Molecular Pathogenesis and Development of a Genetically Engineered Vaccine for Type-2 Porcine Circovirus

By

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Abstract

Porcine circovirus type 2 (PCV2) is the primary causative agent of postweaning multisystemic wasting syndrome (PMWS), whereas the ubiquitous porcine circovirus type 1 (PCV1) is nonpathogenic for pigs. Since its initial detection in a Canadian commercial swine herd in 1991, PMWS has been detected in all swine producing regions of the world and is now a serious economic problem to the swine industry. The objectives of this dissertation were to biologically, genetically and experimentally characterize both PCV1 and PCV2, to identify the genetic determinant(s) for virulence and replication, and to develop an effective genetically-engineered vaccine against PCV2 infection and PMWS.

The genetic heterogeneity of PCV2 and PCV1 isolates from different geographic origins were determined. We found that, although PCV1 and PCV2 genomes were very conserved, some minor genomic variation exists among PCV1 isolates and PCV2 isolates. The nonpathogenic PCV1 and pathogenic PCV2 share only about 76% nucleotide sequence identity but have similar genomic organization. The highest sequence variability among PCV isolates is found in the immunogenic ORF2 capsid gene. Based on the sequence data in this dissertation, a universal polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay was developed

that is capable of detecting all known PCV isolates and differentiating between infections by nonpathogenic PCV1 and pathogenic PCV2.

In order to study the structural and functional relationship of PCV genes and to develop a genetically-engineered vaccine, we constructed infectious DNA clones of both PCV1 and PCV2. By using the PCV2 infectious clone, we showed that pigs can be infected by direct intrahepatic injection of PCV2 infectious DNA clone. The pathological lesions and clinical disease associated with PCV2 infection were more definitively characterized by using the infectious DNA clone. We found that PCV2 is the primary but not the sole causative agent of PMWS, as the full spectrum of clinical PMWS was not reproduced by the infectious PCV2 DNA clone although pathological lesions characteristic of PMWS were reproduced.

A chimeric vaccine was constructed by cloning the immunogenic capsid gene of the pathogenic PCV2 into the genomic backbone of the non-pathogenic PCV1 virus. We showed that the resulting chimeric PCV1-2 vaccine virus, retained the non-pathogenic nature of PCV1 but induced a protective immune response against a wild-type PCV2 challenge. In vaccinated pigs, the chimeric PCV1-2 vaccine reduced PCV2 viremia length and serum virus loads and reduced pathological lesions such as lymphoid depletion (LD) and histiocytic replacement (HR) in lymphoid tissues, inflammation and discoloration of the lymph nodes. The amounts of PCV2 antigen and PCV2 genomic copy loads in lymph node tissues were also significantly reduced. Our results indicated that the attenuated chimeric PCV1-2 virus induces protective immunity against PCV2 infection and thus could serve as an effective vaccine against PCV2 and PMWS.

To improve the safety of the vaccine, we attempted to identify the genetic determinant(s) for PCV2 virulence. An isolate of PCV2 was serially passaged for 120 times in PK-15 cells. After 120 passages, a total of two amino acid mutations were identified in the capsid protein of the passage 120 virus (VP120), P110A and R191S. Compared to other known PCV1 and PCV2 sequences, the two amino acid mutations in PCV2 VP120 are unique. The VP120 virus was biologically characterized in vitro and experimentally characterized in specific-pathogen-free (SPF) pigs. The two amino acid mutations resulted in an enhanced replication ability of PCV2 VP120 in PK-15 cells and an attenuated phenotype in infected pigs. The P110A and R191S mutations in the capsid protein either alone or collectively are likely important for PCV2 virulence and replication.

In summary, we genetically characterized PCV2 isolates from different geographic regions and developed a PCR-RFLP assay. We constructed and characterized infectious DNA clones of PCV1 and PCV2, and developed a genetically engineered vaccine against PCV2 infection. We also identified the genetic determinants for PCV2 virulence and replication. The vaccine developed in this study, when it becomes available, will help the swine industry control this important pathogen.

Dedication

I dedicate my dissertation to my wife, Jill.

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General Introduction

Postweaning multisystemic wasting syndrome (PMWS) was initially discovered in Canada in 1991 (32). Since then it has been reported virtually in all swine producing nations and is increasingly becoming a serious economic problem to the porcine industry. PMWS affects young piglets between 5 to 12 weeks of age. Clinical signs include progressive weight loss, dyspnea, tachypnea, anemia, diarrhea, jaundice and death. PMWS affected herds have low morbidity rates around 4-30% but high mortality rates of 70-80% (9, 18, 73).

Porcine circovirus was discovered in 1974 as a persistent noncytopathic contaminant of the porcine kidney cell line PK-15 (ATCC CCL-33) (79, 81, 82). PK-15 cell line derived PCV does not cause clinical disease in pigs and is generally considered to be non-pathogenic (2, 77, 78, 80). Over the next two decades there was very limited interest in PCV until the discovery that PMWS-affected pigs were infected by a pathogenic form of PCV (3, 7, 22, 29, 54, 56). The PMWS-associated porcine circovirus was designated type 2 (PCV2) to differentiate it from the nonpathogenic PK-15 cell line derived porcine circovirus type 1 (PCV1).

The mechanisms of PCV2 pathogenesis and its role in PMWS are poorly understood. Published studies indicate that PCV2 targets the lymphoid tissues, resulting in the hallmark PMWS lesions of histocytic replacement and lymphocyte depletion of the lymphoid follicles (2, 4, 6, 8, 9, 11, 23, 26, 33, 37, 41, 43, 49). From there the virus spreads systemically with onset of high levels of viremia and leukopenia (19, 48, 59, 72). Within a few days, pigs are severely wasted, disengaged and death quickly follows (9, 32). Improved management techniques aimed to lower the herd stress levels by

minimizing animal mixing and prompt removal of ill animals has had some success in reducing losses to PMWS (28). There is an urgent need for the development of an effective vaccine against PCV2.

Chapter 1

Literature Review

PMWS and PCV

History of PMWS

Postweaning multisystemic wasting syndrome (PMWS) was first described in a high health herd in Canada in 1991 affecting weaning piglets (32). Due to unspecific clinical signs, the syndrome was initially unrecognized in many countries outside of Canada (73). With the more definitive characterization of the syndrome by Harding et al., (32), it was determined that PMWS was the cause of ever increasing losses in commercial herds in both North America and especially in Europe (5, 7, 17, 22, 61, 70, 74, 87). The disease is usually found in commercial swine herds, but recently a wild boar was found dead in a forest outside of Berlin (70). Pathologic analysis revealed that the boar had severe PMWS lesions. PCV2 was detected from the boar and found to have high sequence identity with known PCV2 infecting local commercial herds. Therefore, the virus infecting the wild boar is suspected to be foreign to the wild boar population and was most likely introduced into the environment by the commercial swine facilities in the area (70). PMWS has a low rate of morbidity but causes a relatively high case fatality rate among 5 to 12 week old pigs in Europe and 7 to 16 week old pigs in North America (9, 18, 73). The reason for the difference in PMWS affected age is suspected due to differences in management methods and vaccination timing employed in the North

America and Europe (63). During most outbreaks the mortality rate associated with PMWS usually peaks at about 10% (9, 32, 73).

