ML, MP and NJ methods reported congruent results, and the groupings were supported by high confidence values. NJ tree with 1000 bootstrap is shown in Figure 2. The 87 sequences obtained could be divided into two main clusters supported by high confidence values that matched the two genotypes defined above: genotype 1 (n=75) and genotype 2, (n=12), equivalent to groups 1 and 2 previously reported by Olvera et al. (2007). Interestingly, PCV2 sequences detected in animals coming from PMWS affected farms were mainly included within genotype 1,

248 corresponding more specifically to cluster 1A of Olvera et al. (2007) classification, and
249 were randomly distributed among this group. On the other hand, genotype 1 was never
250 found in non-PMWS affected farms. All sequences obtained from healthy pigs from
251 non-PMWS farms (n=6) were included within genotype 2, corresponding to clusters 2C
252 and 2D of Olvera et al. (2007) classification. The other 6 sequences included within
253 genotype 2 corresponded to sequences obtained from 3 animals (one PMWS case, one
254 wasted non-PMWS and one healthy pigs) from PMWS affected farms. At the same
255 time, these 3 animals also harboured at least one sequence included in genotype 1.
256 Therefore, none of the PCV2 infected pigs coming from PMWS affected farms
257 contained only ORF2 PCV2 sequences genotype 2, since all of them were always co-
258 infected with PCV2 genotype 1.
259
260 Two ORF2 PCV2 sequences obtained from two sows from the same PMWS affected
261 farm (No. 1) were also included in genotype 1 and had a sequence identity of 100%.
262 Moreover, from one of those sows, two ORF2 PCV2 sequences were obtained from two
263 of its offspring (one PMWS and one wasted non-PMWS affected pigs). Interestingly,
264 sequences obtained from both pigs had a 100% of identity between them and were
265 different from that obtained from its mother (sequence identity: 99.4%, having 4
266 nucleotide differences, resulting in 4 amino acid predicted differences).
267
268 The lowest nucleotide and amino acid homology observed between all ORF2 PCV2
269 studied sequences was 90.2% and 86.5%, respectively. Three different regions with
270 high heterogeneity were observed in amino acid positions 57-91, 121-136 and 185-191
271 and the amino acid alignment showed the existence of specific patterns to each group.
272 Thus, up to 15 amino acid substitutions were observed in all the 12 sequences present in

genotype 2, all of them fairly conserved within genotype 1. Eleven out of 15 positions suffered always the same substitution, and 8 out of those 11 positions were located within the first described heterogenic region (residues 57-91). The other three constant substitutions were located at positions 190, 210 and 232 (figure 3).

Phylogenetic analysis among populations (farms)

The high level of population structuring observed in the AMOVA analyses (φst=0.79494, $p<<0.001$) indicates that the pattern of population relationships is related with the presence or absence of PMWS at farm level (φct=0.74418, $p<<0.001$). In addition, the low level of variation detected among farms within groups (φsc=0.19840, $p<<0.001$) emphasizes the uniformity of the groups described (Table 3).

Additional support to the differences detected among farms is provided by the population tree (Figure 4), where clustering also followed the presence or absence of PMWS disease. Moreover, farms 2 to 6 were more closely related between them than with farm No. 1. Despite PMWS could not be diagnosed in the batch of studied animals from farm No. 6, that farm was grouped with PMWS affected, being in agreement with the clinical picture observed and the previously diagnosed history of PMWS.

Discussion

In the present study, we characterized and compared 87 ORF2 PCV2 sequences obtained from 60 animals from 7 different farms with the main aim to elucidate if ORF2 PCV2 sequences could be correlated with different health/disease status of PCV2 infected pigs and/or farms. Present results demonstrate the existence of two different genotypes within PCV2 sequences: genotypes 1 and 2. While pigs from PMWS affected

