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Experimental reproduction of postweaning multisystemic wasting syndrome (PMWS) in pigs in Sweden and Denmark with a Swedish isolate of porcine circovirus type 2

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Abstract

An experimental model using 3-day-old snatch-farrowed colostrum-deprived piglets co-infected with porcine circovirus type 2 (PCV2) and porcine parvovirus (PPV) is at present one of the best methods to study factors affecting development of postweaning multisystemic wasting syndrome (PMWS). A Swedish isolate of PCV2 (S-PCV2) retrieved in 1993 from a healthy pig has been used in this model to reproduce PMWS in pigs from Northern Ireland. This virus has been present in the Swedish pig population for at least a decade without causing any known PMWS disease problems, despite its potential pathogenicity. The reasons for this are unknown, but could be related to genetics, absence of triggers for PCV2 upregulation (infectious agent and/or management forms) within Swedish pig husbandry. In order to confirm the pathogenicity of S-PCV2, Swedish and Danish pigs were experimentally infected with this isolate according to the established model. Swedish pigs were also infected with a reference isolate of PCV2 (PCV2-1010) to compare the severity of disease caused by the two isolates in Swedish pigs. Both Danish and Swedish pigs developed PMWS after the experimental infection with S-PCV2. Antibodies to PCV2 developed later and reached lower levels in serum from pigs infected with S-PCV2 than in pigs inoculated with PCV2-1010. In general, pigs infected with S-PCV2 showed more severe clinical signs of disease than pigs infected with PCV2-1010, but pigs from all PCV2-inoculated groups displayed gross and histological lesions consistent with PMWS. All pigs inoculated with PPV, alone or in combination with PCV2, displayed interleukin-10 responses in serum while only pigs infected with PPV in combination with

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PCV2 showed interferon- α in serum on repeated occasions. Thus, the pathogenicity of S-PCV2 was confirmed and a role for cytokines in the etiology of PMWS was indicated.

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1. Introduction

Porcine circovirus type 2 (PCV2) is now accepted as the causal agent of postweaning multisystemic wasting syndrome (PMWS). However, it is also recognised that other additional infectious (Allan et al., 1999; Allan et al., 2000a; Krakowka et al., 2000) or non-infectious factors (Rose et al., 2003) are necessary for the full clinical expression of the disease. PMWS was first observed in high health herds in Canada in 1991 (Allan et al., 1998; Ellis et al., 1998), and has since then rapidly become a major problem in many pig-producing countries throughout the world. Retrospective studies have demonstrated that a PCV2 virus has been present in pigs for many years prior to the recognition of PMWS without being associated with any specific disease syndrome (Rodriguez-Arrioja et al., 2003; Walker et al., 2000). The global epizootic spread of PMWS since 1996 suggests that the PCV2 virus in pigs may have mutated to a more pathogenic form or that another agent in combination with PCV2 is necessary for the development of PMWS. Alternatively, it has also been suggested that the susceptibility of the host to PCV2-associated clinical disease has, in some way, changed due to alterations in the pig industry. Efforts to identify new pathogenic genotypes of PCV2 (de Boisseson et al., 2004) or new common co-infecting micro-organism (Ellis et al., 2004) have, to date, failed and epidemiological studies including numerous aspects of husbandry forms have not yet revealed any particular factor(s) that predispose for PMWS (Larochelle et al., 2003; Pogranichniy et al., 2002; Rose et al., 2003).

Dual infection with PCV2 and porcine parvovirus (PPV) or porcine reproductive and respiratory syndrome virus (PRRSV), as well as immunomodulators, have been used to successfully reproduce PMWS experimentally in pigs (for review see Allan et al., 2004) and one of the most reproducible infection models involves co-infection with PCV2 and PPV

in 3-day-old snatch-farrowed colostrum-deprived (SFCD) piglets (Allan et al., 1999). This model was recently used in Northern Ireland to demonstrate the potential pathogenicity of a Swedish isolate of PCV2 from 1993 (Allan et al., 2003). The Swedish PCV2 (S-PCV2) was isolated from a clinically healthy pig, which was raised in a SPF-herd that seroconverted to PCV2 at that time (Wattrang et al., 2002).

Sweden remained free from PMWS until December 2003, and as of October 2004, only 12 farms have been diagnosed as affected by the disease. It has been suggested that differences in pig husbandry practices, animal genetics and/or viral pathogenesis could have contributed to relative freedom of Swedish pigs from disease. The present experimental infection with S-PCV2 and PPV was conducted using Swedish and Danish pigs. To allow comparison between various PCV2 isolates, one group of Swedish pigs was also infected with a reference isolate of PCV2 (Imp. 1010). Clinical manifestations of disease, histological lesions, levels of virus antigen in affected tissues and development of antibodies to PCV2 were recorded and the IL-10 and interferon- α responses were determined in serum obtained from Swedish pigs.