History of PCV

Porcine circovirus was initially discovered as a persistent non-cytopathic contaminant of the porcine kidney cell culture (PK-15) in 1974 (82). Experimental inoculations of piglets with PCV1 did not lead to disease nor has PCV1 been associated with pathogenic disease in the field and is generally considered to be non-pathogenic (2, 20, 21, 80). In 1991, PMWS was first described and found to be associated with a pathogenic type of porcine circovirus, designated porcine circovirus type 2 (PCV2) (22, 32, 54). The non-pathogenic PCV isolated from PK-15 cells was designated porcine circovirus type 1 (PCV1) (3).

Biology of PCV

Morphology

Both PCV1 and PCV2 are small non-enveloped icosahedral viruses, with a diameter of 17 nm (79, 82). PCV1 and PCV2 have a single-stranded circular DNA genome of about 1.76 kb. PCVs are members of the *Circoviridae* family, genus *Circovirus*. Other members of the genus include psittacine beak and feather disease virus, pigeon or columbid circovirus, goose circovirus, gull circovirus and canary circovirus (10, 52, 64, 83, 84). The recently discovered human circoviruses include the

TT virus, TTV-like mini virus and the SEN virus. Human circoviruses have as of yet not been linked to any diseases (12, 55, 60, 76).

Genomic organization

Pathogenic PCV2 and non-pathogenic PCV1 share about 76% nucleotide sequence identity and have a similar genomic organization (25, 44). There are two characterized open reading frames (ORFs): ORF1 or the replication (Rep) gene that encodes proteins required for viral replication (16), and ORF2 encodes the immunogenic capsid protein (46, 57, 58). The Rep gene mRNA transcript is spliced into 8 and 5 spliced products of the Rep gene during productive infection by PCV1 and PCV2, respectively (16). Of these multiple splice products, only two, the Rep and Rep', are essential for PCV protein synthesis (16). Phylogenetic analysis of PCV1 and PCV2 sequences from different geographic origins revealed the existence of minor genotypes in the PCV2 population depending on the geographic origin of the PCV2 (25). PCV2 originating from the US and Canada form a minor genotype. Another minor genotype is made up of Canadian and European PCV2 isolates, and a third minor genotype consists of only French PCV2 isolates. Non-pathogenic PCV1 isolates form a distinct genotype (25).

Replication

PCV1 and PCV2 have genomic organizations very similar to that of the plant circoviruses (53). The PCVs origin of DNA replication (Ori) is enclosed in a 111 bp sequence with a stem loop structure, suggesting that PCVs replicate through the rolling

circle mechanism (50, 53). The PCV2 Rep and Rep' proteins form a complex with the stem loop structure and are assumed to function as promoters of rolling circle replication (16, 75). The infected cells must be in the S-phase of the cell cycle before PCV replication can occur (81). PCV replication takes place in the nucleus and the capsid protein has a nuclear localization signal sequence, which directs the protein migration to the nucleus for virion assembly (15).

Both PCV1 and PCV2 replicate in PK-15 cells (ATTC CCL-33) but do not induce cytopathic effect (CPE) (79, 81). PCV1 initially isolated from PK-15 cells, replicates more efficiently in PK-15 cells compared to pathogenic PCV2 (26). PCV infection in PK-15 cells is persistent and gradually increases over passage number (15, 81). PCV1 and PCV2 antigens are usually detected within the nucleus of infected cells. However, as the infection progresses, PCV antigen can also be detected in the cytoplasm (15). It has been shown that PCV1 replication in PK-15 cells can be drastically increased when inoculated cells are cultivated in 300 mM of glucosamine (81). However, a similar study on PCV2 replication in the presence of glucosamine has not been reported yet. PCV1 has also been successfully replicated in the human Vero cell culture (1) but CPE was not induced in Vero cells and persistent infection occurred after about 25 passages.

Pathogenesis

PMWS pathology

PMWS primarily affects newly-weaned pigs. Initial clinical signs may include reduced weight gain, unthriftness, paleness of the skin and a rough hair coat (32). The

initial signs are ambiguous and are easily overlooked or misdiagnosed. As the disease progresses, more severe clinical signs such as dyspnea, tachypnea, anemia, diarrhea and jaundice are manifested (32). An average of 70-80% (73) of the pigs manifesting these PMWS clinical signs will either succumb to the disease or are euthanized. Characteristic microscopic lesions associated with PMWS include granulomatous interstitial pneumonia, lymphocytic depletion and histiocytic replacement of the lymph node follicles, hepatitis, nephritis and pancreatitis (9, 22, 32).

PMWS and PCV2 etiology

The mechanisms of PCV2 pathogenesis and its role in PMWS are poorly understood. It has been shown that PCV2 are present within monocytes, macrophages and follicular dendritic cells but does not replicate within those cells (27, 39, 85). The presence of PCV2 in dendritic cells does not seem to be due to endocytosis since there is no detectable modulation of the MHC class I and II surface protein (85). It has been suggested that PCV2 may take advantage of the phagocytic and fusogenic nature of macrophages to facilitate cell-to-cell spread (39, 85). The infected monocytes may therefore function as the vehicle allowing the virus to spread throughout the body and infecting lymphoid organs and tissues and therefore inaccessible by PCV2 specific antibodies (85). Lymphocytic depletion of lymphoid organs is not caused by direct cytopathic effect of PCV2, since PCV2 does not replicate in lymphocytes (27, 40, 49, 85). However, lymphocytic depletion and leukopenia are the hallmark histopathologic lesions found both in subclinically PCV2 infected pigs and PMWS-affected pigs (9, 23,43). Mandrioli et al., (49) suggests that PCV2 induced lymphocyte depletion is not

due to apoptosis but rather because of the decreased lymphocyte proliferation. Thus, PCV2 uses an alternate route to degrade the immune system of pigs, allowing the occurrence of PMWS. A recent study by Darwich et al., (19) showed that PCV2 induced immunomodulation resulted in a decreased expression of the B-cell growth factor IL-4, the cytotoxic T and macrophage cell activating cytokine IL-2, IFNγ antiviral response and increased expression of pro-inflammatory cytokines IL-1β and IL-8. Hasslung et al., (34) observed that 1 out of 5 promising oligodeoxynucleotides (ODNs) with a central CpG motif found in the PCV2 genome completely inhibited IFNα, which is critical for antiviral responses. Based on the results from these studies, it seems that the mechanism of PCV2 pathogenesis is immunomodulation induced by the virus and, which in turn prevents the appropriate anti-viral response.

