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Examination for a viral co-factor in Postweaning Multisystemic Wasting Syndrome (PMWS)

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Abstract

In order to test the hypothesis that a putative co-factor for the development of PMWS (Postweaning Multisystemic Wasting Syndrome) in pigs could be of viral origin, we performed extensive virological examinations on organ material from pigs diagnosed with PMWS originating from within a Danish PMWS-transmission study. Virus isolation attempts were carried out on a large panel of different cell types including primary pig kidney cells and lung macrophages, primary rabbit kidney cells and 7 established cell lines (MARC-145, ST117, PK15, BHK21, HeLa, Vero, and MDCK). Although these represent cells with susceptibility to a wide range of known viruses, the results did not provide evidence for a specific virus other than PCV2 contributing to the development of PMWS. Furthermore, in order to test whether specific genotypes of PCV2 may trigger the

switch from PCV2 infection to clinical disease, we compared complete DNA genome sequences of PCV2 derived from PMWS-positive as well as PMWS-negative pigs. On the basis of the DNA sequences, the PCV2 isolates were divided into two groups. Group 1 consisting of one isolate originating from a herd unaffected by PMWS, with group 2 consisting of 9 isolates originating from 4 PMWS-affected herds, 4 PMWS positive pigs plus 1 unaffected herd. The PCV2 genomes from the 2 groups showed 95.5 % identity. Alignment analyses of the sequences encoding the replicase and capsid protein from group 1 and group 2 PCV2 isolates showed 2 amino acid differences encoded in the replicase protein, while 19 amino acid differences were predicted among the capsid protein sequences. The PCV2 DNA sequence analysis supports recent observations from studies in USA as well as Europe, which suggest that strain variations may influence the clinical outcome of PCV2 infection.

Keywords: PMWS; PCV2; co-factor; virus isolation; strain variation

1. Introduction

Postweaning Multisystemic Wasting Syndrome (PMWS) is causally associated with porcine circovirus type 2 (PCV2) (Allan et al., 1998; Ellis et al., 1998). The disease is characterised by a constellation of clinical signs including wasting, jaundice and respiratory disease with increased mortality in growing pigs between 5-14 weeks of age (Harding and Clark, 1997). PMWS was first reported from high-health herds in Canada in 1991 (Clark, 1997). Since then, the disease has spread worldwide (Allan et al., 1998; Ellis et al., 1998; Allan et al., 1999a; Onuki et al., 1999; Choi et al., 2000). In Denmark, almost

all swine herds are positive for antibodies against PCV2, thus indicating the widespread occurrence of PCV2 in Danish pigs. On the other hand, the observation that only some herds experience clinical problems with the disease (Vigre et al., 2005) suggests a complex etiology.

Infection with PCV2 is crucial for the development of PMWS (Kennedy et al., 2000; Krakowka et al., 2000; Ladekjær-Mikkelsen et al., 2002), but apparently this virus is not the only factor triggering the disease. Various studies have provided assessments of risk factors for development of PMWS (Pogranichniy et al., 2002; Rose et al., 2003; López-Soria et al., 2004; Wallgren et al., 2004; Vigre et al., 2005). Notably, a recent Danish study demonstrated the risk reducing effects of certain biosecurity measures in herds and supports an unknown infectious component in PMWS (Enøe et al., 2006). However, specific co-factors involved in generating the disease complex remain to be identified.

Several studies aiming at establishing a challenge model for PMWS indicate that other virus infections, e.g. porcine parvovirus (PPV) and porcine reproduction and respiratory syndrome virus (PRRSV), could eventually potentiate PMWS (Allan et al., 1999b; Allan et al., 2000; Kennedy et al., 2000; Krakowka et al., 2000; Rovira et al., 2002; Vigre et al., 2006). In order to test the hypothesis that a putative co-factor could be of viral origin, we performed extended virological examinations on organ material from pigs diagnosed with PMWS originating from a Danish PMWS-transmission study (Kristensen et al., 2004).

This study wished to investigate whether healthy pigs from herds unaffected by PMWS would develop PMWS after close contact with pigs which displayed clinical symptoms of PMWS. Two to 4 weeks after mingling, clinical symptoms of PMWS were observed in pigs from the PMWS-unaffected herds, indicating that the disease complex had been

transmitted from diseased to healthy individuals. Our efforts to identify and characterise a viral co-factor in PMWS were based on virus isolation attempts and electron-microscopic examination of tissue material from a number of these pigs which became PMWS-positive after mingling.

The role of a specific genotype of PCV2 for the development of full PMWS is currently debated. Although a number of studies have reached the conclusion that the PMWS epidemics do not originate from the appearance of a new PCV2 variant (Pogranichniy et al., 2002; de Boissésou et al., 2004), a recent Swedish study, comparing capsid coding sequences determined for material collected from PMWS-positive and PMWS-negative pigs in Sweden, revealed differences at the nucleotide as well as the amino acid level (Timmusk et al, 2005). With this background, PCV2 DNA derived from a number of PMWS-positive and PMWS-negative pigs within the Danish PMWS-transmission study were sequenced in order to elucidate whether an association between specific genotypes and disease could be found.

