Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Available online at www.sciencedirect.com
ScienceDirect
Veterinary Microbiology 129 (2008) 97–107
veterinary microbiology
www.elsevier.com/locate/vetmic

Examination for a viral co-factor in postweaning multisystemic wasting syndrome (PMWS)

Louise Lohse [a], Anette Bøtner [a], Anne-Sofie Ladekjær Hansen [a], Tina Frederiksen [a], Kitt Dupont [b], Charlotte S. Christensen [c], Poul Bækbo [c], Jens Nielsen [a,*]

[a] *National Veterinary Institute, Technical University of Denmark, Lindholm, DK-4771 Kalvehave, Denmark*
[b] *National Veterinary Institute, Technical University of Denmark, Bülowsvej 27, DK-1790 Copenhagen V, Denmark*
[c] *Danish Pig Production, Danish Meat Association, Vinkelvej 11, 8620 Kjellerup, Denmark*

Received 19 September 2007; received in revised form 14 November 2007; accepted 20 November 2007

Abstract

In order to test the hypothesis that a putative co-factor for the development of postweaning multisystemic wasting syndrome (PMWS) 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 seven 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 nine isolates originating from four PMWS-affected herds, four PMWS-positive pigs plus one unaffected herd. The PCV2 genomes from the two 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 two 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

* Corresponding author. Tel.: +45 72347836.
E-mail address: jn@vet.dtu.dk (J. Nielsen).

0378-1135/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.vetmic.2007.11.018

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 and 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, 1999a; Ellis et al., 1998; 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, 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 four 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 Boisséson 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 four PCV2-positive, PMWS-affected herds (herds A, B, C, and D). For virological examination, we selected five 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 two 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 1900 × *g* for 10 min. 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 used for diagnostic virus examination of field samples at our institute and are known to support the replication of swine influenza virus (SIV), porcine parvovirus (PPV), haemagglutinating encephalomyelitis virus (HEV) and Aujezsky's disease virus (ADV). (2) Porcine pulmonary alveolar macrophages (PPAM) – these are considered as one of the few cells sensitive to porcine cytomegalovirus (Edington, 1989) and are known to support the replication of PRRSV (Wensvoort et al., 1991; Bøtner et al., 1994). The PPAM used in the present experiment were obtained by lung lavage of euthanized 4–6-week-old pigs from the institute's own high-health herd, which is free from mycoplasmas and a wide range of bacterial and viral infections, as previously described (Ladekjær-Mikkelsen et al., 2002; Lauritsen, 2005). The cells obtained contained >95% alveolar macrophages. (3) MARC-145 cells (kindly provided by Dr. Ferrari, IZSBS, Brescia, Italy), which are susceptible to the American type of PRRSV (Kim et al., 1993; Bøtner et al., 1994). These cells are generally accepted as one of the most sensitive cell lines for propagation of porcine rotavirus (Debouck, 1989). (4) ST117 cells (ATCC, CLR-1746^{TM}), these are susceptible to swine enterovirus, porcine coronavirus (McClurkin and Norman, 1966) and adenoviruses (Derbyshire, 1989). (5) PK15 cells which are susceptible to several viruses, including PCV type 2 (Allan et al., 1998) and these PK15 cells (kindly provided by Dr. G. Allan, DARDNI, Belfast, UK) were free from PCV type 1, which is a known contaminant of some PK15 cell line variants (Tischer et al., 1974). (6) BHK 21 cells (ATCC, CCL-10^{TM}), these are susceptible to a number of porcine enteroviruses, porcine encephalomyocarditis virus (EMC) (Acland, 1989), and foot-and-mouth disease virus (Mowat and Chapman, 1962). (7) HeLa (ATCC, CCL-2^{TM}) and Vero cells (ATCC, CCL-81^{TM}) support the replication of several viruses, including porcine enteroviruses (Derbyshire, 1989). Furthermore, the Vero A cells are characterised 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-34^{TM}) 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 (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% CO_2–air atmosphere. After 3–4 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 two new plates. These were incubated as above and after another 3–4 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 min 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 min 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 min at 37 °C. Finally, the substrate (ethylcarbazol) was added and the plates were left for 20 min 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 3 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 six 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 3–4 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 1 h of incubation at 37 °C in a 5% CO_2–air atmosphere, 1 ml cell culture material from the first passage was added. After 3–4 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 1 h before. After incubation the two plates were fixed and stained and the cell cultures were passaged as stated above in total for six passages.