Interestingly, the majority of field piglets become infected by PCV2 during the weaning stage but only a minority will progress to clinical PMWS (14, 48, 71). A recent study by Nielson et al., (59) showed that pigs eventually developing PMWS are significantly more viremic than those with only subclinical PCV2 infections. More importantly, these animals also develop leukopenia shortly before the onset of clinical PMWS. Leukopenia coupled with high levels of PCV2 viremia seems to be the most reliable precursor to PMWS in experimentally inoculated animals. Therefore, if PCV2 viremia and leukopenia can be prevented, the progression to clinical PMWS in PCV2 infected animals may be halted.

Experimental reproduction of PMWS and immunostimulation

Initial attempts to experimentally reproduce clinical PMWS by inoculating pigs with PCV2 alone were unsuccessful (47, 65). In more recent studies pigs were coinfected with PCV2 and porcine parvovirus (PPV) or porcine reproductive and respiratory syndrome virus (PRRSV) resulted in the development of clinical PMWS (33, 41, 62). Therefore, it is believed that the immunostimulation by PPV or PRRSV may aid the replication of PCV2 and the development of PMWS. This theory was further supported when severe clinical PMWS signs were reproduced in PCV2 inoculated piglets previously immunostimulated with keyhole limpet hemocyanin in incomplete Freund's adjuvant (40, 41). Although it is generally believed that immunostimulation plays a critical role in the development of PMWS, the exact mechanism is not known.

It has been suspected that the various vaccinations against viruses and bacteria given to young pigs could function as immunostimulants in the development of PMWS. A field study by Kyriakis et al., (42) studied the role of immunomodulation by *Mycoplasma hyopneumoniae* vaccine on the development of clinical PMWS in herds endemic for PMWS. A significant drop in the occurrence of PMWS was found in non-vaccinated animals. Experimental reproduction of the results from this field study by Opriessnig et al., (63) confirmed that *Mycoplasma hyopneumoniae* and *Actinobacillus pleuropneumoniae* vaccinated SPF pigs, when infected by PCV2, had a significant increase in viremia length and more severe histopathological lesions than those non-vaccinated pigs. Therefore, immunostimulation either by non-specific vaccination or secondary viral and bacterial infection may play an important role in the occurrence of PMWS.

However, Ladekjear-Mikkelsen et al., (43) successfully reproduced clinical PMWS in non-*M. hyopneumoniae* or *A. pleuropneumoniae* vaccinated 3-week old piglets with PCV2 alone, suggesting that immunostimulation is not absolutely necessary for the development of clinical PMWS.

Zoonosis

The potential zoonotic nature of PCV viruses was initially suggested by Tisher et al who reported the detection of PCV1 antibodies in humans, mice and cattle (78). Also, Kuipel et al reportedly infected BALB/c mice with PCV2. The infected mice showed characteristic histopathologic lesions comparable those found in PCV2 subclinically infected swine (38). Xenotransplantation with porcine organs, cells and other porcine-derived products has received considerable attention in the last few years, and could potentially alleviate the problem associated with the shortage of human organ donors. However, inadvertent transmission of potentially zoonotic porcine viruses to humans is of great concern. More studies are needed to evaluate the potential risk of PCV zoonosis.

Epidemiology

Both PCV1 and PCV2 have been detected in all porcine producing regions of the world. PCV2 specific antibodies have been retrospectively detected in serum samples collected in 1969 from Belgian pigs (69). PCV2 nucleic acid was also retrospectively detected in Spanish pigs with PMWS lesions using in situ hybridization (ISH) in tissues collected in 1986 (66). These results indicate that PCV2 has been present in the swine

population for some time but with a low prevalence of PMWS (66). The nonspecific clinical signs and the common occurrence of coinfecting agents such as porcine parvovirus (PPV) and porcine respiratory and reproductive syndrome virus (PRRSV) may explain why PMWS remained unrecognized until 1991 (73). That, however, does not explain the sudden explosive appearance of severe PMWS worldwide in the mid 90's.

Studies of French commercial herds showed that the seroprevalance rate of PCV2 is near 100% (67, 71), and studies in other countries reveal similar findings, suggesting that PCV2 infection is ubiquitous (35, 36, 45, 51, 69, 77, 78). However not all farms are affected by the disease, and in PMWS affected herds usually only 5-30% of susceptible pigs become affected whereas the majority of pigs may become PCV2 viremic without showing any clinical signs (14, 71). PCV1 and PCV2 are difficult to remove from the environment due to their resistance to inactivation by common disinfectants (68).

Prevention and control

Management practices

The influx of new infected pigs was initially suspected to be responsible for the introduction of PMWS to naïve farms. However, farms following a strict isolated breeding practice also became affected with PMWS after 2 years of strict isolation (73). Immunostimulation either by coinfecting viruses or vaccination may have a role in the onset of PMWS in PCV2 infected animals. Eliminating vaccination regimen of piglets with *M. hyopneumoniae* and *A. pleuropneumoniae* vaccine would lead to significantly increased losses due to respiratory disease caused by bacteria. It has been reported that, if the timing of vaccination was delayed to 2 to 4 weeks prior to exposure with PCV2,

pigs had significantly less severe histopatholgical lesions, decreased viremia length and reduced viral genomic copy loads in sera (63). Swine producers would have to determine the time of PCV2 exposure of the herd and then accordingly alter the timing of vaccinations to minimize losses due to PMWS (63).

Changes in herd management have shown some success in reducing the occurrence of PMWS in affected farms. These changes include general reduction of stress levels of the herd, improved hygiene, limitation of age group mixing, use of all in–all out procedures, increasing the air quality, and quick segregation of ill animals (28). The use of serum-therapy has reduced the loss to PMWS in test herds (86); however, this procedure requires the collection of PCV2 immune serum from older pigs to be given to younger animals, which is laborious and also increases the risk of reintroducing pathogenic agents in the herd at a critical age.

Attempts to remove PCV2 from a farm by depopulation have been unsuccessful since PCV2 and PMWS quickly returned following the arrival of the new animals (73). This approach failed probably due to PCV2's resistant nature to inactivation by commercial disinfectants (68) and its ubiquitous presence in the swine population (67, 71).

Vaccine

Since PMWS affected pigs are often coinfected with PCV2 and PPV, it was theorized that by vaccinating pig with a killed PPV vaccine at early age could prevent PMWS. Field studies showed promising results with significant reduction in loses due to PMWS (30, 31). However, when this study was repeated under experimental conditions,

similar results were not achieved. PPV and PCV2 coinfected pigs receiving PPV vaccine showed mild to severe clinical signs (62). The reason why the field study was not supported by the experimental study is not known.

Recently, a PCV2 capsid protein subunit candidate vaccine has shown promise to induce protective immunity to a wild type PCV2 challenge (13). However, the high cost associated with the production of a subunit vaccine may make the use of such a vaccine in commercial herds prohibitive.