298 farms harboured PCV2 genotype 1 always (with or without sequences from genotype
299 2), pigs from non-PMWS affected farms had exclusively sequences from genotype 2.
300 Moreover, we examined if multiple PCV2 different sequences can be present in the
301 same animal at the same time, which was demonstrated in 7 out the 60 PCV2 infected
302 studied pigs.
303
304 Globally, in the seven studied farms, we observed a higher prevalence of PCV2 in pigs
305 coming from PMWS affected farms than in pigs coming from non-PMWS affected
306 farms, being in agreement with previous reports (Larochelle et al., 2003; Shibata et al.,
307 2003; Sibila et al., 2004). At the same time, within PMWS affected farms, the
308 prevalence of PCV2 in PMWS affected pigs was also higher than in healthy pigs (Liu et
309 al., 2000; Larochelle et al., 2003).
310
311 It is known that the highest amount of virus in PCV2 infected pigs is found in lymphoid
312 tissues (Mankertz et al., 2000). However, we decided to perform the present study on
313 serum samples based on previous works that indicated blood as the most suitable
314 sample for PCV2 detection by PCR without the need of euthanizing the animal (Shibata
315 et al., 2003). Despite we sacrificed either diseased and healthy pigs from PMWS
316 affected farms, the use of serum samples allowed us to analyze a high number of
317 healthy animals from non-PMWS affected farms without euthanizing them.
318
319 The mandate of the International Committee on Taxonomy of Viruses (ICTV) does not
320 include any consideration below the species level and there is no formally accepted
321 definition for any taxa below it; hence, this has been left to the initiative of specialty
322 groups (Fauquet and Stanley, 2005). In the present work, an effort was made to evaluate

the presence of distinct PCV2 genotypes and to unify the variable terminology that is nowadays reported in the literature. Obtained results demonstrate the existence of two clearly distinct PCV2 genotypes: genotypes 1 and 2 supported by molecular and biological features. Both genotypes corresponded to two different groups that were already described in different studies but usually using different nomenclature. Thus, genotype 1 and 2 correspond respectively to groups 1 and 2 of Olvera et al. (2007), patterns 321 and 422 reported by Carman et al. (2006), I and II reported by De Boisséson et al. (2004), SG3 and SG1/SG2 reported by Timmusk et al. (2005), and A and B reported by Martins Gomes de Castro at al. (2007).

Our results support the hypothesis of differences in pathogenicity between PCV2 genotypes, since differences between ORF2 PCV2 sequences obtained from animals from PMWS affected farms (mainly from genotype 1) and from animals from non-PMWS affected farms (all of them from genotype 2) were observed. Therefore, present results suggest that viruses from genotype 1 may potentially be more pathogenic than those from genotype 2 (Timmusk et al., 2005; Cheung et al., 2007a, 2007b; Stevenson et al., 2007). In addition, the major variability found inside genotype 2 using the data from NCBI until September 2005 suggest that this genotype is older than genotype 1 (Carman et al., 2006; Cheung et al, 2007a). Although in favour, if this explains the emergence of PMWS by late 90s in many European and Asiatic countries as well as the recent re-emergence of the disease in North-America remains to be elucidated. On the other hand, present data confirmed that within PMWS affected farms there is no association between the PCV2 sequences contained in individual pigs and its clinical status, being in agreement with some previous case-control studies (Larochelle et al., 2002; Pogranichniy et al., 2002).

There are different possible explanations because some previous works were not able to find such association between PMWS affected and non-PMWS affected farms (de Boisseson et al., 2004; Martins Gomes de Castro et al., 2007): on one hand, the presence of multiple sequences in the same animal can produce the sequencing of only one of the virus present in the pig. Moreover, obtaining PCV2 from non-PMWS affected farms requires exhaustive sampling due to the low viral load in serum of the animals (Olvera et al., 2004). On the other hand, it is very difficult to establish a farm as a non-PMWS affected due to the ubiquitous presence of PCV2 and that low number of cases of PMWS can be unnoticed (for example, one of the sampled animals in de Boissesson et al. (2004) initially considered as a non-PMWS farm was finally diagnosed as PMWS). Moreover, the lack of confident information about the farm sanitary status in the public available Genbank data makes practically impossible to compare both populations using Genbank data (Olvera et al., 2007).

Since the first description of the disease (Harding and Clark, 1998), many experimental infections have been performed and just a few of them have been successful in reproducing the disease using either PCV2 genotype 1 (Albina et al. 2001; Grasland et al., 2005; Wang et al., 2007) and genotype 2 (Allan et al., 1999, Krakowka et al., 2000; Harms et al., 2001; Allan et al., 2003). Therefore, both PCV2 genotypes seem to be able to reproduce PMWS under the appropriate circumstances. However, one recent experimental infection with PCV2 from both groups described more severe PMWS clinical signs and lesions in pigs infected with PCV2 genotype 1 than those infected with PCV2 genotype 2 (Cheung et al., 2007b). On the other hand, using PCV2 genotype 2, Opriessnig et al. (2006) were not able to reproduce PMWS, but they demonstrated