2. Materials and methods

2.1. Experimental model and virus

An experimental model, using a dual infection with porcine circovirus type 2 (PCV2) and porcine parvovirus (PPV) for induction of PMWS was used as previously described (Allan et al., 1999). Two isolates of PCV2 were used: PCV2-1010 (PCV2 Stoon) isolated from an outbreak of PMWS in a high health herd in Canada (Ellis et al., 1998) and S-PCV2 isolated from a lymph node collected in 1993 from a clinically healthy pig reared in a Swedish SPF-herd (Allan et al., 2003; Wattrang et al., 2002). For co-infection, PPV (isolate 1005 pool 7) recovered from

PMWS-affected pigs was used (Allan et al., 2000b; Krakowka et al., 2000). All viruses were propagated in a PCV2-free cell line (PK/15A) as previously described (Allan et al., 2003).

2.2. Experimental animals

The experimental infections were performed in two sets, one using Swedish pigs and the other using Danish pigs. In the first set, Swedish pigs were snatched-farrowed (SF) and hand-reared on colostrum and milk substitutes (snatch-farrowed colostrum-deprived (SFCD)), whereas the Danish pigs were caesarean-derived colostrum-deprived (CDCD). The Swedish pigs were obtained from a conventional piglet-producing herd. To date, the farm has not reported any PCV2-associated disease problems and the herd is free from infections with *Salmonella* spp, *Sarcoptes scabiei*, *Serpulina hyodysenteriae* and toxin producing strains of *Pasteurella multocida*. The sows were vaccinated against *Escherichia coli*, *Erysipelothrix rhusiopathiae* and PPV. The piglets, originating from five litters (crossed Hampshire, Yorkshire and Swedish Landrace) designated A–E. The piglets were transported within 1 h after birth to the Animal Department at the National Veterinary Institute (NVI), Uppsala, Sweden, and distributed into four groups that were housed in separate rooms with individual ventilation in a clean but not sterile environment. During the first days of life, the piglets were hand reared on bovine colostrum substitute (Calf Volostrum, Volac International Ltd., UK) and subsequently on commercial pig milk substitute (Piggi milk, Manufacturer No. 2077, UK) and pellets (Primary Elite, Primary Diets Ltd., UK). All animals were treated with intramuscular injections of antibiotics (Nuflor, Florfenicol 30 g/100 ml 0.2 ml/pig, day) for the first 3 days, and thereafter orally (Baytril, enrofloxacin 25 mg/ml 0.2 ml/pig, day) once daily throughout the experimental period. Pigs showing severe signs of disease were euthanised and 35 of the initially 42 piglets were included in the experiment, which was approved by the Ethical Committee for Animal Experiments, Uppsala, Sweden.

In the second set, eight Danish piglets (nos. 1–8) Danish Landrace–Yorkshire crossbreeds were derived from two sows originating from the SPF-herd at the Danish Institute for Food and Veterinary Research

(DFVF), Kalvehave, Denmark. This herd is free from PCV1 and PCV2, PRRSV, swine influenza virus (SIV), porcine respiratory coronavirus (PRCV), as well as mycoplasmas. The sows were vaccinated against PPV. The experimental infection of the CDCD piglets was carried out at the isolation facilities of DFVF and the pigs were housed in a separate section of the building. In all other aspects, rearing of the pigs including colostrum and milk substitutes used and antibiotic treatments was the same as described for the Swedish SFCD pigs.

2.3. Experimental infection

On the 3rd day of life, all piglets were inoculated with 1.0 ml per nare of cell lysates from indicated cell culture. Pigs in Swedish group 1 ($n = 8$) were mock inoculated with cell culture supernatant from uninfected PK/15A cells; pigs in Swedish group 2 ($n = 8$) were inoculated with PPV $10^{7.0}$ TCID₅₀ alone; pigs in Swedish group 3 ($n = 10$) were inoculated with a mixture of PCV2-1010 $10^{5.5}$ TCID₅₀ and PPV $10^{7.0}$ TCID₅₀; and pigs in Swedish group 4 ($n = 9$), as well as the Danish pigs in group 5 ($n = 8$), were inoculated with S-PCV2 $10^{5.5}$ TCID₅₀ and PPV $10^{7.0}$ TCID₅₀.

2.4. Sampling procedures

The health status of all individual pigs was assessed twice daily and pigs developing signs of severe disease were euthanised and necropsied (see below). For Swedish pigs, weights and rectal temperatures were measured twice every week. Blood samples were collected from *vena cava cranialis* in evacuated test tubes prior to infection, and on days 8, 15, 22 and 28 (groups 1–4) or 4, 7, 14, 21 and 27 post infection (group 5). At blood sampling, individual faecal samples were collected from pigs in groups 1–4, and samples of faeces were collected from the floor of each of these groups daily.

