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Coinfection by porcine circoviruses and porcine parvovirus in pigs with naturally acquired postweaning multisystemic wasting syndrome

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Abstract. Postweaning multisystemic wasting syndrome (PMWS) is an emerging disease in swine. Recently, the disease has been reproduced with inocula containing a newly described porcine circovirus (PCV), designated PCV 2, and porcine parvovirus (PPV). In order to determine if these viruses interact in naturally acquired PMWS, affected tissues from field cases were examined by immunohistochemistry (IHC) and polymerase chain reaction (PCR) for PCV 2 and PPV, as well as by PCR for the other recognized porcine circovirus, PCV 1. Porcine circovirus 2 was detected by PCR or IHC in affected fixed or frozen tissues from 69 of 69 cases of PMWS collected over 3 years from 25 farms. Porcine parvovirus was detected in 12 of the same cases, and PCV 1 was detected in 9 of 69; however, an apparent decrease was found in the sensitivity of the PCRs used to detect the latter 2 viruses when fixed tissue from the same cases were compared with the use of frozen tissues. Porcine circovirus 2 was not detected by PCR in affected tissues from 16 age-matched pigs that had *Streptococcus suis*-associated disease. Electron microscopic examination of plasma pooled from 15 pigs with PMWS revealed the presence of PCV and PPV, whereas these viruses were not observed in pooled plasma from 5 age-matched clinically normal pigs. These results confirm and extend previous findings documenting a consistent association of PCV 2 with PMWS. As well, infection by PPV or PCV 1 or both may be an important cofactor in the pathogenesis of some, but apparently not all, cases of PMWS.

Postweaning multisystemic wasting syndrome (PMWS) is an emerging disease in swine herds in Canada,[10] the United States,[4] and Europe.[4] This syndrome occurs most commonly in 5–12-week-old pigs.[4,10] Clinically, affected pigs present with progressive weight loss, tachypnea, dyspnea, and jaundice. Gross lesions include lymphadenopathy, interstitial pneumonia, hepatitis, and nephritis.[4,10] Histologically, multifocal lymphohistiocytic cellular infiltrations, often with a prominent eosinophilic component, are found in affected organs. Multinucleated giant cells are commonly found in lymph nodes from pigs with PMWS.[4,10]

A consistent association between PMWS and infection by an apparently new virus, tentatively designated ''porcine circovirus 2'' (PCV 2), in pigs with naturally acquired disease has been demonstrated.[4,10] Moreover, PCV 2 isolates obtained from affected pigs in several countries are virtually identical genetically and are distinctly different from the PCV (CCL33; PCV-I) that was originally identified in the 1970s as a noncytopathic contaminant of porcine kidney (PK/15) cell lines.[17,25] Previous serological surveys documented an apparently high prevalence of seroconversion to PCV 1 in pig populations in North America and Europe[9,14,23]; however, to date, PCV 1 infection of pigs with PMWS has not been documented.

Pigs with PMWS are often infected with a variety of other pathogens, including bacteria and, less frequently, viruses, mycoplasma, and protozoa, in addition to PCV 2.[10] In previous studies, the involvement of porcine parvovirus (PPV) in PMWS was not exhaustively ruled out, largely because PPV was not identified in low-passage cell cultures derived from the organs of pigs with PMWS, and its potential involvement in severe multisystemic disease in weanling pigs was not anticipated. Although PPV is thought to be endemic in pig populations throughout the world,[18] it has only rarely been associated with any disease in pigs other than fetal death.[7,8,15] Most available data indicate that the apparently universal exposure to PPV in pigs was almost exclusively associated with subclinical infection and development of immunity.[18] Recently, the lesions of PMWS have been reproduced in pigs with inocula that, retrospectively, were found to contain low concentrations of PPV in addition to PCV

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2.[11] Subsequent prospective studies confirmed the apparent synergistic effect of the 2 viruses in producing severe clinical disease.[1] These observations raised questions concerning the role of PPV in naturally acquired PMWS.