References

1. **Allan, G.M., D.P. Mackie, J. McNair, B.M. Adair, and M.S. McNulty.** 1994. Production, preliminary characterization and applications of monoclonal antibodies to porcine circovirus. Vet. Immunol. Immunopathol. **43**:357-371.
2. **Allan, G.M., F. McNeilly, J.P. Cassidy, G.A. Reilly, B. Adair, W.A. Ellis, and M.S. McNulty.** 1995. Pathogenesis of porcine circovirus; experimental infections of colostrum deprived piglets and examination of pig foetal material. Vet. Microbiol. **44**:49-64.
3. **Allan, G.M., B. Meehan, D. Todd, S. Kennedy, F. McNeilly, J. Ellis, E.G. Clark, J. Harding, E. Espuna, A. Botner, and C. Charreyre.** 1998. Novel porcine circoviruses from pigs with wasting disease syndromes. Vet. Rec. **142**:467-468.
4. **Allan, G.M., F. McNeilly, J. Ellis, S Krakowka, B. Meeham, I. McNair, I. Walker, and S. Kennedy.** 2000. Experimental infection of colostrums deprived piglets with porcine circovirus 2 (PCV2) and porcine reproductive and respiratory syndrome virus (PRRSV) potentiates PCV2 replication. Arch. Virol. **145**:2421-2429.
5. **Allan, G.M., F. McNeilly, S. Kennedy, B. Daft, E.G. Clarke, J.A. Ellis, D.M. Haines, B.M. Meehan, and B.M. Adair.** 1998. Isolation of porcine circovirus-like viruses from pigs with a wasting disease in the USA and Europe. J. Vet. Diagn. Invest. **10**:3-10.
6. **Allan, G.M., F. McNeilly, B.M. Meehan, J.A. Ellis, T.J. Conner, I. McNair, S. Krakowka, and S. Kennedy.** 2000. A sequential study of experimental infection of pigs with porcine circovirus and porcine parvovirus: immunostaining of cryostat sections and virus isolation. J.Vet. Med. **B47**:81-94.
7. **Allan, G.M., F. McNeilly, B.M. Meehan, S. Kennedy, D.P. Mackie, J.A. Ellis, E.G. Clark, E. Espuna, N. Saubi, P. Riera, A. Botner, and C.E. Charreyre.** 1999. Isolation and characterisation of circoviruses from pigs with wasting syndromes in Spain, Denmark and Northern Ireland. Vet. Microbiol. **66**:115-23.
8. **Allan, G.M., S. Kennedy, F. McNeilly, J.C. Foster, J.A. Ellis, S.J. Krakowka, B.M. Meehan, and B.M. Adair.** 1999. Experimental reproduction of severe

wasting disease by co-infection of pigs with porcine circovirus and porcine parvovirus. J. Comp. Pathol. **121**:1-11.

9. **Allan, G.M. and J.A. Ellis.** 2000. Porcine circoviruses: a review. J. Vet. Diagn. Invest. **12**:3-14.
10. **Bassami, M.R., D. Berryman, G.E. Wilcox, and S.R. Raidal.** 1998. Psittacine beak and feather disease virus nucleotide sequence analysis and its relationship to porcine circovirus, plant circoviruses, and chicken anaemia virus. Virology **249**:453-459.
11. **Balasch, M., J. Segales, C. Rosell, M. Domingo, A. Mankertz, A. Urniza, and J. Plana-Duran.** 1999. Experimental inoculation of conventional pigs with tissue homogenates from pigs with post-weaning multisystemic wasting syndrome. J. Comp. Pathol. **121**:139-148.
12. **Biagini, P., P. Gallian, H. Attoui, M. Touinssi, J-F. Cantaloube, P. de Micco, and X. de Lamballerie.** 2001**.** Genetic analysis of full-length genomes and subgenomic sequences of TT virus-like mini virus human isolates. J. Gen. Virol. **82**: 379-383.
13. **Blanchard, P., D. Mahe, R. Cariolet, A. Keranflec'h, M.A. Baudouard, P. Cordioli, E. Albina, A. Jestin.** 2003. Protection of swine against post-weaning multisystemic wasting syndrome (PMWS) by porcine circovirus type 2 (PCV2) proteins. Vaccine **21**: 4565-4575.
14. **Calsamiglia, M.**, **M. Siblia, J. Segales, C. Rosell, and M. Domingo.** 2002. Detection of porcine circovirus type 2 in different routes of excretion: possible transmission routes and correlation with presence of postwenaing multisystemic syndrome characteristic lesions. Proceedings of the 17th International Pig Veterinary Society Congress. **1**: 28.
15. **Cheung, A.K., S.R. Bolin**. 2002. Kinetics of porcine circovirus replication. Arch. Virol. **147**: 43-58
16. **Cheung, A.K**. 2003. Transcriptional analysis of porcine circovirus. Virology **305**: 168-180.
17. **Choi, C., C. Chae, and E.G. Clark.** 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.

18. **Clark, E.**G. 1997. Postweaning multisystemic wasting syndrome, p. 499-501 *In* Proceedings of American Association of Swine Practitioners. Quebec City, Canada
19. **Darwich L., P. Sandrine, A. Rovira, J. Segales, M.Domingo, I.P. Oswald, and E. Mateu.** 2003. Cytokine mRNA expression profiles in lymphoid tissues of pigs naturally affected by postweaning multisystemic wasting syndrome. J. Gen. Virol. **84**:2117-2125.
20. **Dulac, G.C. and A. Afshar.** 1989. Porcine circovirus antigens in PK-15 cell line (ATCC CCL-33) and evidence of antibodies to circovirus in Canadian pigs. Can. J. Vet. Res. **53**:431-433.
21. **Edwards, S. and J.J. Sands.** 1994. Evidence of circovirus infection in British pigs. Vet. Rec. **134**:680-1.
22. **Ellis, J., L. Hassard, E. Clark, J. Harding, G. Allan, P. Willson, J. Strakappe, K. Martin, F. McNeilly, B. Meehan, D. Todd, and D. Haines.** 1998. Isolation of circovirus from lesions of pigs with postweaning multisystemic wasting syndrome. Can. Vet. J. **39**:44-51.
23. **Ellis, J., S. Krakowka, M. Lairmore, D. Haines, A. Bratanich, E. Clark, G. Allan, C. Konoby, L. Hassard, B. Meehan, K. Martin, J. Harding, S. Kennedy, and F. McNeilly.** 1999. Reproduction of lesions of postweaning multisystemic wasting syndrome in gnotobiotic piglets. J. Vet. Diagn. Invest. **11**:3-14.
24. **Ellis, J.A., A. Bratanich, E.G. Clark, G. Allan, B. Meehan, D.M. Haines, J. Harding, K.H. West, S. Krakowka, C. Konoby, L. Hassard, K. Martin, and F. McNeilly.** 2000. Coinfection by porcine circoviruses and porcine parvovirus in pigs with naturally acquired postweaning multisystemic wasting syndrome. J. Vet. Diagn. Invest. **12**:21-27.
25. **Fenaux M, P.G. Halbur, M. Gill, T.E. Toth, and X.J. Meng.** 2000**.** Genetic characterization of type 2 porcine circovirus (PCV-2) from pigs with postweaning multisystemic wasting syndrome in different geographic regions of North America and development of a differential PCR-restriction fragment length polymorphism

assay to detect and differentiate between infections with PCV-1 and PCV-2. J. Clin. Microbiol. **38**: 2494-503.