At the end of the trial, 4 weeks post infection, all remaining pigs were sacrificed, autopsied and gross lesions recorded. General bacteriological examination of samples from spleen, liver and lung tissues was performed by routine methods at the Bacteriological Departments at NVI and DFVF, respectively. Tissue samples were collected from lymph nodes and tonsils,

lungs, heart, liver, kidneys, spleen, small intestine and any other tissues showing gross pathological changes. Tissues were fixed in 4% paraformaldehyde or buffered formalin for histopathological analysis or rapidly frozen in isopropanol on dry ice and stored in liquid nitrogen for immunohistochemical staining.

2.5. Histopathological examinations and immunostaining of cryostat sections

Sections of fixed, paraffin-embedded tissue were stained with hematoxylin and eosin for morphological evaluation. The severity of lesions was scored as none visible (–), mild (+), moderate (++) or severe (+++). Blocks of tissue from spleen, liver and lymph node were stained with PCV2-specific polyclonal (Ellis et al., 1998) or monoclonal antibodies (McNeilly et al., 2001) as previously described. The presence of PCV2 was determined on cryostat sections and scored as negative (–) or containing minimal (+) up to abundant (+++++) amount of PCV2 antigen.

2.6. Quantitative PCR for estimation of viral DNA in serum

Levels of PCV DNA were determined in serum samples collected from piglets in group 5 by quantitative PCR (Q-PCR) as described elsewhere (Ladekjaer-Mikkelsen et al., 2002). Results were presented as mean values of duplicate reactions.

2.7. PCR for detection of PCV2 in organs

Two sets of PCR methods for detection of PCV2 were designed. One that detected both PCV2 isolates used in this study, and one that discriminates between PCV2-1010 and S-PCV2. For the first set, PCR primers were selected based on sequence alignments of the genomes of several PCV isolates, and two primers, 5' CAG CAA GAA GAA TGG AAG 3' and 5' TAT GTG GTT TCC GGG TCT 3' were selected for the initial PCR product. For the discriminative PCR, primers were chosen according to sequence differences within the ORF2 regions, 5' AAG TAA TCA ATA GTT CTA 3' being specific for S-PCV2 and 5' AAG TAA TCA ATA GTG GAG 3' being specific for PCV2-1010. These primers were used in combination with an ORF2 full-length primer. The specificity of both PCR methods was

verified using purified viral DNA from each isolate as control. Samples of DNA were purified from splenic tissue of experimental pigs according to standard protocols, using proteinase-K digestion, phenol–chisam extraction and ethanol precipitation.

2.8. Sequence analysis of PCV2 isolates

The full-length DNA sequence of the S-PCV2 isolate used in the experimental infection was compared to the sequence of PCV2 from the original lymph node preserved in liquid nitrogen since 1993. Both genomes were amplified by PCR using ORF1 R (5' AAA GGATCC TCA GTA ATT TAT TTC ATA 3') and ORF2 R (5' TTT AAG CTT CCA TGA CGT ATC CAA GGA GG 3') primers. The PCR products were subsequently ligated into the T-Vector (pGEM-T Easy Vector System I, Promega) according to the manufacturer's instructions.

Sequence analysis of the PCR products was performed by standard procedures used at the Department of Animal Breeding and Genetics, Section of Disease Genetics, at the Swedish University of Agricultural Sciences, Uppsala, and at Uppsala Genome Centre, Uppsala University, Sweden. Alignments were made using DNASTAR and pairwise comparison of nucleotide and amino acid sequence identities was performed using MEGALIGN software version 1.13 (DNASTAR, Wisconsin). The nucleotides were numbered in analogy with Meehan et al., 1998.

2.9. Detection of antibodies to PCV2 and PPV in serum

Antibodies to PCV2 were detected in an immunoperoxidase monolayer assay (IPMA) as described elsewhere (Allan et al., 2000b; Ladekjaer-Mikkelsen et al., 2002). Sera were examined for antibodies to PPV by blocking ELISA (Madsen et al., 1997) or by a competitive ELISA (Svanovir[®] PPV-Ab, Svanova Biotech, Uppsala, Sweden).

2.10. Detection of IFN- α and IL-10 in serum

Serum IFN- α was determined in samples from the piglets in groups 1–4 by a dissociation-enhanced lanthanide fluoroimmunoassay (DELFI), as described earlier (Artursson et al., 1995). The DELFIA, which is

based on two mAbs directed to porcine IFN- α , had a lower detection limit of 0.3 U IFN- α /ml. Presence of IL-10 was determined in serum samples diluted 1:2 by ELISA (Biosource, Camarillo, CA) according to the manufacturers description. The sensitivity of the ELISA is 3 pg/ml which corresponded to an absorbance value ($A_{450\text{ nm}}$) of approximately 0.2.