The objectives of this study were to determine the prevalence of PCV 1, PCV 2, and PPV in lesions of pigs with naturally acquired PMWS and to compare the efficacy of immunohistochemistry (IHC) and polymerase chain reaction (PCR) and in detecting PCV 2 in frozen and fixed tissue.

Materials and methods

Case selection. One hundred six weanling pigs ranging in age from 4 to 12 wk were the subjects of this study. Sixty-nine pigs were presented to the referring clinical veterinarian or the diagnostic laboratory at the Western College of Veterinary Medicine during 1995–1998 with clinical signs and multisystemic lesions[4,10] characteristic of PMWS.[4,10] These pigs were from 25 farms, comprising high health and commerical herds in Alberta and Saskatchewan. Pigs on these farms are routinely vaccinated for parvovirus as gilts prior to entry into the breeding herd. ''Booster'' vaccines for parvovirus are usually administered every 5–6 mo. Tissues were collected at necropsy, fixed in formalin, and paraffin-embedded or frozen at −70 C. Paraffin blocks containing samples of diseased organs from 16 pigs with *Streptococcus suis*-related illness from 13 farms in Saskatchewan from which PMWS has never been diagnosed were examined by PCR. Blood samples from 15 pigs with PMWS from 1 farm were examined by electron microscopy for circulating viruses. Tissues from 6 clinically normal weanling pigs from a farm where PMWS has not been diagnosed were used as controls.

PCR for PCV and PPV. Primers were designed that allowed the amplification of a 347-bp fragment specific to the PCV I (for, 5′-GCGCCATCTGTAACGGTTTC-3′; rev, 5′-TCCAAACCTTCCTCTCCGC-3′) and a 481-bp fragment specific to PCV II (1443, 5′-CGGATATTGTAGTCCTGGTCG-3′; 150, 5′-ACTGTCAAGGCTACCACAGTCA-3′).[16] DNA was extracted from 10 10-μm sections of paraffin-embedded tissue blocks or 50 mg of frozen tissue with a commercial kit according to the manufacturer's instructions.[a] The reaction mixture contained 200 μM dNTPS, 1.5 mM $MgCl_2$, 50 pmoles of each primer, 1× Taq buffer,[b] 1.25 units Taq polymerase,[b] and 100 ng DNA in a final volume of 50 μl. Reaction conditions were 94 C for 1 min (1 cycle), followed by 35 cycles of 94 C for 1 min, 55 C for 1 min, and 72 C for 1 min, and a final cycle at 72 C for 10 min. A PCR for PPV was performed with primers that amplfy a 158-bp fragment from the VP2 gene.[19] Reaction conditions were as previously described except that the annealing temperature was increased from 45 to 50 C and 1 μg DNA was used per 50-μl reaction. The identity of amplified fragments was confirmed by digestion with *Eco*R1.[19]

IHC. Immunohistochemical identification of PCV 2 in tissues was performed as previously described.[10] Briefly, sections cut from blocks of embedded tissue were reacted with either diluted rabbit anti-PCV antiserum, diluted porcine immune serum, or a monoclonal antibody specific for PCV 2.[10] After reaction with the primary antibody, tissues were incubated with appropriate secondary antisera before visualizing the reaction product by an avidin–biotin complex technique as previously described.[10] For immunohistochemical detection of PPV, sections were cut from blocks of frozen tissue with a cryostat and similarly stained with a monoclonal antibody specific for PPV.[12] Negative controls included serial sections of each block stained with the omission of primary antisera and with the substitution of primary antisera with irrelevant polyclonal antisera from the appropriate species. Positive control tissue from a pig with naturally acquired PMWS was also stained. Tissues tested included lymphoid organs, liver, kidney, lungs, stomach, intestines, and pancreas.