26. **Fenaux M., T. Opriessnig, P.G. Halbur, and X.J. Meng**. 2003. Immunogenicity and pathogenicity of the chimeric infectious DNA clones between pathogenic type 2 porcine circovirus (PCV2) and non-pathogenic PCV1 in weaning pigs. J. Virol. **77**:11232-11243.
27. **Gilpin D.F., K. McCullough, B.M. Meeham, F. McNeilly, I. McNair, L.S. Stevenson, J.C. Foster, J.A. Ellis, S. Krakowka, B.M. Adair, and G.M. Allan**. 2003. In vitro studies on the infection and replication of porcine circovirus type 2 in cells of porcine immune system. Vet. Immunol. Immunopathol. **94**:149-161.
28. **Guilmoto, H., and S. Wessel-Robert**. 2002. Control of PMWS in Brittany: a mainly zootechnical approach. In: PMWS: a new emerging disease of swine. Merial Symposium, new emerging disease of swine. Merial Symposium, Melbourne, Australia, p. 45-55.
29. **Hamel, A.L., L.L. Lin, and G.P. Nayar.** 1998. Nucleotide sequence of porcine circovirus associated with postweaning multisystemic wasting syndrome in pigs. J. Virol. **72**:5262-5267.
30. **Halbur, P.**G. 2000. Proceedings of the swine disease conference for swine practitioners, **8**:113-123.
31. **Halbur, P.**G. 2001. Proceedings of the swine disease conference for swine practitioners, **9**:162-166.
32. **Harding, J.C., and E.G. Clark.** 1997. Recognizing and diagnosing postweaning multisystemic wasting syndrome (PMWS). Swine Health and Production. **5**:201-203.
33. **Harms P.A., S.D. Sorden, P.G. Halbur, S.R. Bolin, K.M. Lager, L. Morozov, and P.S. Paul.** 2001. Experimental reproduction of severe disease in CD/CD pigs concurrently infected with type 2 porcine circovirus and porcine reproductive and respiratory syndrome virus. Vet. Pathol. **38**:528-539.
34. **Hasslung, F.C., M. Berg, G.M. Allen, B.M. Meeham, F. McNeilly and C. Fossum.** 2003. Identification of a sequence from the genome of porcine circovirus

type 2 with an inhibitory effect on IFNα production by porcine circovirus. J Gen Virol. **84**: 2937-2945.

35. **Hines, R.K., and P.D. Lukert.** 1995. Porcine circovirus: a serological survey of swine in the United States. Swine Health and Production. **3**:71-73.
36. **Kennedy, S., G. Allan, F. McNeilly, B.M. Adair, A. Hughes, and P. Spillane.** 1998. Porcine circovirus infection in Northern Ireland. Vet. Rec. **142**:495-6.
37. **Kennedy, S., D. Moffett, F. McNeilly, B. Meehan, J. Ellis, S. Krakowka, and G.M. Allan.** 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.
38. **Kiupel, M., G.W. Stevenson, S.K. Mittal, E.G. Clark, and D.M. Haines.** 1998. Circovirus-like viral associated disease in weaned pigs in Indiana. Vet. Pathol. **35**:303-307.
39. **Krakowka, S., J.A. Ellis, F. McNeilly, D. Gilpin, B. Meeham, K. McCullough and G. Allan.** 2002. Immunological features of porcine circovirus type 2 infection. Viral Immunol. **15**:567-582.
40. **Krakowka, S., J.A. Ellis, F. McNeilly, S. Ringler, D.M. Rings, and G. Allen.** 2001. Activation of the immune system is the pivotal event in the production of wasting disease in pigs infected with porcine circovirus-2 (PCV-2). Vet. Pathol. **38**:31-42.
41. **Krakowka, S., J.A. Ellis, B. Meehan, S. Keenedy, F. McNeilly, and G. Allan.** 2000. Viral wasting syndrome of swine: experimental reproduction of postweaning multisystemic wasting syndrome in gnotobiotic swine by coinfection with porcine circovirus 2 and porcine porvovirus. Vet Pathol. **37**:254-263.
42. **Kyriakis, S.C., K. Saoulidis, S. Lekkas, Ch. C. Miliotis, P.A. Papoutsis, and S. Kennedy.** 2002. The effects of immuno-modulation on the clinical and pathological expression of postweaning multisystemic wasting syndrome. J. Comp. Pathol. **126**:38-46.
43. **Ladekjaer-Mikkelsen, A.S., J. Nielsen, T. Stadejek, T. Storgaard, S. Krakowka, J. Ellis, F. McNeilly, G. Allan, and A. Botner.** 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.

44. **Larochelle, R., R. Magar, S. D'Allaire.** 2002. Genetic characterization and phylogenetic analysis of porcine circovirus type 2 (PCV2) strains from cases presenting various clinical conditions. Virus Res. **90**:101-112
45. **Larochelle, R., M. Morin, M. Antaya, and R. Magar.** 1999. Identification and incidence of porcine circovirus in routine field cases in Quebec as determined by PCR. Vet. Rec. **145**:140-142.
46. **Lekcharoensuk, P., I. Morozov, P.S. Paul, N. Thangthumniyom, W. Wajjawalku, and X.J. Meng.** 2004. Epitope mapping of the major capsid protein of type 2 porcine circovirus (PCV2) using chimeric PCV1 and PCV2 viruses. J. Virol. Submitted.
47. **LeCann ,P., E. Albina, F. Madec, R. Cariolet and A. Jestin**. 1997. Piglet wasting disease. Vet Rec. **141**:660.
48. **Liu, Q., L. Wang, P. Willison, and L.A. Babiuk.** 2000. Quantitative, competitive PCR analysis of porcine circovirus DNA in serum from pigs with postweaning multisystemic wasting syndrome. J. Clin. Microbiol. **38**:3474-3477.
49. **Mandrioli, L., G. Sarli, S. Panarese, S. Balboni, P.S. Marcato**. 2004. Apoptosis and proliferative activity in lymph node reaction in postweaning multisystemic wasting syndrome (PMWS). Vet. Immun. Immunopathol. **97**: 25-37.
50. **Mankertz, J., H.J. Buhk, G. Blaess, and A. Mankertz.** 1998. Transcription analysis of porcine circovirus (PCV). Virus Genes **16**:267-276.
51. **Mankertz, A., M. Domingo, J.M. Folch, P. LeCann, A. Jestin, J. Segales, B. Chmielewicz, J. Plana-Duran, and D. Soike.** 2000. Characterization of PCV-2 isolates from Spain, Germany and France. Virus Res. **66**:65-77.
52. **Mankertz A., K. Hattermann, B. Ehlers, and D. Soike.** 2000**.** Cloning and sequencing of columbid circovirus (CoCV), a new circovirus from pigeons. Arch. Virol. **145**:2469-2479.
53. **Meehan, B.M., J.L. Creelan, M.S. McNulty, and D. Todd.** 1997. Sequence of porcine circovirus DNA: affinities with plant circoviruses. J. Gen. Virol. **78**:221-227.