373 differences in pathogenicity even within genotype 2, further supporting such variability
374 among PCV2 sequences.
375
376 Population phylogenetic analysis showed significant differences between ORF2 PCV2
377 sequences from PMWS affected and non-PMWS affected farms. Those findings were
378 strongly supported by the population tree, where non-PMWS affected farms were
379 closed to each other and separately from PMWS affected farms. These two groups fitted
380 perfectly the two genotypes obtained in the nucleotide sequence analysis. Moreover, the
381 low variation observed in the population phylogenetic tree between PMWS affected
382 farms could apparently be explained by the geographic location of breeding and/or
383 weaning facilities, being in agreement with previous reports suggesting that minor
384 genetic differences among PCV2 sequences could be accounted by their geographic
385 origin (Fenaux et al., 2000; Meehan et al., 2001). Thus, sequences obtained from farm 1
386 (with breeding and weaning facilities separated approximately by 180 Km from the rest
387 of the studied farms, data not shown) had less similitude with those obtained from farms
388 2 to 6 (located all five in a radium of 40 km). On the contrary, there was no apparent
389 relation with the geographic distribution of fattening facilities. On the other hand, data
390 from one sow and its two offspring showed that ORF2 PCV2 sequences obtained from
391 both pigs were identical between them and different from that obtained from the mother.
392 Considering the possibility here demonstrated that different sequences can be present in
393 the same animal at the same time, we can not rule out that both pigs would have got
394 PCV2 from its sow. Moreover, the fact that both ORF2 PCV2 sequences obtained from
395 two different sows located in the same farm and in the same room were identical could
396 be explained easily by horizontal transmission of PCV2 (Albina et al., 2001; Sibila et
397 al., 2004).

Previous reports suggested that one animal can contain different PCV2 sequences at the same time (de Boisseson et al., 2004; Opriessnig et al., 2006) but this is the first work that have exhaustively studied this issue. Present results demonstrate that this is a relatively frequent finding in PMWS affected farms and that occurs in PMWS affected as well as in healthy animals. Interestingly, three animals (one PMWS, one wasted non-PMWS and one healthy) harboured sequences from genotypes 1 and 2 at the same time. Curiously, clones obtained from the PMWS affected pig contained predominantly sequences from genotype 1 while clones obtained from the healthy pig contained sequences predominantly from genotype 2. The biological importance of this fact is nowadays unknown, but it may reinforce the hypothesis that sequences from genotype 1 could be more pathogenic than those from genotype 2.

The amino acid alignment identified three major heterogenic regions between different ORF2 PCV2 sequences in regions 57-91, 121-136 and 185-191, similarly to those already described (Larochelle et al., 2002) and corresponding to two out of the three antigenic domains described by Mahe et al (2000) (65-87, 113-139 and 193-207). Larochelle et al. (2002) pointed out the possible implication of these immunoreactive regions of the capsid protein of PCV2 as potential candidate regions involved in the emergence of PCV2 variants, data supported also by other recent reports (Wen et al., 2005). Specifically, up to 8 amino acid positions located in positions 57-91 were constantly different between sequences included in genotype 2 in respect those included in genotype 1. These data suggest that specific primers within this region could be designed to differentiate both genotypes by PCR techniques instead of using sequencing, resulting in a less expensive and time consuming diagnostic method.

In summary, the present work contributes to the understanding of PCV2 epidemiology, including the establishment of a genotype definition for PCV2 that should help unifying different nomenclatures and classifications used. Taking into account the present results and other recent data from USA and Canada (Carman et al., 2006; Cheung et al., 2007a) it seems evident that nowadays PCV2 sequences included in genotype 1 are more related to PMWS occurrence than PCV2 sequences included in genotype 2. Further *in vivo* and *in vitro* studies are needed to confirm the suggested differences in pathogenicity between genotypes and to establish relationship between PCV2 genotypes and PMWS occurrence.

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Figure 1. PLOT: Frequency distribution of pairwise distances between ORF2 PCV2. TREE: Collapsed PCV1 rooted NJ tree deduced from the comparison of 235 PCV2 ORF2 sequences. Vertical arrows indicate the cutt-off value to distinguish both genotypes. Sequences belonging to distinct genotypes show genetic distances > 0.035.

Figure 2. Phylogenetic tree based on the NJ method for 87 ORF2 PCV2 sequences and PCV1 rooted using 1000 bootstraps. Numbers along the branches refer to the percentages of confidence in the NJ, MP and ML analyses respectively. Minor branches values are hidden. References means: number of farm-number of sow-number of piglet-weeks of age. ● =PMWS case, ○ = wasted non-PMWS case, □ = Healthy from PMWS affected farm, Δ = Healthy from non-PMWS affected farm, ◊ = sow. * =sequences obtained from different clones of same animal.