3. Results

3.1. Clinical observations and gross lesions at necropsy

Among the Swedish pigs, 10 animals in total belonging to all four experimental groups developed transient diarrhoea within the first 4 days post infection (DPI). Five of these pigs were sacrificed within the first 5 DPI due to moribund conditions. From three of these animals, enrofloxacin and florphenicol resistant *Klebsiella pneumoniae* were isolated from liver, intestine, spleen and peritoneal cavity. From one pig, *Pseudomonas aeruginosa* intermediate sensitive to enrofloxacin and florphenicol resistant were isolated from lungs, intestine, liver and spleen. Among pigs in group 5, no pathogenic bacteria were detected at necropsy, except for lung tissue of piglet 4 from which non-haemolytic *E. coli* was isolated. Apart from these early manifestations of disease, no pigs in groups 1 (mock) or 2 (PPV) developed signs of clinical disease during the

experimental period. At necropsy, only three pigs in the control groups (groups 1 and 2) displayed minor lesions as specified in Table 1.

In group 3 (PCV2-1010 and PPV), one pig (D8) developed congestive heart failure and was found dead on day 24 PI. This pig also had overall enlarged lymph nodes, some with subcapsular haemorrhages, and patchy kidneys due to haemorrhages. Another pig (D2) was sacrificed due to weakness and bleeding from the nose. This pig displayed severe haemorrhages in the ventral neck region, enlarged axillary lymph nodes and was slightly jaundiced. In addition, three pigs were slightly weak during the last 4 days of the experiment. Gross lesions indicating PMWS were found in all pigs at necropsy (Table 1).

In group 4 (S-PCV2 and PPV), two pigs developed clinical signs indicating PMWS. One pig (D3) was found listless on day 20 post infection, and was sacrificed the following day in moribund condition. Necropsy demonstrated severe jaundice, overall enlarged lymph nodes and liver, and haemorrhages in lungs, liver and kidneys. The other pig (D9) became weak and lethargic 24 DPI and was subsequently sacrificed. Necropsy revealed haemorrhagic lesions in the kidneys and haematuria, as well as jaundice and overall enlarged lymph nodes with subcapsular haemorrhages. No other animals in group 4 showed clinical signs consistent with PMWS, but at necropsy, all pigs showed varying degrees of gross lesions consistent with PMWS (Table 1).

Table 1
Percent of pigs displaying gross lesions at necropsy

Lesions	Mock (group 1, n = 6)	PPV (group 2, n = 7)	PCV2-1010 + PPV (group 3, n = 10)	S-PCV2 + PPV Swe (group 4, n = 7)	S-PCV2 + PPV Den (group 5, n = 8)
Lymphadenopathy	17	14	90	100	100
Jaundice			30	57	13
Enlarged liver		14	40	71	63
Hypotrophic liver			10		
Enlarged spleen			30	43	13
Thymic atrophy			30	43	25
Gastric ulcer					13
Ascites containing fibrin					25
Pulmonary lesions			30		100
Congestive heart failure			10		
Enlarged kidneys			10		13
Haemorrhagic kidneys/haematuria		29			

The pigs were either controls, inoculated with virus-free cell lysate (mock) or PPV, or dually infected with PPV and a Canadian (PCV2-1010 + PPV) or Swedish (S-PCV2 + PPV) isolate of PCV2.

In group 5 (S-PCV2 and PPV), three pigs (nos. 2, 4 and 8) either died or were euthanised during the experimental period. Piglet 4 appeared pale and lethargic 5 DPI, experienced severe respiratory distress, and was euthanised 8 DPI. Piglet 2 was found dead on 19 DPI. Prior to this, it had suffered from respiratory distress and appeared lethargic with intermittently observed tremors. It was also pale and anorexic 2–3 days prior to dying. Pig 8 was euthanised 19 DPI in a moribund condition after showing pronounced respiratory distress since almost 2 weeks. In addition, this pig showed inappetence, appeared pale and lethargic and suffered from tremor attacks 2 days prior to euthanasia. The remaining five piglets (nos. 1, 3, 5, 6 and 7) showed mild clinical symptoms primarily characterized by periods of respiratory distress and lethargic appearance intermittently during the entire experimental period. All pigs in group 5 displayed gross lesions consistent with PMWS at necropsy (Table 1).

3.2. Histological lesions

Histological lesions found in organs tested from all experimentally infected pigs are summarized in Table 2. In brief, all but one of the pigs in group 2, infected with PPV alone, had mild to moderate lesions

mainly affecting myocardial tissue and kidneys. In groups 3, 4 and 5, a majority of the pigs displayed mild to severe lesions consistent with PMWS in most of the tissues tested. Among the uninfected control pigs (group 1), three animals displayed mild histological lesions at necropsy 28 DPI.

3.3. Detection of PCV2

Immunohistochemical staining of cryostat sections revealed that all pigs in group 3, 4 and 5 were positive for PCV2 in two or more tissues tested (Table 3). Furthermore, discriminative PCR analysis confirmed that pigs in group 3 were infected with PCV2-1010 only, whereas pigs in group 4 were only infected with S-PCV2. No PCV2 antigen was detected in cryostat sections of liver, spleen or lymph node from any of the pigs in the control groups 1 or 2.