54. **Meehan, B.M., F. McNeilly, D. Todd, S. Kennedy, V.A. Jewhurst, J.A. Ellis, L.E. Hassard, E.G. Clark, D.M. Haines, and G.M. Allan.** 1998. Characterization of novel circovirus DNAs associated with wasting syndromes in pigs. J. Gen. Virol. **79**:2171-2179.
55. **Miyata, H., H. Tsunoda, A. Kazi, A. Yamada, M.A. Khan, J. Murakami, T. Kamahora, K. Shiraki, and S. Hino.** 1999. Identification of a novel GC-rich 113-nucleotide region to complete the circular, single-stranded DNA genome of TT virus, the first human circovirus. J. Virol. **73**:3582-3586.
56. **Morozov, I., T. Sirinarumitr, S.D. Sorden, P.G. Halbur, M.K. Morgan, K.J. Yoon, and P.S. Paul.** 1998. Detection of a novel strain of porcine circovirus in pigs with postweaning multisystemic wasting syndrome. J. Clin. Microbiol. **36**:2535-2541.
57. **Nawagitgul, P., P.A. Harms, I. Morozov, B.J. Thacker, S.D. Sorden, C. Lekcharoensuk, and P.S. Paul.** 2002. Modified indirect porcine circovirus (PCV) type 2-based and recombinant capsid protein (ORF2)-based ELISA for the detection of antibodies to PCV. Clin. Diagn. Lab. Immunol. **9**, 33-40.
58. **Nawagitgul, P., I. Morozov, S.R. Bolin, P.A. Harms, S.D. Sorden, and P.S. Paul.** 2000. Open reading frame 2 of porcine circovirus type 2 encodes a major capsid protein. J. Gen. Virol. **81**:2281-2287.
59. **Nielson, J., I.E. Vincent, A. Bøtner, A-S. Ladekjaer-Mikkelsen, G. Allan, A. Summerfield, and K.C. McCullough**. 2002. Association of lymphopenia with porcine circovirus type 2 induced postweaning multisystemic wasting syndrome (PMWS). Vet. Immunol. Immunopathol. **92**:97-111.
60. **Nishizawa, T., H. Okamoto, K. Konishi, H. Yoshizawa, Y. Miyakawa, and M. Mayumi.** 1997. A novel DNA virus (TTV) associated with elevated transaminase levels in posttransfusion hepatitis of unknown etiology. Biochem. Biophys. Res. Commun. **241**:92-97.
61. **Onuki, A., K. Abe, K. Togashi, K. Kawashima, A. Taneichi, and H. Tsunemitsu.** 1999. Detection of porcine circovirus from lesions of a pig with wasting disease in Japan. J. Vet. Med. Sci. **61**:1119-1123.

62. **Opriessnig, T., M. Fenaux, S. Yu, R.B. Evans, D. Cavanaugh, J.M. Gallup, F.J. Pallares, E.L. Thacker, K.M. Lager, X.J. Meng, and P.G. Halbur.** 2003. Effect of porcine parvovirus vaccination on the development of PMWS in segregated early weaned pigs coinfected with type 2 porcine circovirus and porcine parvovirus. Vet. Microbiol. **98**: 209-220.

63. **Opriessnig, T., S. Yu, J.M. Gallup, R.B. Evans, M. Fenaux, F.J. Pallares, E.L. Thacker, C.W. Brockus, M.R. Ackermann, P. Thomas, X.J. Meng, and P.G. Halbur**. 2003. Effects of vaccination with selective bacterins on conventional pigs infected with wild type 2 porcine circovirus. Vet. Pathol. **40**:521-9.

64. **Phenix, K.V., J.H. Weston, I. Ypelaar, A. Lavazza, J.A. Smyth, D. Todd, G.E. Wilcox and S.R. Raidal.** 2001. Nucleotide sequence analysis of a novel circovirus of canaries and its relationship to other members of the genus circovirus of the family Circoviridae. J Gen Virol **82**: 2805-2809.

65. **Pogranichnyy, R.M., K-J. Yoon, P.A. Harms, S.L. Swenson, J.J. Zimmerman, and S.D. Sorden.** 2000. Characterization of immune response of young pigs to porcine circovirus type 2 infection. Viral. Immunol. **13**:143-153.

66. **Rosell, C., J. Segales, J.A. Ramos-Vara, J.M. Folch, G.M. Rodriguez-Arrioja, C.O. Duran, M. Balasch, J. Plana-Duran, and M. Domingo.** 2000. Identification of porcine circovirus in tissues of pigs with porcine dermatitis and nephropathy syndrome. Vet. Rec. **146**:40-43.

67. **Rose, N. G. Larour, G. Le Diguerher, P. Blanchard, M. Le Dimma, E. Eveno, J.P. Jolly, A. Jestin and F. Madec.** 2001. Serological profile of porcine circovirus type 2 (PCV2) in PMWS affected and non-affected farms. Proceedings of the ssDNA Viruses of Plants, Birds, Pigs and Primates: porcine postweaning multisystemic wasting syndrome. 117.

68. **Royer R.L., P. Nawagitgul, P.G. Halbur and P.S. Paul.** 2001**.** Susceptibility of porcine circovirus type 2 to commercial and laboratory disinfectants. Swine Health Prod **9**: 281-284.

69. **Sanchez, R., H. Nauwynck and M. Pensaert**. 2001. Serological survey of porcine circovirus 2 antibodies in domestic and feral pig population in Belgium.

Proceedings of the ssDNA viruses of plants, birds, pigs and primates. European Society of Veterinary Virology : 122.