Electron microscopy. Plasma samples from 15 pigs affected with PMWS and 5 clinically normal pigs were pooled separately, filtered (0.2 μm), and centrifuged at 100,000 × *g* at 4 C for 24 hr. Each pellet was resuspended in 1 ml of Tris–NaCl–ethylenediaminetetraacetic acid (EDTA) buffer (50 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, pH 7.4) and passed through a 20–50% sucrose gradient prepared in the same buffer. The gradient was fractionated and the fractions were measured for protein concentration. Peak fractions were pooled and measured for refractive index. Pooled fractions with a refractive index of approximately 1.17 g/cm^3 were pelleted as above for 4 hr at 4 C. The resulting pellets were resuspended in 100 μl of Tris-NaCl-EDTA buffer. One drop was applied to carbon-coated formvar (0.3%) grids that had been wetted, with a drop of water containing 2% fetal bovine serum and dried. After 30 sec, excess liquid was removed, and the specimen was air dried and stained with 0.5% phosphotungstic acid in phosphate-buffered saline (pH 7.4).

Results

Detection of PCV and PPV in PMWS cases. Porcine circovirus 2 was detected by PCR or IHC or both (Fig. 1; Table 1) in 69 of 69 cases that were previously diagnosed as PMWS on the basis of clinical and pathologic examination. Porcine circovirus 2 DNA was detected in 65 of 69 cases tested. Porcine circovirus 2 antigens were detected by IHC in fixed tissue from 35 of 35 PMWS cases that were tested by this method. Circoviral antigens were detected most consistently in lymphoid tissue and variably in other tissues examined. Four cases were IHC+ for PCV 2 and PCR negative for PCV 2. Histologically, these PCR-negative cases had low numbers of PCV 2+ cells in affected tissues. With the same extracted DNA samples, PCV 1 was detected in 9 of 69 cases of PMWS, 8 of 34 frozen samples, and 1 of 35 fixed, embedded samples. Detection of PCV 1 antigens by IHC was not attempted. Parvoviral DNA was detected in 9 of 69 cases of PMWS from 7 farms, 11 of 34 frozen samples, and 1 of 35 fixed, embedded samples. Immunohistochemical examination of frozen sections of 4 randomly selected

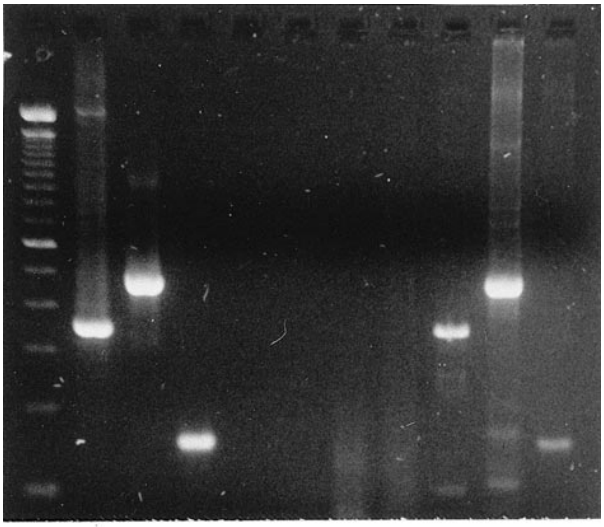

Figure 1. Detection of porcine circoviruses (PCV) 1 and 2 and porcine parvovirus (PPV) in porcine tissues by polymerase chain reactions. **a**, DNA ladder, **b**, PCV 1 positive control, **c**, PCV 2 positive control, **d**, PPV positive control, **e**, blank, **f**, PCV 1 PCR with liver from normal pig, **g**, PCV 2 PCR with liver from normal pig, **h**, PPV PCR with liver from normal pig, **i**, PCV 1 PCR with affected liver from pig with PMWS, **j**, PCV 2 PCR with affected liver from pig with PMWS, **k**, PPV PCR with affected liver from pig with PMWS.

PMWS samples that were PCR+ for both PCV 2 and PPV revealed coinfection by both viruses in the same lesion sites in liver, kidney (Figs. 2–4), lung, or lymphoid tissue. In contrast, neither PCV 1 or PCV 2 was detected by PCR in lesions from 16 pigs that had *S. suis*-associated disease; 1 of these pigs was PCR positive for PPV (Table 1). No apparent relationship was found between accession date and results of tests for virus detecton.