2. Materials and Methods

2.1. Origin of organ material used in the study

The organ material was obtained from pigs included in the Danish PMWS-transmission study (Kristensen et al., 2004). These pigs originated from two PCV2 positive but PMWS-unaffected herds (herds 1 and 2). After weaning, the pigs were transferred to animal experiment facilities and kept in close contact with other pigs showing clinical symptoms of PMWS. The latter pigs originated from 4 PCV2-positive, PMWS-affected herds (herds A, B, C, and D). For virological examination, we selected 5 PMWS-positive

pigs, which in the course of the experiment developed clinical symptoms consistent with PMWS and typical histopathological lesions, i.e. lymphoid depletion together with histiocytic infiltration and/or giant cells and/or inclusion bodies as well as detection of PCV2 in moderate to massive quantity in lymphoid tissues (Jorsal et al., 2006): Pig no. 134 originating from herd 1 having contact with pigs from herd A. Pigs no. 152 and 157 (tissues from these 2 pigs were pooled) originating from herd 1 having contact with pigs from herd B. Pig no. 179 originating from herd 2 having contact with pigs from herd C. Finally, pig no. 206 originating from herd 2 having contact with pigs from herd D. Prior to examination, tissue samples were minced and diluted 1:10 in Eagle's Minimum Essential Medium (EMEM) containing 0.5 mg/ml neomycin and 1.0 mg/ml streptomycin, homogenized (stomacher 80) and clarified at 1900g for 10 minutes. The supernatants were filtered through 0.45 µm filters and stored at -80°C . Two samples were prepared from each pig: Sample 1 was prepared from lung and liver, while sample 2 was derived from lymphoid tissue (spleen, mesenteric and inguinal superficial lymph nodes). PCV2 samples were also isolated from lymphoid tissues obtained from the donor herds, i.e. herds 1, 2, A, B, C, D.

2.2. Cell culture material

For virus isolation studies, samples derived from the tissues were incubated for a minimum of two passages on a panel of cell culture monolayers. Since the virus target was unknown, a range of different cell types were chosen to provide extensive opportunity for virus growth with regard to the availability of target cells and virus receptors. The cells used were: 1) Primary porcine kidney cells (PK) – these are routinely

120 used for diagnostic virus examination of field samples at our Institute and are known to
 121 support the replication of swine influenza virus (SIV), porcine parvovirus (PPV),
 122 haemagglutinating encephalomyelitis virus (HEV) and Aujeszky's disease virus (ADV).
 123 2) Porcine pulmonary alveolar macrophages (PPAM) – these are considered as one of the
 124 few cells sensitive to porcine cytomegalovirus (Edington, 1989) and are known to support
 125 the replication of PRRSV (Wensvoort et al., 1991; Bøtner et al., 1994). The PPAM used
 126 in the present experiment were obtained by lung lavage of euthanized 4- to 6-week-old
 127 pigs from the Institute's own high health herd, which is free from mycoplasmas and a
 128 wide range of bacterial and viral infections, as previously described (Ladekjær-Mikkelsen
 129 et al., 2002; Lauritsen, 2005). The cells obtained contained > 95% alveolar macrophages.
 130 3) MARC-145 cells (kindly provided by Dr. Ferrari, IZSBS, Brescia, Italy), which are
 131 susceptible to the American type of PRRSV (Kim et al., 1993; Bøtner et al., 1994). These
 132 cells are generally accepted as one of the most sensitive cell lines for propagation of
 133 porcine rotavirus (Debouck, 1989). 4) ST117 cells (ATCC, CLR-1746TM), these are
 134 susceptible to swine enterovirus, porcine coronavirus (McClurkin and Norman, 1966) and
 135 adenoviruses (Derbyshire, 1989). 5) PK15 cells which are susceptible to several viruses,
 136 including PCV type 2 (Allan et al., 1998) and these PK15 cells (kindly provided by Dr.
 137 G. Allan, DARDNI, Belfast, UK) were free from PCV type 1, which is a known
 138 contaminant of some PK15 cell line variants (Tischer et al., 1974). 6) BHK 21 cells
 139 (ATCC, CCL-10TM), these are susceptible to a number of porcine enteroviruses, porcine
 140 encephalomyocarditis virus (EMC) (Acland, 1989), and foot-and-mouth disease virus
 141 (Mowat and Chapman, 1962). 7) HeLa (ATCC, CCL-2TM) and Vero cells (ATCC, CCL-
 142 81TM) support the replication of several viruses, including porcine enteroviruses

(Derbyshire, 1989). Furthermore, the Vero A cells are characterized by lacking the ability to produce interferons. The lack of this cytokine, which causes susceptible cells to express potent antiviral mechanisms, limiting further viral growth and spread (Stark et al., 1998), is likely to increase the general virus susceptibility of Vero cells. 8) MDCK cells (ATCC, NBL-2, CCL-34TM) are susceptible to a range of viruses, e.g. vesicular stomatitis virus, vesicular exanthema virus of swine, reovirus and SIV and 9) Primary rabbit kidney cells (RK), are often used for propagation of various viruses, e.g. human adenovirus (Jogler et al., 2006), bovine viral diarrhoea virus (L. Rønsholt, personal communication), and herpes simplex virus (Landry et al., 1982).

2.3. *Virus isolation*

Tissue homogenate suspensions were inoculated onto cell cultures at 50-75% and 100% confluency using 200 µl/well in 2 ml growth medium (EMEM supplemented with 5% fetal calf serum) in 24-well multidish plates (Nunc, Denmark) and cultivated for two passages. After inoculation the plates were incubated at 37°C in a 5% CO₂-air atmosphere. After three to four days of incubation, one plate was fixed with alcohol for subsequent staining, while the second plate was freeze/thawed and inoculated onto fresh cell cultures on 2 new plates. These were incubated as above and after another 3 to 4 days, one plate was fixed whereas the other was frozen for putative additional analysis. For identification of viruses, the cells during the first and second passage were observed for cytopathic effect (CPE) and after fixation stained in an immunoperoxidase monolayer assay (IPMA) according to the method described by Bøtner et al. (1994). Briefly, after the supernatants were discarded and the cells were fixed in ice-cold absolute ethyl alcohol

for 45 minutes at 5°C and washed with PBS containing 0.1 % Tween-20 (PBST), the cell cultures were incubated with the immune sera listed in Table 1. Serum was diluted 1:100 or 1:200 in PBST supplemented with 5 % skimmed milk powder (PBST-5 %SMP), was added to the wells and incubated for 30 minutes at 37°C. Following washing, peroxidase conjugated rabbit anti-swine IgG (P0164, DAKO, Denmark) diluted 1:200 in PBST-5 %SMP was added and the plates were incubated for 30 minutes at 37°C. Finally, the substrate (ethylcarbazol) was added and the plates were left for 20 minutes at room temperature.