70. **Schulze, C., G. Neuman, I. Grutze, A. Engelhardt, C. Mirle. F. Ehlert. A. Hlinak.** 2003. Case report: Porcine circovirus type 2 infection in an European wild boar (Sus scrofa) in the state of Brandenburg, Germany. Dtsch Tierarztl Wochenschr. 2003 Oct; **110**(10):426-8.
71. **Sibila, M., M. Calsamiglia, J. Segales,, A. Jestin and M. Domingo.** 2001. Detection of porcine circovirus type 2 genome in nasal swabs and serum samples from naturally infected pigs using polymerase chain reaction. Proc of the ssDNA Viruses of Plant, Birds, Pigs and Primates. European Society of Veterinary Virology: 115.
72. **Segales J., C. Alfonso, C. Rosell, J. Pastor, F. Chianini, E. Campos, L. Lopez-Fuertes, J. Quinana, G. Rodriguez-Arrioja, M. Calsamiglia, j. Pujols, J. Dominguez, and M. Domingo.** 2001. Changes in peripheral blood leukocyte populations in pigs with natural postweaning multisystemic wasting syndrome (PMWS). Vet. Immunol. Immunopathol. **81**:37-44.
73. **Segales, J. and M. Domingo**. 2002. Postweaning multisystemic wasting syndrome in pigs. A review. Vet Q. **24**: 109-124.
74. **Spillane, P., S. Kennedy, B. Meehan, and G. Allan**.1998. Porcine circovirus infection in the Republic of Ireland. Vet. Rec. **143**:511-512.
75. **Steinfeldt, T., T. Finsterbusch, A. Mankertz**. 2001. Rep and Rep' protein of porcine circovirus type 1 bind to the origin of replication in vitro. Virology. **291**:152-160.
76. **Takahashi, K., Y. Iwasa, M. Hijikata, and S. Mishiro.** 2000. Identification of a new human DNA virus (TTV-like mini virus, TLMV) intermediately related to TT virus and chicken anemia virus. Arch. Virol. **145**:979-93.
77. **Tischer, I., L. Bode, D. Peters, S. Pociuli, and B. Germann.** 1995. Distribution of antibodies to porcine circovirus in swine populations of different breeding farms. Arch. Virol. **140**:737-743.

78. **Tischer, I., L. Bode, J. Apodaca, H. Timm, D. Peters, R. Rasch, S. Pociuli, and E. Gerike.** 1995. Presence of antibodies reacting with porcine circovirus in sera of humans, mice, and cattle. Arch. Virol. **140**:1427-1439.
79. **Tischer, I., H. Gelderblom, W. Vettermann, and M.A. Koch.** 1982. A very small porcine virus with circular single-stranded DNA. Nature **295**:64-66.
80. **Tischer, I., W. Mields, D. Wolff, M. Vagt, and W. Griem.** 1986**.** Studies on epidemiology and pathogenicity of porcine circovirus. Arch. Virol. **91**:271-276.
81. **Tisher, I., D. Peters, R. Rasch, S. Pociuli.** 1987. Replication of porcine circovirus: induction by glucosamine and cell cycle dependence. Arch. Virol. **96**: 39-57.
82. **Tischer, I., R. Rasch, and G. Tochtermann.** 1974. Characterization of papovavirus and picornavirus-like particles in permanent pig kidney cell lines. Zentralbl. Bakteriol. Hyg. A **226**:153-167.
83. **Todd, D., F.D. Niagro, B.W. Ritchie, W. Curran, G.M. Allan, P.D. Lukert, K.S. Latimer, W.L. Steffens, and M.S. McNulty.** 1991. Comparison of three animal viruses with circular single-stranded DNA genomes. Arch. Virol. **117**:129-135.
84. **Todd, D., J.H. Weston, D. Soike, and J.A. Smyth.** 2001. Genome sequence determinations and analyses of novel circoviruses from goose and pigeon. Virology. **286**:354-362.
85. **Vincent, I.E., C.P. Carrasco, B. Herrmann, B.M. Meeham, G.M. Allan, A. Summerfield and K.C. McCullough**. 2003. Dendritic cells harbor infectious porcine circovirus type 2 in the absence of apparent cell modulation or replication of the virus. J. Virol. **77**:13288-13300.
86. **Waddilove, A.E.J. and E. Marco.** 2002. Assessing serotherapeutic control of PMWS in the field. Proceedings of the 17th International Pig Veterinary Society Congress. **1**: 34.
87. **Wellenberg G.J., S. Pesh, F.W. Berndsen, P.J.G.M. Steverink, W. Hunneman, T.J.K. Van der Vorst, N.H.M.T. Peperkamp, V.F. Ohlinger, R. Schippers, J.T. Van Oirschot, and M.F. de Jong.** 2000. Isolation and characterization of

porcine circovirus type 2 from pigs showing signs of post-weaning multisystemic wasting syndrome in the Netherlands. Vet. Quart. **22**:167-72.

Chapter 2

Genetic characterization of type-2 porcine circovirus (PCV-2) from pigs with PMWS in different geographic regions of North America and development of a differential PCR-RFLP assay to detect and differentiate infections between PCV-1 and PCV-2

Fenaux M, P.G. Halbur, M. Gill, T.E. Toth, and X.J. Meng. 2000. J. Clin. Microbiol. 38: 2494-503.

ABSTRACT

Postweaning multisystemic wasting syndrome (PMWS) is an emerging disease in swine. Increasing evidence indicates that a variant strain of porcine circovirus (PCV), designated as PCV-2, is responsible for PMWS. To determine the extent of genetic heterogeneity of PCV-2 isolates, the complete genome of six PCV-2 isolates from different regions of North America were amplified by PCR and sequenced. Sequence and phylogenetic analyses confirmed that two distinct genotypes of PCV exist: the non-pathogenic PCV-1 and PMWS-associated PCV-2. However, within the major genotype of PCV-2, several minor branches that have been identified appear to be associated with geographic origins. Two French PCV-2 isolates diverge the most in their genomic sequences from other PCV-2 isolates and form a distinct branch. Other minor but distinguishable branches have also been identified for a Taiwan PCV-2 isolate, two of the Canadian PCV-2 isolates. All the U.S. PCV-2 isolates are closely related, but the

Canadian isolates vary, to some extent, in their genomic sequences. The data from this study indicate that, although the genome of PCV-2 is generally stable among different isolates, PCV-2 isolates from different geographic regions vary in their genomic sequences. This variation may have important implications for PCV-2 diagnosis and research. Based on the genetic analyses of available PCV strains, a universal PCR-restriction fragment length polymorphism (PCR-RFLP) assay was developed to detect and differentiate between infections with PCV-1 and PCV-2. This PCR-RFLP assay should be useful for studying the pathogenesis of PCV-2, for detecting PCV-2 infection in pigs from different geographic regions and for screening donor pigs for use in xenotransplantation.

INTRODUCTION

Porcine circovirus (PCV) was originally isolated as a noncytopathic contaminant of the porcine kidney cell line, PK15 (50). PCV is a small nonenveloped virus that contains a single-stranded circular DNA genome of about 1.76 kb (50). Based on the morphology and genomic organization, PCV was classified as a member of *Circoviridae* family (30, 36), which consists of two other animal circoviruses: chicken anemia virus (CAV) and psittacine beak and feather disease virus (PBFDV) and three plant circovirues: banana bunchy top virus (BBTV), coconut foliar decay virus (CFDV) and subterranean clover stunt virus (SCSV). Members of the three recognized animal circoviruses, PCV, CAV and PBFDV, do not share nucleotide sequence homology or antigenic determinants with each other (8, 54). More recently, a human circovirus, designated as TT virus (TTV), was identified from individuals with posttransfusion hepatitis (38, 43). The human TTV is similar to the circovirus CAV in its genomic organization (38). Although antibodies to PCV were found in various animal species including humans, mice, cattle and pigs (11, 12, 22, 23, 41, 52, 53), little is known regarding the pathogenesis of PCV in these animal species. Experimental infection of pigs with the PK15-derived PCV did not produce clinical disease and thus, this virus is not considered to be pathogenic to pigs (1, 51).