Electron microscopic examination of pooled plasma from pigs with PMWS revealed that both PCV and PPV were present in gradient-separated preparations. Although similar in size and physiochemical characteristics, the viruses were distinguishable. Parvoviral particles were slightly larger and often had an electron-dense center surrounded by a radiolucent ''halo'' (Figs. 5, 6). These viruses were not observed in the plasma of the age-matched clinically normal pigs that were sampled.

Effect of fixation on the detection of PCV and PPV by PCR. In order to assess the effect of fixation and routine processing on the PCRs for PCV 1, PCV 2, and PPV, additional PCR tests were conducted with 7 PCV2/PPV PCR-positive PMWS cases for which both frozen and fixed tissues were available. PCV 2 was detected in all 7 cases with both types of tissue samples; however, there was a reduction in the number of PCR-positive results for PCV 1 and PPV comparing frozen and fixed tissue (Table 2). Overall, PCV 1 and PPV were detected in a higher percentage of frozen tissue than in the same fixed tissue from clinically affected pigs.

Table 1. Detection of porcine circoviruses (PCVs) 1 and 2 and porcine parvovirus (PPV) by polymerase chain reaction (PCR) in diseased organs from pigs with and without postweaning multisystemic wasting syndrome (PMWS).

	PCV 1 PCR	PCV 2 PCR	PPV PCR
PMWS (fixed tissue)	1/35	31/35	1/35
PMWS (frozen tissue)	8/34	34/34	11/34
Non-PMWS (*Streptococcus suis*)	0/16	0/16	1/16

Discussion

The results of this study confirm and extend previous observations of consistent association between PCV 2 and lesions in pigs with PMWS.[4,10] This association was detectable by both PCR and IHC. These data are in contrast to a previous study reporting that only 15% of pigs with PMWS were infected with PCV 2 at the time of death or euthanasia because to wasting disease.[20] In contrast to the consistency of infection with PCV 2, a lower prevalence of PCV 1 in affected tissues was determined by PCR. In approximately 17% of randomly selected typical cases of PMWS, coinfec-

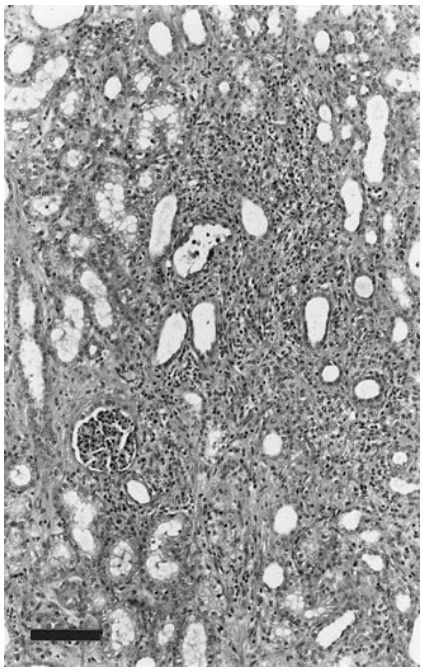

Figure 2. Renal tissue from a pig with PMWS. Note severe lymphogranulomatous interstitial nephritis. Hematoxylin and eosin stain. Bar = 100 μm.

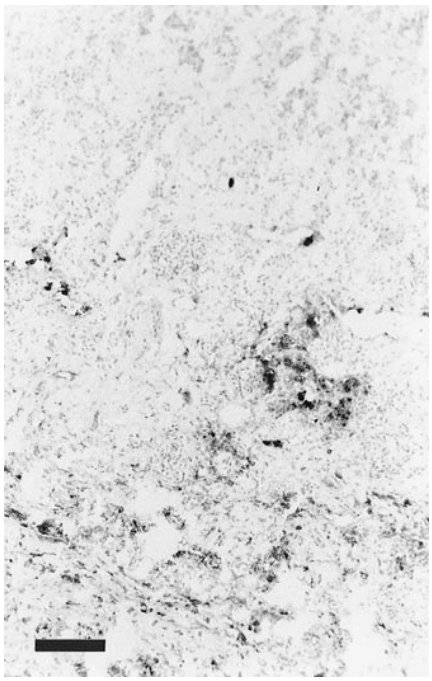

Figure 3. Cryostat section of renal tissue from pig in Fig. 2 with PMWS immunohistochemically stained for PCV 2. Note scattered intensely stained cells. Bar = 100 μm.