Figure 3. Amino acid sequences of capsid protein. All 12 obtained sequences from genotype 2 as well as 12 randomly selected sequences from genotype 1 are represented. Heterogenic regions are marked in black lines. References means: number of farm-number of sow-number of piglet-weeks of age. * =sequences obtained from different clones of same animal.

Figure 4. Population tree (NJ) clustering 8 farms according to the distance matrix of nucleotide divergence. ● =history of PMWS with PMWS diagnosed in the studied batch, ○ = history of PMWS without PWMS diagnosis in studied batch, Δ = non-PMWS affected farm.

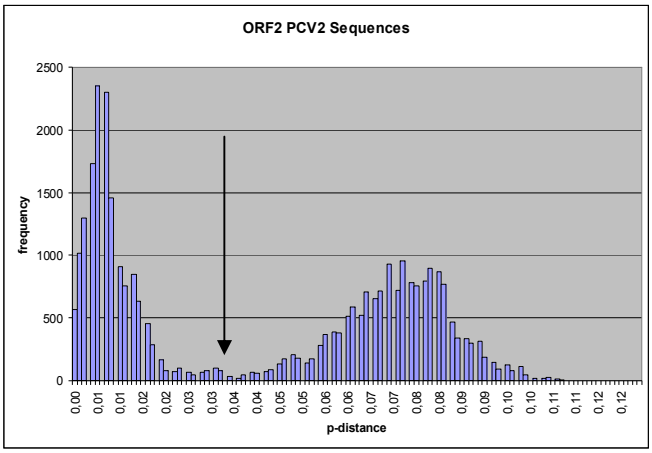
646 **Table 1**. Characteristics of farms and studied animals.

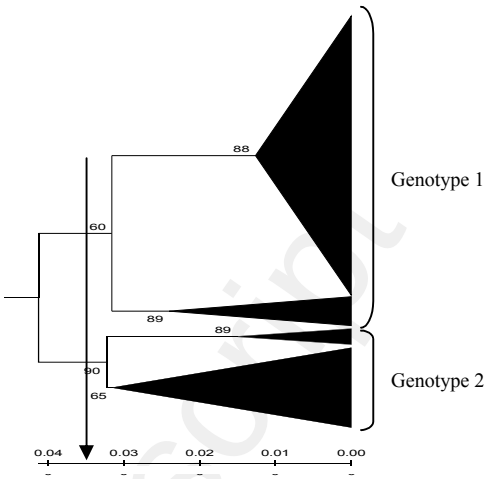
647

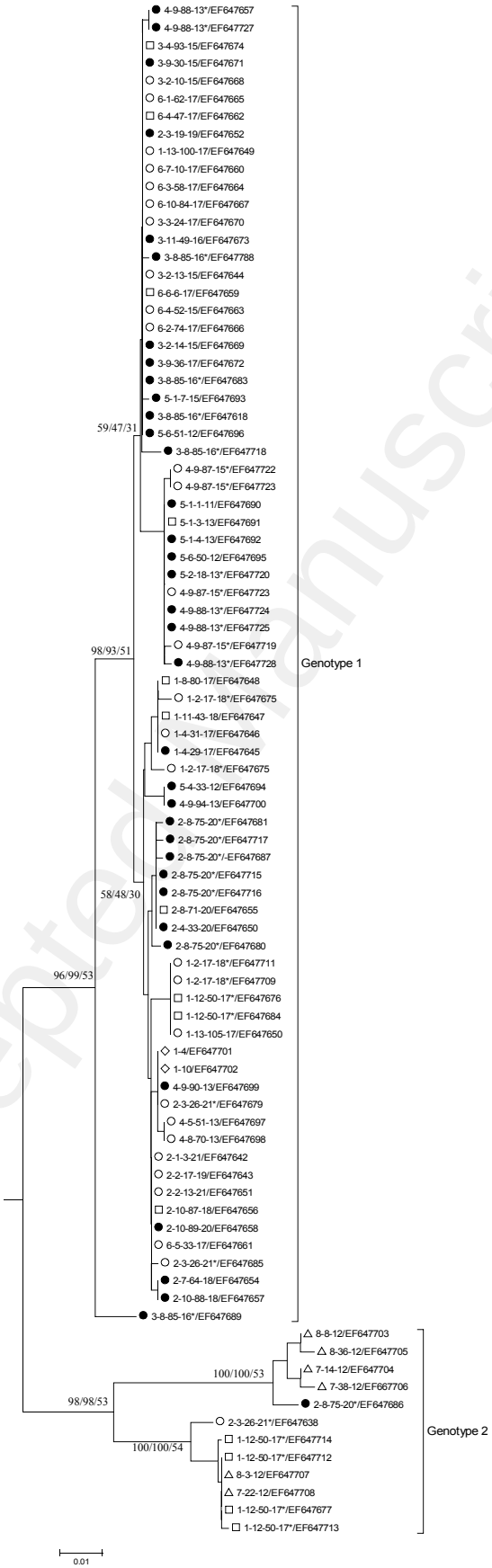
648 **Table 2**. Animals showing more than one ORF2 PCV2 sequence at the same time.