Quantitative PCR performed on serum samples obtained from the Danish pigs infected with S-PCV2 and PPV (group 5) demonstrated PCV2 DNA from 4 days PI and onwards (Fig. 1). The levels of PCV2 DNA increased during the first 3 weeks reaching levels of 10^4 – 10^8 template copies per ml serum. At 27 DPI, however, the amount of PCV2 DNA had declined slightly in four of the five pigs remaining in the experiment.

Table 2
Summary of histological lesions determined in various organs

Organ	Lesion	1 Mock (n = 6)	2 PPV (n = 7)	3 PCV2-1010 + PPV (n = 10)	4 S-PCV2 + PPV Swe (n = 7)	5 S-PCV2 + PPV Den (n = 8)
Liver	Atrophy	–	–	30/+++	57/+++	13/+++
	Non suppurative hepatitis	–	29/+	100/++	100/++	75/++
Spleen	Depletion	17/+	14/+	100/++	60/++	63/+
Heart	Non suppurative myocarditis	33/+	86/+	100/++	83/+	50/+
Lung	Peribronchiolitis	33/+	29/++	100/++	57/++	38/+
	Interstitial pneumonia	–	–	10/++	–	38/++
Kidney	Non suppurative pyelitis	–	–	30/++	–	–
	Non suppurative interstitial pyelitis	17/+	86/++	60/++	100/++	75/+++
Lymph node	Depletion	17/+	–	100/++	100/++	50/++
	Hyperplasia	–	–	–	–	50/+
Intestine	Depletion Peyer's patches	17/+	14/+	40/+	71/+	100/++
Thymus	Depletion	NT	NT	NT	NT	63/++

The results are given as percent of affected pigs of those tested in the group for each lesion and average severity of lesions in affected pigs scored as (+) mild, (++) moderate or (+++) severe; NT = not tested.

Table 3

Immunohistochemical staining for PCV2 antigen in cryostat sections obtained from liver, spleen and lymph nodes (Ly node) of pigs experimentally infected with PCV2-1010 and PPV (no. 3) or S-PCV2 and PPV in Sweden (no. 4) or Denmark (no. 5)

Pig	Liver	Spleen	Ly node
PCV2-1010 + PPV (no. 3 Swe)			
A7	+++	+	++++
B1	+	–	+
B6	+	+/-	++
B11	++	++	+++
B14	+	+/-	++
C1	+	–	+
D1	+	++	+++
D2	+++	++++	+++
D8	+++	++++	++++
E5	+	+	++
S-PCV2 + PPV (no. 4 Swe)			
A3	+++	++	+++
B7	+	++	+++
B8	++	++	+++
C3	+	–	+
D3	+	+++	++
D9	+++	+++	+++
E3	++	+++	+++
S-PCV2 + PPV (no.5 Den)			
1	+	++	++
2	+	++	++++
3	+	++	++
4	–	+	+
5	++	+++	++++
6	++	++++	++++
7	++	++++	++++
8	+	++	+++

Amounts of PCV2 antigen were scored as not detectable (–), or minimal (+/-) to abundant (++++).

3.4. Sequence analysis of S-PCV2

Sequence comparison of the genome of S-PCV2 used in the experimental infection and PCV2 retrieved from the original lymph node revealed differences in five nucleotides at position 103, 346, 374, 478 and 491. One of these exchanges caused a substitution of amino acids (no. 27) located in ORF1. In ORF2, only one exchange of nucleotides (no. 103) was found, not leading to any change in amino acid sequence. In addition, an intronic nucleotide deletion between ORF1 and ORF2 (no. 315) was found. Thus, repeated passages in cell culture had only caused minor changes in the genome of S-PCV2.

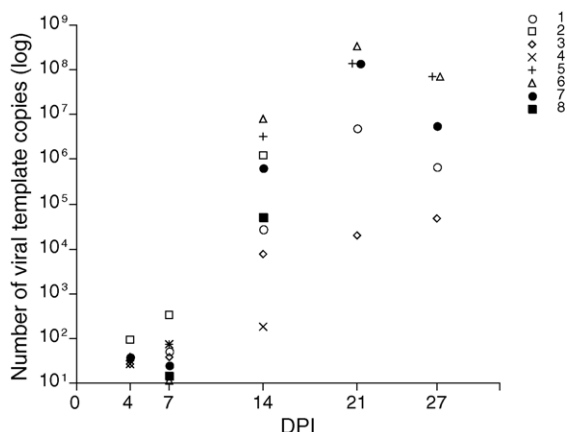


Fig. 1. Levels of PCV2 viral DNA found in sera from Danish pigs in group 5, expressed as number of viral template copies for individual pigs at the indicated time points.