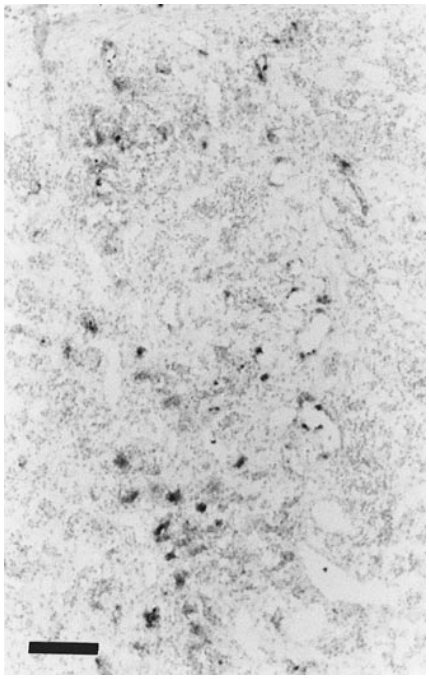

Figure 4. Cryostat section of renal tissue from pig in Fig. 2 with PMWS immunohistochemically stained for PPV. Note scattered intensely stained cells. Bar = 100 μm.

tion by PPV and PCVs was found in organs with lesions typical of PMWS.

The comparative testing results by PCR on frozen and fixed tissues suggest that routine fixation, processing, and extraction apparently have little discernable effect on the ability to detect PCV 2 with these primers under these PCR conditions. Most tissues submitted to the diagnostic lab would have been fixed for 2–3 days prior to processing. Moreover, fixation in formalin of up to 7 days apparently does not affect the detection of PCV 2 by this PCR or IHC (J. Ellis, unpublished results). In contrast, the lower prevalence of PCV 1 positives and PPV positives in fixed tissues as well as the direct comparison of frozen and fixed tissue indicate a decrease in sensitivity (apparent false-negative results) of these PCRs to detect the latter viruses. The reason for the decreased sensitivity was not apparent but could be related to low viral copy number or the direct effect of processing or extraction methods on DNA from PCV 1 and PPV. Nevertheless, these results serve as a caveat to the application of PCRs with these conditions for PCV 1 and PPV for retrospective studies on readily available archival material. Furthermore, these comparative results indicate that infection by PCV 1 or PPV could have been underdiagnosed in the 35 PMWS cases for which only fixed and embedded tissue was available. Application of a recently reported[5] nested PCR may increase the sensitivity of detection of PPV, but applicability of this assay in fixed tissue remains to be demonstrated.

The lower prevalence of PCV 1 compared with PCV 2 infection in pigs with PMWS was unexpected on the basis of previous serological surveys conducted in Canada and other countries.[9,14,23] Those studies documented a high prevalence of seroconversion to PCV 1 in healthy pigs. Those data, together with experimental infections with PCV 1, suggested that infection by PCV 1 was endemic and apparently nonpathogenic.[2] Several explanations are possible for this discordance between past serological data and the present results. One possibility is that previous serological testing that was done by immunofluorescence on PCV 1-infected cells detected group-specific antibodies that cross-reacted with PCV 2. Supportive of this hypothesis a high degree of homology in the sequences of open reading frame (ORF) 1 of both viruses, with less homology in the 3 other ORFs, has recently been documented.[17] This finding would imply that exposure to PCV 2 infection was widespread in clinically normal pigs, which is supported by more recent preliminary serological studies (J. Ellis, unpublished data). Another possibility is that PCV 2 emerged as a pathogenic variant of PCV 1 since previous serological surveys were conducted and is now the predominant PCV infecting domestic swine. Finally, available data indicate that PCV 2 replicates to high titers in cell cultures, whereas PCV 1 infection results in only low copy number.[17] If