After evaluation of the two passages for each cell type, frozen material corresponding to cell cultures where CPE or positive staining was observed were further propagated for six cell culture passages (extended examination). For these studies, we used:

1) Established cell lines (MARC-145, ST117, PK15, BHK21, HeLa, Vero, and MDCK). Frozen cell culture material from the first passage was inoculated 1:10 into a fresh cell culture suspension of the same cell type. The cell suspension was divided into two 10 ml plastic cell culture flasks (Nunc, Denmark) and after incubation for 3-4 days, one of the cell culture flasks was freeze/thawed whereas the cells in the second flask were trypsinized. The cells from the two flasks were pooled, supplemented with fresh cell culture medium, and distributed into two new flasks and two 96-well microtiter plates (Nunc, Denmark). Incubation was performed under the same conditions as mentioned above. After three days, the microtiter plates were fixed with alcohol and stained in IPMA and the two cell culture flasks were passaged as stated above. This procedure was performed in total for 6 passages.

2) Primary cells (PK and RK cells).

Frozen cell culture material from the first passage was inoculated 1:10 into a fresh cell culture suspension of the same cell type in a 10 ml cell culture flask and incubated for three to four days. Hereafter, the flask was freeze/thawed, supplemented with a fresh cell suspension and distributed into one new cell culture flask and two 96-well microtiter plates, respectively. After incubation the two plates were fixed and stained and the cell culture flask was passaged as stated above in total for six passages.

3) PPAM cells.

One 10 ml cell culture flask was seeded with 9 ml cell suspension. After one hour of incubation at 37°C in a 5% CO₂-air atmosphere, 1 ml cell culture material from the first passage was added. After three to four days of incubation, the flask was freeze/thawed and the harvest distributed to a new cell culture flask (5 ml) and two 96-well microtiter plates (50 µl/well) which had been seeded with a fresh suspension of PPAM one hour before. After incubation the two plates were fixed and stained and the cell cultures were passaged as stated above in total for six passages.

All microtiter plates from the extended virological examination were fixed with alcohol and stained in IPMA with the sera used for the first two cell culture passages and also with the following antibodies; 2C6B12C2 (monoclonal antibody against structural protein of PCV2, kindly provided by Dr F. McNeilly, DARDNI, Belfast, UK), LPPV2 (monoclonal antibody against PPV, Lohse et al., 2005), SDOW17 (monoclonal antibody against PRRSV, Nelson et al., 1993), KA8 (serum from rabbit immunized with PCV1, produced at the Institute), PEV1/8/10 (pool of sera from guinea pigs immunized with porcine enterovirus subtype 1, 8 and 10, respectively, produced at the Institute), HEV (polyclonal swine serum with antibodies against HEV, produced at the Institute), F210

(monoclonal antibody against non-structural protein of PCV2, kindly provided by Dr F. McNeilly, DARDNI, Belfast, UK).

2.4. Electron microscopy

First passage cell cultures showing signs of changed morphology or nonspecific staining patterns were further examined by EM after preparation. Cell suspension material was freeze/thawed and then divided into two portions, one being treated with ultrasonography for further particle enrichment. Hereafter both portions were centrifuged at 30,000g for 45 minutes. Cell pellets were resuspended in 10 mM Tris-buffer, pH 7.4, one droplet was put on a microscopic grid (standard square mesh, 300, from EMS, Hatfield, PA) and stained with Sodium silicotungstate 2% (ICN Biomedicals Inc., Ohio) before examination in a Zeiss 10-C electron microscope.

2.5. DNA sequencing

Using the QIAamp DNA minikit extraction procedure according to the manufacturer's instructions (QIAGEN GmbH, Germany), total DNA was extracted from lymphoid tissue of 10 pig samples.

The PCV2 genome was amplified as two overlapping PCR products with the primer pairs 1-ALM1 and 1-ALM2 for PCR product 1, and PCV2R and PCV2 ORF2 SEK2 for PCR product 2 (see Table 2). The PCR product 1 (fragment size 1734bp) was generated using the Expand 20kb^{PLUS} PCR System (Roche, Diagnostics GmbH, Germany) in PCR reactions containing a final concentration of 1X Expand PCR buffer, 0.5 mM dNTP, 0.5 µM of each primer (1-ALM1 and 1-ALM2) with addition of 0.7 µl Expand DNA

polymerase and 2 µl dimethylsulfoxide (DMSO) (Merck, Germany) in a total volume of 50 µl. The cycling conditions were 1 cycle of [94°C for 3 min.], 45 cycles of [94°C for 15 sec., 53°C for 20 sec., 68°C for 2 min. with 1 sec. increase per cycle], and 1 cycle of [68°C for 7 min.]. PCR product 2 (fragment size 577 bp) was generated in PCR reactions (50 µl) containing 2 µl DMSO and a final concentration of 1X PCR Gold buffer (Applied Biosystems, Denmark), 2 mM MgCl₂, 0.2 mM dNTP, 0.5 µM of each primer (PCV2R and PCV2 ORF2 SEK2), and 2.5 U of Ampli Taq Gold DNA polymerase (Applied Biosystems, Denmark). The cycling conditions were 1 cycle of [94°C for 5 min.], 45 cycles of [94°C for 15 sec., 50°C for 20 sec., 72°C for 40 sec.], and 1 cycle of [72°C for 4 min.].

After gel electrophoresis, PCR products of the predicted fragment sizes were excised from the gel and purified using Ultrafree-MC Centrifugal Filter Devices (Millipore, USA) by centrifugation for 5 min. at 8000g.