3.5. Serologic examinations

None of the pigs had antibodies to PCV2 or PPV prior to infection. All pigs in group 1 remained seronegative to both PCV2 and PPV, and pigs in group 2 remained seronegative to PCV2 throughout the experimental period. All pigs in groups 2, 3, 4 and 5 had seroconverted to PPV 1 week PI, and remained positive for PPV at the following sampling occasions (data not shown). The development of antibodies to PCV2 in the pigs dually infected with PPV and PCV2 is shown in Table 4. In brief, the two pigs in group 4 (S-PCV2 + PPV) that developed clinical PMWS, died or were sacrificed before serum antibodies appeared. Three of the other pigs in group 4 displayed low levels of antibodies to PCV2, whereas one (C3) developed high titers of antibodies. In group 5, all animals but two had seroconverted to PCV2 on 14 DPI, and on 21 DPI, all the remaining pigs were seropositive, whereas piglet 4 died before seroconversion occurred. Of the five piglets surviving the 4 weeks of experimental period, piglets 5, 6 and 7 only reached low antibody titers to PCV2. In general, pigs in group 3 (PCV2-1010 + PPV) developed a higher titer of antibodies to PCV2 than those infected with S-PCV2 and PPV. No such difference between these two groups was apparent for the antibody response to PPV (data not shown).

Table 4

Development of antibodies to PCV2 in serum of pigs experimentally infected with PCV2-1010 and PPV (no. 3) or S-PCV2 and PPV in Sweden (no. 4) or Denmark (no. 5)

PCV2-1010 + PPV (no. 3 Swe)						S-PCV2 + PPV (no. 4 Swe)						S-PCV2 + PPV (no. 5 Den)						
Pig	Days post infection					Pig	Days post infection					Pig	Days post infection					
	0	8	15	22	28		0	8	15	22	28		0	4	7	14	21	27
A7	<	<	<	1600	6400	A3	<	<	<	50	<	1	<	<	<	<	250	>6250
B1	<	<	<	1600	12500	B7	<	<	100	200	200	2	<	<	<	50	Dead	Dead
B6	<	<	200	3200	1600	B8	<	<	100	100	100	3	<	<	<	250	1250	>6250
B11	<	<	50	1600	1600	C3	<	<	50	3200	50000	4	<	<	<	Dead	Dead	Dead
B14	<	<	50	6400	25000	D3	<	<	<	Dead	Dead	5	<	<	<	250	250	250
C1	<	<	<	800	12500	D9	<	<	<	<	Dead	6	<	<	<	<	50	250
D1	<	<	200	3200	6400	E3	<	<	<	50	<	7	<	<	<	250	50	50
D2	<	<	100	200	Dead	-	-	-	-	-	-	8	<	<	<	250	Dead	Dead
D8	<	<	400	12500	Dead	-	-	-	-	-	-	-	-	-	-	-	-	-
E5	<	<	NT	50	6400	-	-	-	-	-	-	-	-	-	-	-	-	-

The serum levels of antibodies are given as reciprocal titers, determined in an immunoperoxidase monolayer assay.

3.6. Serum IFN- α and IL-10

All serum samples collected before infection (0 DPI) of the piglets in groups 1–4 were negative for IFN- α (Fig. 2). One week later, however, pigs from all experimental groups, including the mock-inoculated controls, displayed IFN- α in serum. Thereafter, only pigs infected with both PCV2 and PPV had serum

IFN- α , and some of these pigs still had detectable levels of IFN- α upon termination of the experiment.

Serological testing of the Swedish pigs revealed that most of the samples contained less than 3 pg IL-10 per ml serum. However, comparison of absorbance values obtained by the ELISA (Fig. 3) indicated a low IL-10 response at day 8 PI which occurred in the majority of pigs exposed to PPV, alone or in