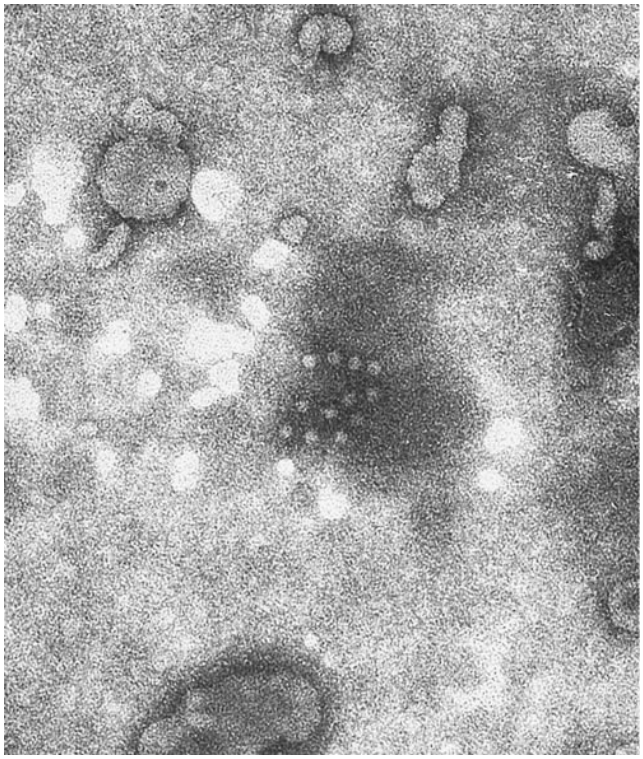

Figure 5. Electron micrograph of PCV in gradient-purified pooled plasma sample from pigs with PMWS. ×98,000.

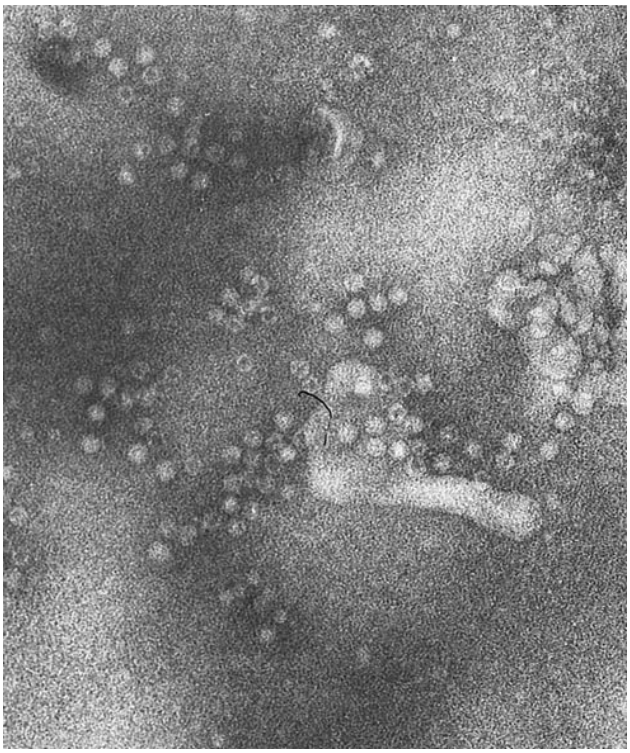

Figure 6. Electron micrograph of PPV in the same gradient-purified pooled plasma sample as Fig. 5 from pigs with PMWS. ×98,000.

a similar phenomenon occurs in vivo, identification of PCV 1 in affected tissue in many cases may be beyond the detection limits of the PCR that was used.

The failure to detect PCV 2 in organs with a variety of lesions from pigs with *S. suis*-associated disease from unaffected (no PMWS diagnoses) farms suggests that there is not endemic persistent infection by PCV 2 in pigs without PMWS. More extensive studies are currently under way to determine the prevalence of exposure to both PCV 1 and PCV 2 in populations of clinically normal pigs and diseased pigs. Nevertheless, presence of PCV 2 in affected organs of pigs with PMWS, and lower prevalence of PCV 1, is consistent with the hypothesis that PCV 2 is at least a necessary cofactor in the pathogenesis of PMWS.