DNA sequencing was performed as cycle sequence reactions using BigDye Terminator v. 1.1 kits (Applied Biosystems, Denmark) according to the manufacturer's instructions and analysed with an ABI 310 genetic analyser (Applied Biosystems, Denmark). Primers used for sequencing are listed in Table 2. Sequences were assembled and edited using the SeqManII software (DNASTAR, Lasergene, USA) and alignments were made using the DS Gene software (Accelrys, USA). Full-length DNA genome sequences from 10 PCV2 samples, representing pigs 134 and 152/157 (herd 1), 179 and 206 (herd 2) and herds 1, 2, A, B, C, D, were analysed (Table 3).

3. Results

3.1. Virological examination

PCV2 was isolated from organ material obtained from all of the examined pigs. With samples derived from 7 out of 8 organ pools (lymphoid pool from pig no. 134 was negative), nuclear staining for PCV2 was detected in the 1st or 2nd cell culture passage of porcine cell lines (PK15 and ST117) as well as in primary PK cells. In addition, PCV2 was isolated in the 1st cell culture passage of PPAM inoculated with the samples obtained from the lymphoid organ pool from 2 pigs (pigs no. 179 and no. 206).

Pronounced non-specific staining was observed in the 1st passages of the Vero A cell line when inoculated with organ material samples. In an attempt to determine the reason for this staining, EM examination was carried out. By this method, PCV2 was detected in these samples but no other particles of viral origin were observed.

Nuclear staining was seen in cultures of HeLa, RK, MDCK and BHK monolayers incubated with lymphoid tissue samples. However, this staining was of a sporadic nature and only detected in the first passage using the polyclonal serum DS pool (Table 1).

Following propagation, the staining was lost, suggesting either non-specific background staining or if specific, low-level infection or the presence of an agent of a non-replicating nature under the culture conditions.

In HeLa, RK and MDCK cell monolayers, CPE, was observed in the 1st cell culture passages of samples from the lung/liver organ pools. However, no antibody staining was observed, and when further passaged, neither CPE nor staining with antibodies could be detected. In BHK monolayer cultures, neither CPE nor specific staining could be detected.

Using MARC-145 cells, PRRSV was detected in the lymphoid tissue samples as well as in the samples from the lung/liver pool from pig no. 179.

Finally, no other viruses could be demonstrated through this extended virological examination.

3.2. PCV2 alignment analysis

PCV2 DNA from the 10 pig samples (Table 3) were amplified by PCR, sequenced and aligned. The sequence data for the examined PCV2 have been deposited in the GenBank database, and the accession numbers are listed in Table 3.

Comparison of the DNA sequence from PCV2 isolates obtained from the PMWS-affected herds (herds A, B, C, D) showed a very high degree of nucleotide identity, ranging from 99.8% to 99.9%. In contrast, the PCV2 isolates representing the herds unaffected by PMWS (herds 1, 2) showed a slightly lower level of sequence identity (95.6%). Interestingly, DNA sequences of PCV2 isolates originating from the two PMWS free herds compared to those from the PMWS affected herds, were 95.5% to 95.6% identical between herd 1 and herds A, B, C, D, while between herd 2 and herds A, B, C, D the sequence identity was higher (ranging from 99.7% to 100%). Finally, PCV2 from the PMWS-positive pigs selected for the virological examination was sequenced and all of these sequences showed a closer relationship to the sequences from the PMWS affected herds (99.6% to 100% nucleotide identity) than to sequences of PCV2 from the herds from where the pigs originated (95.6% to 99.8% nucleotide identity).

Alignment of the coding sequences for the replicase and the capsid protein, respectively, from PCV2 isolates from all herds in this study showed that the predicted amino acid

sequences for both the replicase and the capsid protein was identical for all the isolates except for isolate 1 from herd 1 (PMWS-unaffected herd). The amino acid sequence of the replicase in isolate 1 differed only at positions 34 and 266 as compared to the other PCV2 isolates in this study (Table 4). In contrast, 19 differences were found in the amino acid sequence of the capsid protein from isolate 1 as compared to the other isolates (Table 4).

The absence of a single nucleotide was observed near the 3'-terminus of the capsid protein coding sequence gene (nt 1042) for all the PCV2 sequences except for that from herd 1 (data not shown). However, this modification only resulted in the predicted incorporation of asparagine as the second last amino acid of the capsid-protein instead of lysine (data not shown).

4. Discussion

The present study provides new information with regard to the current attempts to identify a putative viral trigger for the development of PMWS.

In the first part of the study, we searched for a virological agent other than PCV2 that could be linked to the occurrence of clinical disease/PMWS. The definition of an ideal environment for propagation of a yet unknown factor is obviously not possible to determine in advance, and the identification of such a factor may even rely on pure coincidence.

In order to increase the chance of isolating a virus of hitherto unknown specificity, however, a broad variety of primary cell cultures as well as established cell lines originating from porcine tissue and other animal tissues were used for the virus isolation

assays. Using different selected cell types and culture conditions, a diverse range of cell culture environments for virus propagation was provided. The cell panel represented various target tissues for porcine viruses together with receptors for a wide range of virus families. A wide variety of cells including epithelial cells, fibroblasts and macrophages were represented. In addition, naïve as well as transformed cells were included.

Furthermore, the use of two stages of cell confluence, 50-75% and 100%, respectively, at the initial seeding of the tissue material, attempted to take into consideration the different replication properties of specific viruses, e.g. that various viruses require cells at different stages of the cell cycle to replicate. On the basis of this extensive examination, we conclude that

i) The isolation of PCV2 from the examined organ material was expected and supports the pig selection criteria, which included the presence of PCV2.

ii) PRRSV was isolated from one pig as well. This result was not surprising, as it is well known that PRRSV and PCV2 infection often co-exist in individual animals (Segales et al., 2002; Wellenberg et al., 2004; Vigre et al., 2006).

iii) We did not find any evidence for the presence of a specific virus that triggers PMWS. Despite the use of a wide array of variable cell culture monolayers for propagation of a putative co-factor, no candidate could be found. The sporadic staining observed in some of the first passage cultures remains obscure, since further passages did not reveal the nature of this staining. In order to strengthen our attempts to identify a hitherto undetected virus, we used a broad range of different antibodies (Table 1). Specifically, a difference in staining pattern between anti-sera originating from herds unaffected by PMWS versus PMWS-affected herds could give interesting information, since a specific

virus would be expected to stain with sera from PMWS-affected herds, but not with sera from PMWS-unaffected herds. Although no such difference was observed, the involvement of a yet unknown virological factor cannot be completely excluded, as alternative cell culture conditions may have been necessary for virus propagation. Finally, it may be speculated that a co-factor important for initiation of PMWS could be present only in the early phase of the infection and then be degraded or otherwise eliminated when clinical and pathological manifestations are evident.