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[a]	Pig 15[b]	DS pool[c]	69930[d]	67268[e]
HEV	+	−	+	−	+
PEV1	+	+	+	+	+
PEV8	+	+	+	+	+
PRRSV/EU	+	−	+	−	−
PRRSV/US	+	−	+	−	−
PCV1	+	−	+	+	+
PCV2	+	−	+	+	+
Adenovirus III	+	+	+	+	+
SIV	+	−	+	−	−
PPV	+	−	+	−	−

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

[a] Convalescence serum from sows in Danish swine herds 1998/1999.

[b] Serum from a PCV2 negative pig.

[c] Serum pool from pigs originating from PMWS-affected herds A, B, C, D.

[d] Serum pool from a PMWS-affected herd.

[e] Serum pool from a herd unaffected by PMWS.

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 30,000 × *g* for 45 min. 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 1734 bp) was generated using the Expand 20 kb^{PLUS} PCR System (Roche, Diagnostics GmbH, Germany) in PCR reactions containing a final concentration of 1× 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 one cycle of (94 °C for 3 min), 45 cycles of (94 °C for 15 s, 53 °C for 20 s, 68 °C for 2 min with 1 s increase per cycle), and one 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 1× PCR Gold buffer (Applied Biosystems, Denmark), 2 mM $MgCl_2$, 0.2 mM dNTP, 0.5 μM of each primer (PCV2R and PCV2 ORF2

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[a])	TGC CGA GGC CTA CGT GG	1086
1-A LM 2 (PCR1 Rev[b])	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[c] Forw)	TCA TTG TGG GGC CAC CTG	511
1-A LM 24 (Seq Forw)	TAG TAT ATC CGA AGG TGCGG	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

[a] Forw = forward orientation.
[b] Rev = reverse orientation.
[c] Seq = sequencing.

SEK2), and 2.5 U of Ampli Taq Gold DNA polymerase (Applied Biosystems, Denmark). The cycling conditions were one cycle of (94 °C for 5 min), 45 cycles of (94 °C for 15 s, 50 °C for 20 s, 72 °C for 40 s), and one 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 8000 × *g*.

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 seven out of eight 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 two pigs (pigs no. 179 and 206).

Table 3
Identification and information concerning PCV2 samples from examined pigs

Virus identification	GenBank accession number	PMWS status[a]	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

[a] + or −, respectively, describes the PMWS status at pig or herd level.

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.

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

[a] Representing isolate 1 from herd 1.
[b] Representing all other PCV2 isolates in this study.

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 two 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 group 1 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 outcompetes 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 groups 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 a 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 characterisation of isolates from PMWS-affected and unaffected herds (de 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 groups 1 and 2. Although none of those could be associated with disease status, the definition of two groups of PCV2 as such supports our indications of the existence of two groups in our study. Thus, groups 1 and 2 in our study match PCV2 groups 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 phylogenic 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.

Acknowledgements

The technical assistance of J. Holm Hansen, H. Rasmussen, P. Norman, J. Nowicki, and the cell culture laboratory staff, DTU, Lindholm, is highly appreciated. We are also grateful to G. Belsham for critical reading of the manuscript and valuable suggestions. The study was financially supported by The Danish Veterinary and Agricultural Research Council, grant no. 23020079.

References

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

Allan, G.M., McNeilly, F., Kennedy, S., Daft, B., Clarke, E.G., Ellis, J.A., Haines, D.M., Meehan, B.M., Adair, B.M., 1998. Isolation of porcine circovirus-like viruses from pigs with wasting disease in the USA and Europe. J. Vet. Diagn. Invest. 10, 3–10.

Allan, G.M., McNeilly, F., Meehan, B.M., Kennedy, S., Mackie, D.P., Ellis, J.A., Clark, E.G., Espuna, E., Saubi, N., Riera, P., Bøtner, A., Charreyre, C.E., 1999a. Isolation and characterization of circoviruses from pigs with wasting syndromes in Spain Denmark and Northern Ireland. Vet. Microbiol. 66, 115–123.

Allan, G.M., Kennedy, S., McNeilly, F., Foster, J.C., Ellis, J.A., Krakowka, S.J., Meehan, B.M., Adair, B.M., 1999b. Experimental reproduction of severe wasting disease by co-infection of pigs with porcine circovirus and porcine parvovirus. J. Comp. Pathol. 121, 1–11.

Allan, G., McNeilly, F., Ellis, J., Krakowka, S., Meehan, B., McNair, I., Walker, I., Kennedy, S., 2000. Experimental infection of colostrum deprived piglets with porcine circovirus (PCV2) and porcine reproductive and respiratory syndrome virus (PRRSV) potentiates PCV2 replication. Arch. Virol. 145, 2421–2429.

Bøtner, A., Nielsen, J., Bille-Hansen, V., 1994. Isolation of porcine reproductive and respiratory syndrome (PRRS) virus in a Danish swine herd and experimental infection of pregnant gilts with the virus. Vet. Microbiol. 40, 351–360.