Previously, PCV 2 and PPV have been isolated from naturally acquired cases of PMWS.[1] This coinfection would not necessarily be unexpected, given the reported endemic nature of PPV infection;[18] however, how the current management systems employing stringent biosecurity and sanitation may have affected the prevalence and epidemiology of parvoviral infections is not documented. Both PPV and PCV are small, single-stranded DNA viruses that have a small coding capacity and are, therefore, highly dependent on host cell functions.[17,24] The predilection of autonomous parvoviruses from a variety of species for rapidly dividing cells is well recognized and well documented both in vivo and in vitro.[6,13,24] Although it has been documented in vitro that the replication of at least the original isolates of PCV are dependent on the S, or synthesis phase, of the cell cycle, currently little is known about the requirements for PCV growth in vivo.[23] Replication of both PPV and PCV depends on cellular enzymes expressed during S phase of the cell cycle,[24] suggesting that PPV and PCV may target the same cells in vivo. They may interact in some way to synergistically enhance replication, or the same physiologic factors may enhance replication of both viruses. Therefore, the presence of both PCV 2 and PPV in lesions sites could be causally related to disease. Alternatively, viral antigens in phagocytic cells in lesion sites may have been engulfed at extralesional sites and transported intracellularly into sites of tissue damage, or replication of both PCV 2 and PPV may occur in rapidly replicating (repairative) cells in sites of preexistent tissue damage.

Table 2. Comparision of detection of porcine circoviruses (PCVs) 1 and 2 and porcine parvovirus (PPV) by polymerase chain reaction (PCR) in fixed and frozen tissues.

	PCV 1 PCR	PCV 2 PCR	PPV PCR
Frozen tissue	7/7	7/7	4/7
Formalin fixed	1/7	7/7	3/7

The presence of cell-free PPV in the circulation of pigs with PMWS would not necessarily in and of itself be an indication that the virus is causally involved in the disease process. Pigs with PMWS generally present with clinical signs and corresponding lesions at an age when maternal antibodies would be waning.[21] Pigs exposed to PPV at this time could be subclinically infected and transiently viremic prior to the development of immunity.[18] Although the pathogenesis of the newly described strains of PCV is not currently known, a similar scenario could also occur after infection of postweaning age pigs with this virus. However, the detection of both viruses in the typical lesions of PMWS, in which necrosis or inflammation or both are associated with infection of cells, indicates that the viremia detected was not simply indicative of an inocuous or subclinical infection, as has usually been reported for PPV infections in postnatal pigs.[18] These data support the concept that PPV may be one of several cofactors, including other infectious agents such as PRRSV,[11] that act synergistically with PCV 2 in the pathogenesis of PMWS.

Both PPV and PCV have a predilection for lymphoid tissue,[1,3,10,12,13] therefore replication of these agents in synchrony or in concert could have profound immunomodulating consequences and predispose to debility and secondary infections. PPV also has extralymphoid targets, including pulmonary and renal epithelium, hepatocytes, and endothelium.[21] Although controversial, it has been proposed on the basis of in vitro and in vivo studies that observed differences in virulence among PPV isolates may relate to differences in tissue tropism.[6,16] Although similar studies have yet to be performed with PCV isolates, it appears, on the basis of immunohistochemical analyses of lesions in naturally and experimentally infected pigs,[1–3,10,12] that, compared with PCV 1, the recent isolates of PCV 2 have a wider tropism than just lymphoid tissue, which may account, in part, for its virulence in vivo.

In conclusion, the results of this study confirm and extend previous work documenting a consistent association between active infection by PCV 2 and the development of PMWS in weanling pigs. Together with data from recent experimental infections with PCV 2,[1,12] this study is consistent with the notion that PPV may serve as an important cofactor in the development of naturally acquired PMWS. Further investigation is required to identify additional cofactors that, together with PCV 2, interact in the pathogenesis of PMWS.

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Sources and manufacturers

a. Qiagen Inc., Mississauga, Ontario, Canada.
b. Gibco-BRL, Burlington, Ontario, Canada.

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