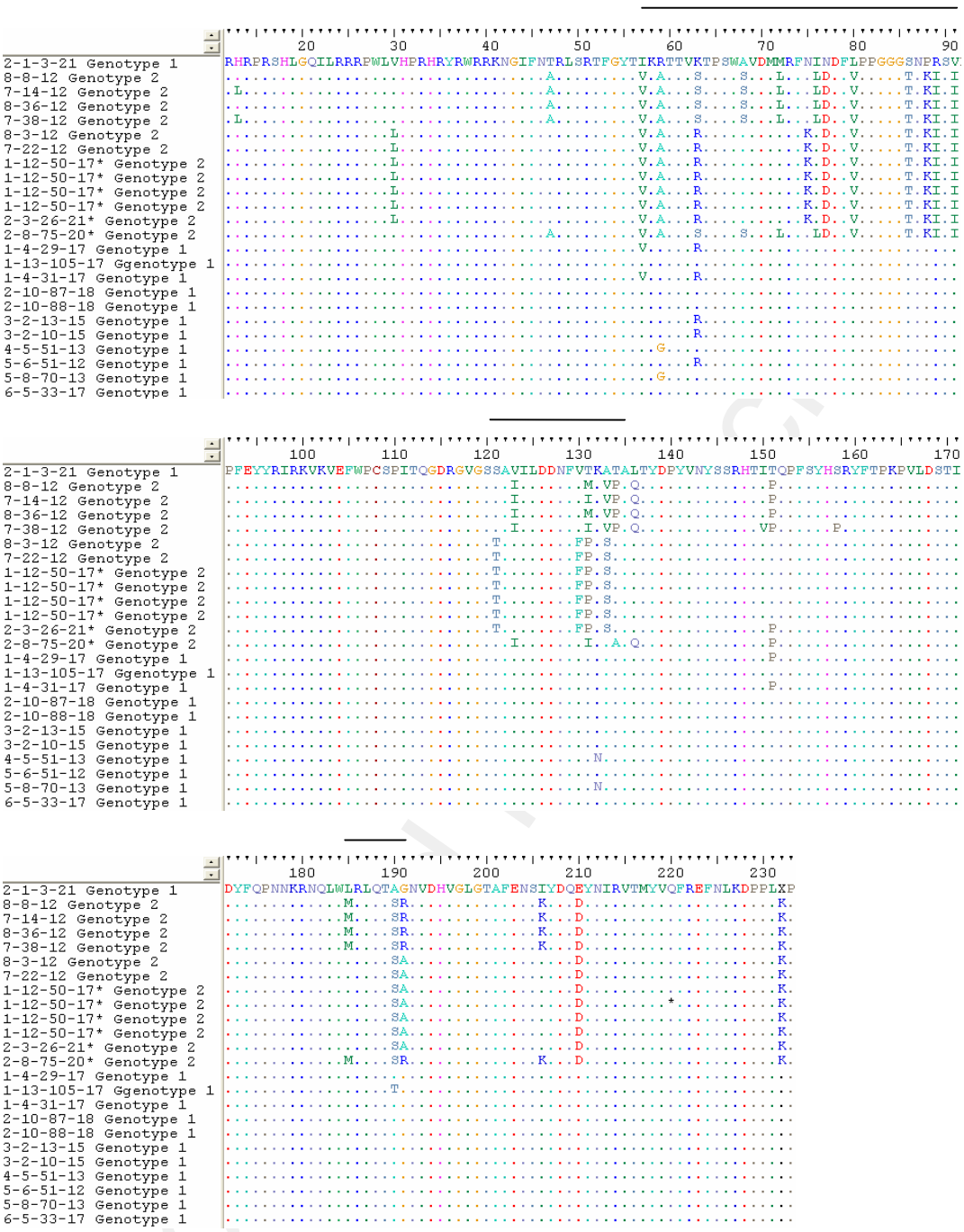
649

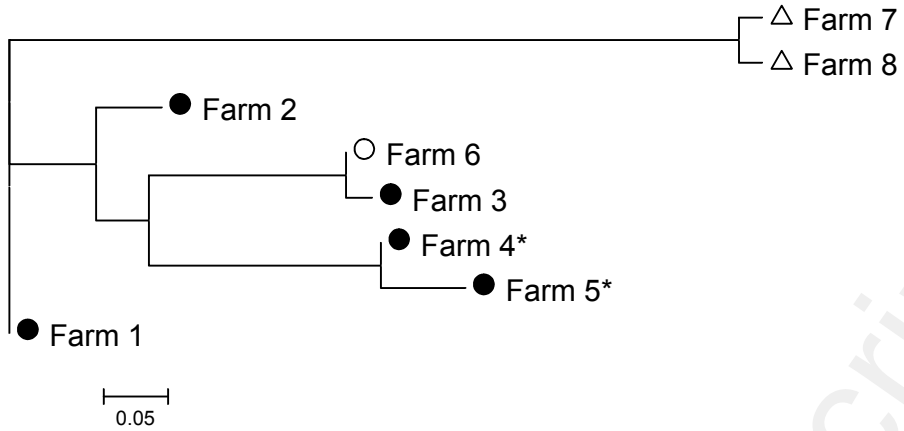
650 **Table 3.** Hierarchical nested analysis of molecular variance (AMOVA) results.











*Farms 4 and 5 corresponded to two independent studied batches coming from the same farm.

Farm	History of PMWS	Clinical PMWS compatible picture	Weeks of Age	PMWS Confirmation	Number of studied animals (number of animals with available ORF2 PCV2 sequence)				% Mortality*
					PMWS	Wasted non-PMWS	Healthy	sows	
1	Yes	Yes	17-18	Yes	2 (1)	6 (4)	4 (3)	14 (2)	6.0
2	Yes	Yes	18-21	Yes	7 (6)	4 (4)	5 (2)	12 (0)	8.0
3	Yes	Yes	15-17	Yes	6 (5)	6 (3)	4 (1)	12 (0)	10.0
4	Yes	Yes	12-15	Yes	4 (3)	6 (3)	5 (0)	12 (0)	17.4
5	Yes	Yes	11-15	Yes	9 (7)	2 (0)	5 (1)	12 (0)	17.4
6	Yes	Yes	15-17	No	0 (0)	12 (7)	5 (2)	12 (0)	7.0
7	No	No	12	No	0	0	40 (3)	0	4.3
8	No	No	12	No	0	0	40 (3)	0	4.9
Total					28 (22)	36 (21)	108 (15)	74 (2)	

* = percentage of mortality during the fattening period (from 8 to 24 weeks of age).