Thus, our comprehensive, but at the same time also unsuccessful, attempts to isolate a virus other than PCV2 from PMWS-positive pigs suggest that the development of PMWS is not triggered by one particular virus other than PCV2. This conclusion is supported by the results of other virus isolation attempts in a recent Danish case-control study (Enøe et al. 2006; Vigre et al., 2006).

In order to elucidate whether specific genotypes may trigger the switch from PCV2 infection to clinical disease, the second part of the study compared DNA sequences of PCV2 obtained from organ material originating from PMWS-affected herds as well as from herds unaffected by PMWS. We found the PCV2 isolates to be divided into 2 groups (Table 4): Group 1, consisting of one isolate, originating from donor herd 1 (a herd unaffected by PMWS) and group 2, consisting of all isolates from PMWS-affected herds (herds A, B, C, D), donor herd 2 (another unaffected herd) and all individual pigs examined, i.e. the pigs from healthy donor herds which became PMWS-positive after contact with pigs from one of the PMWS-affected herds. Comparison of the genomes showed that all sequences obtained from PMWS-affected herds shared an almost identical PCV2 nucleotide sequence (99.6 to 100% nucleotide identity) whereas the

relationship to the healthy donor herd 1 was less close, about 95.5% nucleotide identity. The donor herd 2 sequence (a herd unaffected by PMWS) did not fit into this pattern as it showed a PMWS-linked PCV2 sequence. However, since this herd experienced clinical problems consistent with PMWS about 3 months after initiation of the transmission study, it may be speculated that this herd was not truly PMWS free at examination, explaining the PMWS-linked PCV2 sequence in this herd.

Based on the above stated observations we therefore suggest that, to develop PMWS a pig must have a PMWS-linked PCV2 sequence consistent with the group 2 PCV2 sequences. Although based on a small number of samples, our observations support the conclusion of a recent Swedish study that identified a typical amino acid ‘signature’ at 10 positions in a PMWS-linked PCV2 sequence, which differed from the sequence of non-PMWS linked PCV2 (Timmusk et al., 2005). Thus, the amino acid sequence found in our group 2 is identical to the amino acid sequence found to be linked to PMWS in the Swedish study whereas the amino acid sequence found in group1 is identical to the Swedish non-PMWS linked sequence.

An interesting observation was that PCV2 from pig 134 and pig 152/157, although originating from herd 1 (unaffected by PMWS), after contact with pigs from affected herds, had a replicase and a capsid protein, which was 100% identical to those from the PMWS affected herds (herds A, B, C and D), and as such differed from isolate 1 from herd 1. We speculate that the PMWS-linked PCV2 strain (group 2) binds more effectively to the host cell surface and therefore out-competes PCV2 isolate 1 found in herd 1. The level of pathogenicity of the PMWS-linked PCV2 strain may possibly be connected to this observation. However, this needs to be investigated further.

The PCV2 alignment analyses of the replicase and the capsid protein showed that most amino acid differences between group 1 and 2 were found in the capsid protein. This observation indicates that if strain variation is important for development of disease, it is likely that the capsid protein is responsible for the pathogenicity but this remains to be established. Furthermore, we identified a deletion in the nucleotide sequence of the capsid-gene from group 2. The loss of a single nucleotide at the 3' terminus of the capsid protein coding sequence does not influence the length of the capsid protein. Instead, the change results in incorporation of asparagine as the second last amino acid in the capsid protein instead of lysine. Asparagine is an uncharged, polar amino acid, whereas lysine is a positively charged, polar amino acid. Whether different properties of these two amino acids are important to the function of the capsid protein is unknown.

Previous studies on molecular characterization of isolates from PMWS affected and unaffected herds (Boisséson et al., 2004; Larochelle et al., 2003) did not demonstrate a grouping of PCV2 isolates associated with PMWS among French and Canadian pig herds. The immediate discrepancy between these observations and our results may be related to various factors, e.g. different criteria for defining a herd as PMWS positive. Recently, Olvera et al. (2007) demonstrated that phylogenetically PCV2 genomes can be divided in two major groups defined as PCV2 group 1 and 2. Although none of those could be associated with disease status, the definition of 2 groups of PCV2 as such supports our indications of the existence of 2 groups in our study. Thus, groups 1 and 2 in our study match PCV2 group 2 and 1, respectively, by Olvera et al. (2007). A similar observation was made by Cheung et al. (2007), who further demonstrated a pattern in phylogenetic match and disease. They found that the American PCV2 group 1 isolate was

detected in all PMWS-diseased animals, while only two of the diseased animals harbored the PCV2 group 2 isolate.

In conclusion, the present study does not provide evidence that a specific virus other than PCV2 is involved in the development of PMWS. However, our results indicate a link between different isolates of PCV2 and disease and therefore support the hypothesis that strain variations may constitute a crucial factor for triggering the switch from PCV2 infection to PMWS disease as proposed by Timmusk et al. (2005) and Cheung et al. (2007). Ongoing sequence analyses of a large spectrum of PCV2 DNA from PMWS-affected and unaffected swine herds included in the Danish case-control study are expected to enlighten a putative role of strain variations for the development of PMWS.

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References

Acland, H.M., 1989. Encephalomyocarditis Virus. In: Pensaert, M.B. (Ed.), Virus Infections of Porcines, Elsevier, Amsterdam, pp. 259-264.