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, 1035–1044.

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 Boisséson, 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.

Edington, N., 1989. Porcine cytomegalovirus. In: Pensaert, M.B. (Ed.), Virus Infections of Porcines. Elsevier, Amsterdam, pp. 65–70.

Ellis, J., Hassard, L., Clark, E., Harding, J., Allan, G., Willson, P., Strokappe, J., Martin, K., McNeilly, F., Meehan, B., Todd, D., Haines, D., 1998. Isolation of circovirus from lesions of piglets with postweaning multisystemic wasting syndrome. Can. Vet. J. 39, 44–51.

Enøe, C., Vigre, H., Nielsen, E.O., Larsen, P.B., Bøtner, A., Hansen, V.B., Jorsal, S.E., Baekbo, P., 2006. A Danish case–control study on risk factors for PMWS—biosecurity in the herd. In: Proceedings of the 19th IPVS Congress, Copenhagen, Denmark, p. 163.

Harding, J.C.S., Clark, E.G., 1997. Recognizing and diagnosing postweaning multisystemic wasting syndrome (PMWS). Swine Health Prod. 5, 201–203.

Jogler, C., Hoffmann, D., Theegarten, D., Grunwald, T., Überla, K., Wildner, O., 2006. Replication properties of human adenovirus in vivo and in cultures of primary cells from different animal species. J. Virol. 80, 3549–3558.

Jorsal, S.E., Bille-Hansen, V., Vigre, H., Larsen, P.B., Bøtner, A., Nielsen, E.O., Enøe, C., Bækbo, P., 2006. PMWS—laboratory diagnosis on herd and pig level in a Danish case–control study. In: Proceedings of the 19th IPVS Congress, Copenhagen, Denmark, p. 270.

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

Kim, H.S., Kwang, J., Yoon, I.J., Joo, H.S., Frey, M.L., 1993. Enhanced replication of porcine reproductive and respiratory syndrome (PRRS) virus in a homogeneous subpopulation of MA-104 cell line. Arch. Virol. 133, 477–483.

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

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

Ladekjær-Mikkelsen, A.S., Nielsen, J., Stadejek, T., Storgaard, T., Krakowka, S., Ellis, J., McNeilly, F., Allan, G., Bøtner, A., 2002. Reproduction of postweaning multisystemic 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., Ďallaire, 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, 489–496.

López-Soria, S., Segalés, J., Nofrarías, M., Calsamiglia, M., Ramírez, H., Míngues, 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, 1119–1123.

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 post-weaning multisystemic wasting syndrome. J. Vet. Diagn. Invest. 14, 449–456.

Rose, N., Larour, G., Le Diguerher, G., Eveno, E., Jolly, J.P., Blanchard, P., Oger, A., Le Dimma, M., Jestin, A., Madec, F., 2003. Risk factors for porcine post-weaning multisystemic wasting syndrome (PMWS) in 149 French farrow-to-finsh herds. Prev. Vet. Med. 61, 209–225.

Rovira, A., Balasch, M., Segales, J., Garcia, L., Plana-Duran, J., Rosell, C., Ellerbrok, H., Mankerts, A., Domingo, M., 2002. Experimental inoculation of conventional pigs with porcine reproductive and respiratory syndrome virus and porcine circovirus 2. J. Virol. 76, 3232–3239.

Segales, J., Calsamiglia, M., Rosell, C., Sloer, M., Maldonado, J., Martin, M., Domingo, M., 2002. Porcine reproductive and respiratory syndrome virus (PRRSV) infection status in pigs naturally affected with post-weaning multisystemic wasting syndrome (PMWS) in Spain. Vet. Microbiol. 85, 23–30.

Stark, G.R., Kerr, I.M., Williams, B.R., Silverman, R.H., Schreiber, R.D., 1998. How cells respond to interferons. Annu. Rev. Biochem. 67, 227–264.

Timmusk, S., Wallgren, P., Bélak, K., Berg, M., Fossum, C., 2005. Genetic analysis of PCV2 capsid protein sequences reveals two main groups of Swedish isolates. In: International Conference on Animal Circoviruses and Associated Diseases, Belfast, N. Ireland, UK, p. 82.

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, 170–187.

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.

Wensvoort, G., Terpstra, C., Pol, J.M., ter Laak, E.A., Bloemraad, M., de Kluyver, E.P., Kragten, C., van Buiten, L., den Besten, A., Wagenaar, F., et al., 1991. Mystery swine disease in the Netherlands: the isolation of Lelystad virus. Vet. Q. 13, 121–130.