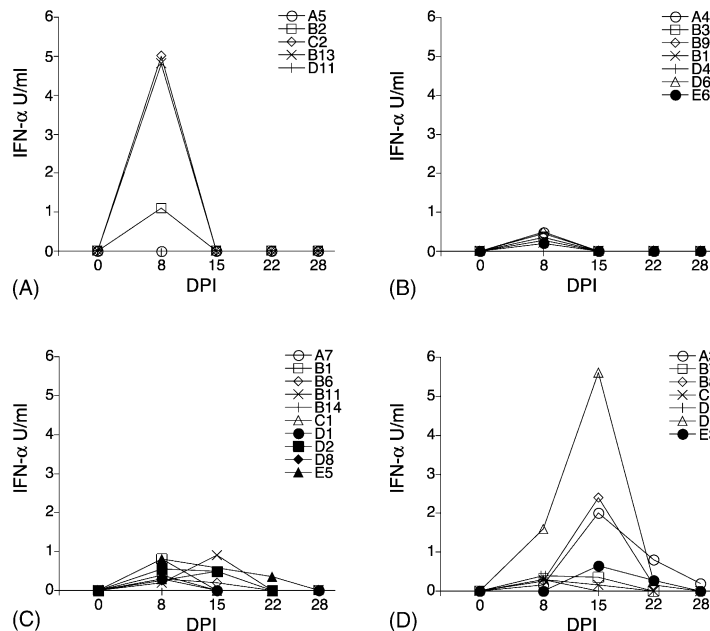


Fig. 2. Serum levels of IFN- α in Swedish pigs inoculated with mock (A), PPV (B), PCV2-1010 and PPV (C) and S-PCV2 and PPV (D). Levels of IFN- α were determined by immunoassay and expressed as units per ml.

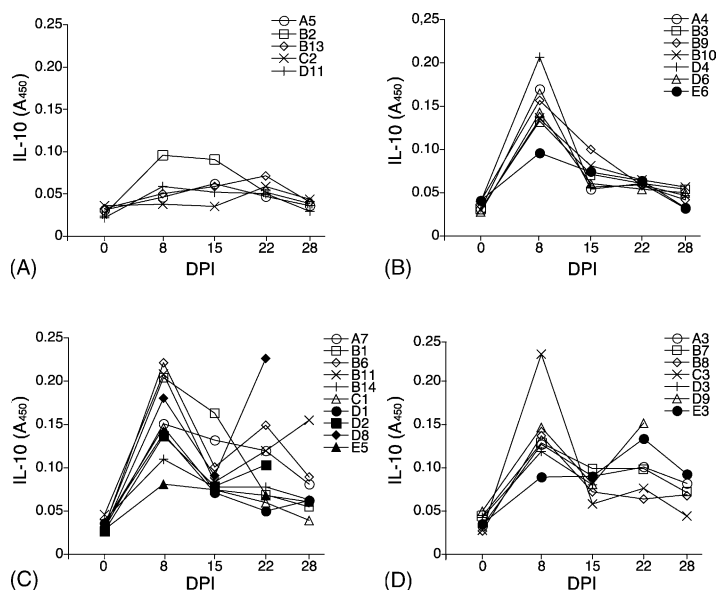


Fig. 3. Indicated IL-10 responses in sera from Swedish pigs inoculated with mock (A), PPV (B), PCV2-1010 and PPV (C) and S-PCV2 and PPV (D). Levels of IL-10 are expressed as absorbance values (A_{450}).

combination with PCV2. In groups 3 and 4, elevated levels of IL-10 were also detected in serum collected 22 DPI.

4. Discussion

PMWS could be reproduced in both Swedish and Danish pigs with a Swedish isolate of PCV2 in combination with PPV, using a previously established model for experimental infection (Allan et al., 1999). The Swedish PCV2 used (S-PCV2) was isolated from a pig raised in a SPF-herd at the time of herd seroconversion to PCV2 more than 10 years ago (Wattrang et al., 2002). At that time, transient reproductive problems were noticed in the herd, but later no effects of the infection could be observed. The present study thus confirms that a strain of PCV2 virus can persist in a pig population without causing clinical PMWS, and still be pathogenic under certain conditions. This potential of S-PCV2 has previously been demonstrated in the same experimental model using pigs derived from an SPF unit in Northern Ireland (Allan et al., 2003), and the present study shows that Swedish pigs are susceptible to experimental reproduction of PMWS. Moreover, it was

confirmed that the pathogenicity of S-PCV2 is indistinguishable from that of the reference strain PCV2-1010 in this experimental model. PCV2-1010 was initially recovered from a diseased pig in Canada.

At the time of this study, PMWS had not been diagnosed in Sweden, whereas in Denmark, the disease spread rapidly after it was first discovered in 2001 (<http://www.dfvf.dk>). PMWS was diagnosed in Sweden in late 2003 and so far the spread of disease is moderate with only few animals showing clinical disease in affected farms. It is to date not possible to predict the final outcome of PCV2-related diseases in Sweden, but it seems that the prevalence and mortality within each herd is comparably low, approximately 7% mortality and runts after weaning. Thus, the situation is more similar to what has been reported from North America than that reported from the rest of Europe (Harding, 2004). Generally, postweaning mortality in Sweden is low (2.5% of weaned pigs in 2003, <http://www.qgenetics.com>) and the country is free from many porcine pathogens such as PRRSV, ADV and enteric corona virus. Also, rearing of pigs according to the “Swedish model” includes a relatively high weaning age (4–5 weeks), batch-wise rearing from birth to slaughter, and a reduction of transportation and/or mixing of pigs from different

litters. All of these factors may contribute to the relative resistance of the Swedish pig industry to PCV2-related diseases, although it is now clear that Swedish pigs can develop PMWS both experimentally and naturally. Notably, the first PMWS outbreaks in Sweden occurred in herds where high production intensity had resulted in deviations from this “Swedish model”.