Pig Reference*	Clinical status	No. of different sequences in the same pig (No of clones sequenced)	No. of different sequences within genotype 1 (number of sequenced clones)	No. of different sequences within genotype 2 (number of sequenced clones)	Sequence identity between different sequences
1-17	Wasted non-PMWS	3(4)	3(4)	0(0)	98.8-99.2
2-26	Wasted non-PMWS	3(3)	2(2)	1(1)	92.9-99.7
4-87	Wasted non-PMWS	3(4)	3(4)	0(0)	99.7-99-8
2-75	PMWS	6(7)	5(6)	1(1)	91.3-99.8
3-85	PMWS	3(5)	3(5)	0(0)	99.4-99.8
4-88	PMWS	3(5)	3(5)	0(0)	99.1-99-8
1-50	Healthy	5(6)	1(2)	4(4)	92.5-99.8
Total		26(34)	20(28)	6(6)	90.2-99.8

*Number of farm-number of pig.

Source of variation	df	Sum of squares	Variance components	% of variation	Related φ-statistics	P value
Within farms	81	440.6	5.43	20.50	φst =0.79494	<<0.001
Among frams within groups	6	128.3	1.34	5.08	φsc=0.19840	<<0.001
Between groups	1	231.5	19.74	74.42	φct=0.74418	<<0.001
Total	88	800.3	26.52	100		