- 440 Allan, G.M., McNeilly, F., Kennedy, S., Daft, B., Clarke, E.G., Ellis, J.A., Haines, D.M.,
441 Meehan, B.M., Adair, B.M., 1998. Isolation of porcine circovirus-like viruses from pigs
442 with wasting disease in the USA and Europe. *J. Vet. Diagn. Invest.* 10 , 3-10.
443
- 444 Allan, G.M., McNeilly, F., Meehan, B.M., Kennedy, S., Mackie, D.P., Ellis, J.A., Clark,
445 E.G., Espuna, E., Saubi, N., Riera, P., Bøtner, A., Charreyre, C.E., 1999a. Isolation and
446 characterization of circoviruses from pigs with wasting syndromes in Spain, Denmark
447 and Northern Ireland. *Vet. Microbiol.* 66, 115-123.
448
- 449 Allan, G.M., Kennedy, S., McNeilly, F., Foster, J.C., Ellis, J.A., Krakowka, S.J., Meehan,
450 B.M., Adair, B.M., 1999b. Experimental reproduction of severe wasting disease by co-
451 infection of pigs with porcine circovirus and porcine parvovirus. *J. Comp. Pathol.* 121, 1-
452 11.
453
- 454 Allan, G., McNeilly, F., Ellis, J., Krakowka, S., Meehan, B., McNair, I., Walker, I.,
455 Kennedy, S., 2000. Experimental infection of colostrum deprived piglets with porcine
456 circovirus (PCV2) and porcine reproductive and respiratory syndrome virus (PRRSV)
457 potentiates PCV2 replication. *Arch. Virol.* 145, 2421-2429.
458
- 459 Bøtner, A., Nielsen, J., Bille-Hansen, V., 1994. Isolation of porcine reproductive and
460 respiratory syndrome (PRRS) virus in a Danish swine herd and experimental infection of
461 pregnant gilts with the virus. *Vet. Microbiol.* 40, 351-360.
462

- Cheung, A.K., Lager, K.M., Kohutyuk, O.I., Vincent, A.L., Henry, S.C., Baker, R.B., Rowland, R.R., Dunham, A.G., 2007. Detection of two porcine circovirus type 2 genotypic groups in United States swine herds. *Arch. Virol.* 152, 1035-44.
- Choi, C., Chae, C., Clark, E.G., 2000. Porcine postweaning multisystemic wasting syndrome in Korean pig: detection of porcine circovirus 2 infection by immunohistochemistry and polymerase chain reaction. *J. Vet. Diagn. Invest.* 12, 151-153.
- Clark, E.G., 1997. Post weaning multisystemic wasting syndrome. In: *Proceedings of the American Association of Swine Practitioners*, pp. 499-501.
- de Boissésou, C., Béven, V., Bigarré, L., Thiéry, R., Rose, N., Eveno, E., Madec, F., Jestin, A., 2004. Molecular characterization of porcine circovirus type 2 isolates from post-weaning multisystemic wasting syndrome-affected and non-affected pigs. *J. Gen. Virol.* 85, 293-304.
- Debouck, P., 1989. Porcine Rotavirus. In: Pensaert, M.B. (Ed.), *Virus Infections of Porcines*, Elsevier, Amsterdam, pp. 97-109.
- Derbyshire, J.B., 1989. Porcine Adenovirus. In: Pensaert, M.B. (Ed.), *Virus Infections of Porcines*, Elsevier, Amsterdam, pp. 73-80.

- 485 Edington, N., 1989. Porcine Cytomegalovirus. In: Pensaert, M.B. (Ed.), Virus Infections
486 of Porcines, Elsevier, Amsterdam, pp. 65-70.
- 487
- 488 Ellis, J., Hassard, L., Clark, E., Harding, J., Allan, G., Willson, P., Strokappe, J., Martin,
489 K., McNeilly, F., Meehan, B., Todd, D., Haines, D., 1998. Isolation of circovirus from
490 lesions of piglets with postweaning multisystemic wasting syndrome. *Can. Vet. J.* 39, 44-
491 51.
- 492
- 493 Enøe, C., Vigre, H., Nielsen, E.O., Larsen, P.B., Bøtner, A., Hansen, V.B., Jorsal, S.E.,
494 Bækbo, P., 2006. A Danish case-control study on risk factors for PMWS - biosecurity in
495 the herd. In: Proceedings of the 19th IPVS Congress, Copenhagen, Denmark, p. 163.
- 496
- 497 Harding, J.C.S., Clark, E.G., 1997. Recognizing and diagnosing postweaning
498 multisystemic wasting syndrome (PMWS). *Swine Health Prod.* 5, 201-203.
- 499
- 500 Jogler, C., Hoffmann, D., Theegarten, D., Grunwald, T., Überla, K., Wildner, O., 2006.
501 Replication properties of human adenovirus in vivo and in cultures of primary cells from
502 different animal species. *J. Virol.* 80, 3549-3558.
- 503
- 504 Jorsal, S.E., Bille-Hansen, V., Vigre, H., Larsen, P.B., Bøtner, A., Nielsen, E.O., Enøe,
505 C., Bækbo, P., 2006. PMWS - Laboratory Diagnosis on herd and pig level in a Danish
506 case-control study. In: Proceedings of the 19th IPVS Congress, Copenhagen, Denmark, p.
507 270.

508

509 Kennedy, S., Moffett, F., McNeilly, F., Meehan, B., Ellis, J., Krakowka, S., Allan, G.M.,
510 2000. Reproduction of lesions of postweaning multisystemic wasting syndrome by
511 infection of conventional pigs with porcine circovirus type 2 alone or in combination with
512 porcine parvovirus. *J. Comp. Path.* 122, 9-24.

513

514 Kim, H.S., Kwang, J., Yoon, I.J., Joo, H.S., Frey, M.L., 1993. Enhanced replication of
515 porcine reproductive and respiratory syndrome (PRRS) virus in a homogeneous
516 subpopulation of MA-104 cell line. *Arch. Virol.* 133, 477-83.