The clinical and pathological evidence of disease in S-PCV2 and PPV co-infected animals was similar in the Swedish and Danish experiments presented herein. To further evaluate the pathogenicity of S-PCV2, one group of Swedish pigs was infected with a reference strain of PCV2 (Imp. 1010, Stoon) in combination with PPV. No pigs in this group died from PMWS, and no animal showed clinical signs of disease related to PMWS during the experimental period. At necropsy, however, all pigs from this group displayed gross lesions consistent with PMWS. The combined manifestation of clinical disease and pathological lesions caused by the two isolates indicates that the pathogenicity of the Swedish isolate is equal to or even higher than that of PCV2-1010. In two previous studies applying the same experimental model, however, the proportions of pigs developing clinical PMWS was somewhat larger, both concerning PCV2-1010 and S-PCV2 (Allan et al., 2003; Allan et al., 1999; Allan et al., 2004). This discrepancy could reflect a diminished pathogenicity of PCV2-1010, possibly caused by several *in vitro* passages of the virus, but variations in the experimental conditions cannot be excluded.

To rule out that S-PCV2 had undergone major changes during the isolation procedure, a comparison of the genomes of S-PCV2 before and after repeated passages in cell culture was performed. This revealed only minor changes, indicating that the virus used in the experimental infection was very similar to the Swedish field isolate from 1993. As previously described, this isolate differs from PCV2-1010 in the nucleotide sequence of ORF2 (Allan et al., 2003) causing eight amino acid substitutions. Nucleotide sequence analysis revealed several differences between S-PCV2 used in the experimental infection and PCV2 isolated in December 2003 from the first natural cases of PMWS in Sweden (data not shown).

The occurrence of IFN- α in serum samples collected on day 8 from pigs in groups 1–4 indicates

that some pigs experienced infections unrelated to the experimental infections. This was also evident from early deaths among pigs in all experimental groups, which in most cases were related to navel infections, and could be expected for pigs snatched-farrowed in a conventional farm. On later sampling occasions, however, IFN- α could only be detected in sera from pigs in groups 3 and 4 that had been inoculated with PCV2. In contrast, elevated serum IL-10 was indicated in pigs infected with PPV alone or in combination with PCV2. This suggests that PPV induces a systemic response of this cytokine, which might be of importance for the development of PMWS in the experimental co-infection with PCV2. In natural cases of PMWS, an over-expression of IL-10 mRNA has been found in PBMC (Sipos et al., 2004) and thymocytes (Darwich et al., 2003b), and PBMC from PMWS affected pigs preferentially produced IL-10 and IFN- γ at *in vitro* re-exposure to PCV2 (Darwich et al., 2003a). If an altered cytokine profile is caused by progressing PMWS or induced by other factors that might predispose for PMWS, remains however to be determined. Interestingly, PRRSV infections, often considered as a co-factor for development of PMWS, induced increased expression of IL-10 mRNA in PBMC, lung tissue and bronchoalveolar cells (Chung and Chae, 2003; Johnsen et al., 2002; Suradhat and Thanawongnuwech, 2003). Thus, if IL-10 expression is important for PMWS development, the PRRSV free status of Sweden may well explain the lower incidence of PMWS recorded within Swedish herds.

The impact of PPV infections in the pathogenesis of PMWS was recently demonstrated under field conditions where PMWS was induced in segregated early-weaned 6–7 week old pigs co-infected with PPV and PCV2 (Opriessnig et al., 2004), which further justifies the current experimental model. It should, however, be noted that the permanent treatment with broad-spectrum antibiotics severely affected the normal bacterial flora of the gut. Pen floor samples revealed less than 10^2 CFU coliforms per gram faeces (data not shown) compared to 10^8 – 10^{10} in samples from non-treated pigs of the same age category (Melin et al., 2000). The importance of a normal microflora for the development of both mucosal and systemic immunity has been described for several species, including gnotobiotic piglets (Tlaskalova-Hogenova et al., 2004). In addition to the antibiotic treatment, piglets

were handfed with cow-milk based colostrum substitutes during the first days of life to avoid passive transfer of porcine immunoglobulins. This treatment might further affect the function of the gut associated lymphoid tissue of the experimental pigs. Since large amounts of PCV2 are secreted through faeces during PMWS, the gut and surrounding tissues are likely to be involved in the replication of PCV2 as well as of PPV. Thus, several non-physiological effects are caused by the present experimental model, which have to be considered for a better understanding of co-factors that contribute to the development of PMWS. Nevertheless, the results presented here clearly show that a virus strain that persisted for 10 years in a SPF-herd without causing clinical PMWS was able to induce this disease under experimental conditions in pigs of different origin. Further studies concerning the effects of husbandry forms as well as genetics of the animals and microorganisms are needed to elucidate why the spread of PMWS differs between regions.

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