517

518 Krakowka, S., Ellis, J.A., Meehan, B., Kennedy, S., McNeilly, F., Allan, G., 2000. Viral
519 wasting syndrome of swine: experimental reproduction of postweaning multisystemic
520 wasting syndrome in gnotobiotic swine by coinfection with porcine circovirus 2 and
521 porcine parvovirus. *Vet. Pathol.* 37, 254-263.

522

523 Kristensen, C.S., Bækbo, P., Bille-Hansen, V., Hassing, A.G., Bøtner, A., 2004.
524 Transmission of PMWS. In: *Proceedings of 18th IPVS Congress, Hamburg, Germany*, p.
525 77.

526

527 Ladekjær-Mikkelsen, A.S., Nielsen, J., Stadejek, T., Storgaard, T., Krakowka, S., Ellis,
528 J., McNeilly, F., Allan, G., Bøtner, A., 2002. Reproduction of postweaning multisystemic
529 wasting syndrome (PMWS) in immunostimulated and non-immunostimulated 3-week-old

- piglets experimentally infected with porcine circovirus type 2 (PCV2). *Vet. Microbiol.* 89, 97-114.
- Landry, M.L., Mayo, D.R., Hsiung, G.D., 1982. Comparison of guinea pig embryo cells, rabbit kidney cells, and human embryonic lung fibroblast cell strains for isolation of herpes simplex virus. *J. Clin. Microbiol.* 15, 842–847.
- Larochelle, R., Magar, R., D'allaire, S., 2003. Comparative serologic and virologic study of commercial swine herds with and without postweaning multisystemic wasting syndrome. *Can. J. Vet. Res.* 67, 114-120.
- Lauritsen, K.T., 2005. The significance of passively transferred and active immunity for the development of *Mycoplasma hyosynoviae* arthritis in pigs. Ph.D. Thesis. The Royal Veterinary and Agricultural University & The Danish Institute for Food and Veterinary Research, Copenhagen, Denmark, p. 65.
- Lohse, L., Nielsen, J., Eriksen, L., 2005. Porcine humoral immune responses to multiple injections of murine monoclonal antibodies. *APMIS* 113, 489-96.
- López-Soria, S., Segalés, J., Nofrías, M., Calsamiglia, M., Ramírez, H., Mínguez, A., Serrano, J.M., Marín, O., Callén, A., 2004. Genetic influence on the expression of PCV disease. *Vet. Rec.* 16, 504.

McClurkin, A.W., Norman, J.O., 1966. Studies on transmissible gastroenteritis of swine.

II. Selected characteristics of a cytopathogenic virus common to five isolates from

transmissible gastroenteritis. *Can. J. Comp. Med. Vet. Sci.* 30, 190-198.

Mowat, G.N., Chapman, W.G., 1962. Growth of Foot-and-Mouth Disease Virus in a

fibroblastic cell line derived from hamster kidneys. *Nature* 196, 253.

Nelson, E.A., Christopher-Hennings, J., Drew, T., Wensvoort, G., Collins, J.E., Benfield,

D.A., 1993. Differentiation of U.S. and European isolates of porcine reproductive and

respiratory syndrome virus by monoclonal antibodies. *J. Clin. Microbiol.* 31, 3184-3189.

Olvera, A., Cortey, M., Segalés, J., 2007. Molecular evolution of porcine circovirus type

2 genomes: Phylogeny and clonality. *Virology* 357(2), 175-185.

Onuki, A., Abe, K., Togashi, K., Kawashima, K., Taneichi, A., Tsunemitsu, H., 1999.

Detection of porcine circovirus from lesions of a pig with wasting disease in Japan. *J.*

Vet. Med. Sci. 61, 1119-23.

Pogranichniy, R.M., Yoon, K.J., Harms, P.A., Sorden, S.D., Daniels, M., 2002. Case-

control study on the association of porcine circovirus type 2 and other swine viral

pathogens with postweaning multisystemic wasting syndrome. *J. Vet. Diagn. Invest.* 14,

449-56.

- 576 Rose, N., Larour, G., Le Diguerher, G., Eveno, E., Jolly, J.P., Blanchard, P., Oger, A., Le
577 Dimma, M., Jestin, A., Madec, F., 2003. Risk factors for porcine post-weaning
578 multisystemic wasting syndrome (PMWS) in 149 French farrow-to-finish herds. *Prev.*
579 *Vet. Med.* 61, 209-25.
- 580
- 581 Rovira, A., Balasch, M., Segales, J., Garcia, L., Plana-Duran, J., Rosell, C., Ellerbrok, H.,
582 Mankerts, A., Domingo, M., 2002. Experimental inoculation of conventional pigs with
583 porcine reproductive and respiratory syndrome virus and porcine circovirus 2. *J. Virol.*
584 76, 3232-3239.
- 585
- 586 Segales, J., Calsamiglia, M., Rosell, C., Sloer, M., Maldonado, J., Martin, M., Domingo,
587 M., 2002. Porcine reproductive and respiratory syndrome virus (PRRSV) infection status
588 in pigs naturally affected with post-weaning multisystemic wasting syndrome (PMWS) in
589 Spain. *Vet. Microbiol.* 85, 23-30.
- 590
- 591 Stark, G.R., Kerr, I.M., Williams, B.R., Silverman, R.H., Schreiber, R.D., 1998. How
592 cells respond to interferons. *Annu. Rev. Biochem.* 67, 227-264.
- 593
- 594 Timmusk, S., Wallgren, P., Bélak, K., Berg, M., Fossum, C., 2005. Genetic analysis of
595 PCV2 capsid protein sequences reveals two main groups of Swedish isolates. *Int. Conf.*
596 *on Animal Circoviruses and Associated Diseases*, Belfast, N. Ireland, UK, p. 82.
- 597
- 598 Tischer, I., Rasch, R., Tochtermann, G., 1974. Characterization of

- papovavirus-and picornavirus-like particles in permanent pig kidney cell lines. Zentralblatt für Bakteriologie Mikrobiologie und Hygiene Serie A 226, 153-167.
- Vigre, H., Bækbo, P., Jorsal, S.E., Bille-Hansen, V., Hassing, A.G., Enøe, C., Bøtner, A., 2005. Spatial and temporal patterns of pig herds diagnosed with postweaning multisystemic wasting syndrome (PMWS) during the first two years of its occurrence in Denmark. Vet. Microbiol. 110, 17-26.
- Vigre, H., Enøe, C., Bøtner, A., Jorsal, S.E., Bækbo, P., Nielsen, E.O., 2006. Association between PMWS and PRRS. In: Proceedings of the 19th IPVS Congress, Copenhagen, Denmark , p. 174.
- Wallgren, P., Hasslung, F., Bergstrom, G., Linder, A., Belak, K., Hard, A.F., Segerstad, C., Stampe, M., Molander, B., Bjornberg Kallay, T., Norregard, E., Ehlorsson, C.J., Tornquist, M., Fossum, C., Allan, G.M., Robertsson, J.A., 2004. Postweaning multisystemic wasting syndrome – PMWS. The first year with the disease in Sweden. Vet. Q. 26, 170-87.
- Wellenberg, G., Stockhofe-Zurwieden, N., Boersma, W., de Jong, M., Elbers, A., 2004. The presence of co-infections in pigs with clinical signs of PMWS in the Netherlands: a case-control study. Res. Vet. Sci. 77, 177-184.

- 622 Wensvoort, G., Terpstra, C., Pol, J.M., ter Laak, E.A., Bloemraad, M., de Kluyver, E.P.,
623 Kragten, C., van Buiten, L., den Besten, A., Wagenaar, F. et al. 1991. Mystery swine
624 disease in the Netherlands: the isolation of Lelystad virus. Vet. Q. 13, 121-30.

Table 1. Antibody specificities to known viruses of pig immune sera used in IPMA for staining of selected cell cultures in the viral examination

Virus	Serum				
	RKV ¹	Pig 15 ²	DS pool ³	69930 ⁴	67268 ⁵
HEV	+	-	+	-	+
PEV1	+	+	+	+	+
PEV8	+	+	+	+	+
PRRSV/EU	+	-	+	-	-
PRRSV/US	+	-	+	-	-
PCV1	+	-	+	+	+
PCV2	+	-	+	+	+
Adenovirus III	+	+	+	+	+
SIV	+	-	+	-	-
PPV	+	-	+	-	-

¹Convalescence serum from sows in Danish swine herds 1998/1999; ²Serum from a PCV2 negative pig; ³serum pool from pigs originating from PMWS-affected herds A,B,C,D; ⁴serum pool from a PMWS-affected herd; ⁵serum pool from a herd unaffected by PMWS.

+ or -, respectively, indicates whether the used serum is either antibody positive or negative against the specific virus.

Table 2. Primers used for amplification and sequencing of PCV2 DNA

Name	Sequence 5' to 3'	PCV2 position 5'
1-A LM 1 (PCR1 Forw ¹)	TGC CGA GGC CTA CGT GG	1086
1-A LM 2 (PCR1 Rev ²)	ACA ATA TCC GTG TAA CCA	1033
1-A LM 22 (PCR2 Rev)	GGC GGT GGA CAT GAT GAG	1467
1-A LM 23 (Seq ³ Forw)	TCA TTG TGG GGC CAC CTG	511
1-A LM 24 (Seq Forw)	TAG TAT ATC CGA AGG TGC GG	1516
1-A LM 25 (Seq Rev)	TCC CAG GGC AGC CAG CC	654
1-A LM 26 (Seq Forw)	CCC CAT GCC CTG ATT TTC C	908
PCV2R (PCR2 Forw)	CCC ATG CCC TGA ATT TCC	908
8-H PN 32 (Seq Rev)	GCA GTA GAC AGG TCA CTC C	346
8-H PN 33 (Seq Rev)	AGC CAT CTT GGC CAG ATC C	1618
8-H PM 34 (Seq Forw)	CTT CCG AAG ACG AGC GCA	70
8-H PN 171 (Seq Rev)	GTT CGT CCT TCC TCA TTA CC	144
8-H PN 213 (Seq Forw)	GGA GCA GGG CCA GAA TTC	1359

¹Forw = Forward orientation; ²Rev = Reverse orientation; ³Seq = sequencing

Table 3. Identification and information concerning PCV2 samples from examined pigs

Virus identification	GenBank accession number	PMWS status ¹	Herd of origin	Contact herd
PCV2/pig 134	EU136712	+	1	A
PCV2/pig 152/157	EU136713	+	1	B
PCV2/pig 179	EU136715	+	2	C
PCV2/pig 206	EU136716	+	2	D
PCV2/herd 1	EU136711	-	1	none
PCV2/herd 2	EU136714	-	2	none
PCV2/herd A	EU136717	+	A	none
PCV2/herd B	EU136718	+	B	none
PCV2/herd C	EU136719	+	C	none
PCV2/herd D	EU136720	+	D	none

¹ + or -, respectively describes the PMWS-status at pig or herd level

Table 4. Amino acid differences in the replicase and in the capsid protein, respectively, between PCV2 isolates from group 1 and group 2

PCV2 isolates from group 1 and group 2																			
Position	34	266																	
Replicase protein																			
Group 1 ^a	E	A																	
Group 2 ^b	D	T																	
Position	8	34	57	59	63	80	86	88	89	91	121	151	169	180	190	191	206	210	232
Capsid protein																			
Group 1 ^a	F	L	V	A	T	V	T	K	I	I	T	P	R	K	S	A	K	D	K
Group 2 ^b	Y	H	I	R	K	L	S	P	R	V	S	T	S	R	A	G	I	E	N

^arepresenting isolate 1 from herd 1; ^brepresenting all other PCV2 isolates in this study