Postweaning multisystemic wasting syndrome (PMWS) is an emerging disease in pigs first described in 1991 (10, 20). The disease occurs in high-health swine herds as a low morbidity but high case fatality disease of 5 to 12 week-old pigs (6, 10). Clinically, PMWS is characterized by progressive weight loss, dyspnea, tachypnea, anemia, diarrhea and jaundice. In an acute outbreak, the mortality rate associated with PMWS may peak at about 10% and can reach up to 50% in some cases (6, 20, 21).

Microscopic lesions of PMWS include granulomatous interstitial pneumonia, lymphadenopathy, hepatitis, nephritis and pancreatitis (20, 21). PMWS has now been recognized in pigs in Canada and most of the United States (2, 3, 6, 13, 18, 26, 27, 37, 39, 40), many European countries (4, 6, 24, 33, 48, 49) and some countries in Asia (6, 44), and potentially has serious economic impact on swine industry worldwide.

The etiology of PMWS is rather complicated, but it is believed that a variant strain of PCV, designated as type-2 PCV (PCV-2), is responsible for PMWS in pigs (2, 3, 4, 6, 13, 18, 37, 39). The nonpathogenic PK15-derived PCV has been designated as PCV-1 to distinguish it from the PMWS-associated PCV-2 (2, 3). PCV-2 was isolated from pigs with clinical and pathological findings consistent with PMWS (2, 3, 4, 6, 13, 19, 27, 39, 40). PCV-2 DNA and antigen were detected in various tissues and organs from natural cases of PMWS in pigs (19, 27, 33, 37, 39, 40, 46). The complete genome of PCV-2 has been determined and surprisingly, the nonpathogenic PCV-1 and the PMWS-associated PCV-2 are found to share only about 75% nucleotide sequence identity (18, 37, 39). Seven open reading frames (ORFs) have been identified for PCV-1 (31, 32, 36), whereas six or eleven ORFs have been identified for PCV-2 (18, 37, 39). Although PMWS has been reported in most of the United States, only a few PCV-2 isolates from the U.S. have been genetically characterized (37, 39). Based on the nucleotide sequence of the U.S. and other PCV-2 isolates sequenced thus far, it appears that there exists only one genotype of PCV-2 worldwide (19, 37, 39). Nevertheless, the value of diagnosing PCV-2 infections and studying the pathogenesis of PCV-2 using PCR and other molecular approaches will depend on the knowledge of the extent of genetic variation among PCV-2 isolates from different geographic

regions. In addition, the development of an effective vaccine against PMWS also requires a better understanding of the extent of genetic variation among PCV-2 isolates. In this study, we genetically characterized six PCV-2 isolates from cases of confirmed PMWS in different geographic regions of North America. The extent of genetic variation among these six PCV-2 isolates and all other known PCV isolates (both PCV-1 and PCV-2) was analyzed. Based on the data generated from this study, we further developed a universal polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay to detect and differentiate between infections with PCV-1 and PCV-2 in pigs from different geographic regions.

MATERIALS AND METHODS

Sample sources. Various tissue samples (liver, spleen, tonsil, lymph nodes, etc.) were collected from pigs with PMWS as confirmed by immunohisochemistry (IHC) (data not shown). The tissues were stored at -80 ˚C until use. The complete PCV-2 genome was amplified, sequenced and characterized from tissue samples of six selected PMWS cases originated from different geographic regions of North America: two cases from Utah, one from Missouri, one from Iowa, one from Illinois, and one from Canada (Table 1). These six PMWS cases, along with four more field cases of PMWS (Table 1) from Iowa, were also characterized by the PCR-RFLP analyses.

Isolation of DNA from tissues. DNA was extracted from various tissue samples with a QIAamp DNA Mini kit (Qiagen, Inc., Valencia, CA) according to the protocol supplied by the manufacturer. For each DNA extraction, 25 mg of tissue samples

were used. The resulting DNA was eluted in DNase, RNase and proteinase-free water (Eppendorf 5 Primer, Inc., Boulder, CO).

PCR amplification of the complete genome of PCV-2. Two sets of PCR primers were designed on the basis of the published PCV-2 sequence. These primers amplify two overlapping fragments that represent the entire genome of PCV-2 (Fig. 1). The first set of primers, CV1 and CV2 (Table 2), amplifies a 989 bp fragment, and the second set of primers, CV3 and CV4 (Table 2), amplifies a 1092 bp fragment. The extracted DNA was amplified by PCR using AmpliTaq Gold polymerase (Perkin Elmer, Norwalk, CT). The PCR reaction consisted of 35 cycles of denaturation at 94 ˚C for 1 min, annealing at 55 ˚C for 1 min, and extension at 72 ˚C for 3 min, followed by a terminal extension at 72 ˚C for 7 min.

Nucleotide sequencing, sequence and phylogenetic analyses. The PCR products of expected sizes were purified by electrophoresis on a 1% agarose gel followed by extraction with a Geneclean Kit (Bio101, La Jolla, CA). Both strands were sequenced with a variety of sequencing primers (Table 2) with an ABI automated DNA Sequencer at Virginia Tech's DNA Sequencing Facility. The sequences of the primers used to sequence the complete genome of PCV-2 are listed in Table 2, and their relative positions in the circular genome are indicated (Fig. 1). The sequences were compiled and analyzed by the MacVector program (Oxford Molecular Ltd., Beaverton, OR). The percentages of sequence identity among different PCV isolates were determined with the Clustal alignment program in the MacVector package. Sequence alignments were performed

with the ALIGN program in the MacVector package. Phylogenetic analyses were conducted with the aid of the PAUP program (David L. Swofford, Smithsonian Institute, Washington, DC, distributed by Sinauer Associates, Inc., Sunderland, MA). Branch-and-bound searching and midpoint rooting options were used to produce a consensus tree.

Development of a PCR-RFLP assay. A PCR-RFLP assay was developed to differentiate between strains of PCV-1 and PCV-2 infecting pigs. Briefly, the complete sequences of the 6 PCV-2 isolates from this study and the complete sequences of all other PCV sequences available in the GenBank (both PCV-1 and PCV-2) were aligned with the Clustal program (data not shown). Based on this alignment, a set of conserved PCR primers (MCV 1 and MCV 2, Table 2) was designed to amplify a fragment of 243 bp from samples containing either PCV-1 or PCV-2 or both. The sequences of the two chosen PCR primers are identical among all known PCV-1 and PCV-2 isolates including the six PCV-2 isolates sequenced in this study (Fig. 2). The PCR reaction consisted of 37 cycles of denaturation at 94 ˚C for 1 min., annealing at 56 ˚C for 1 min. and extension at 72 ˚C for 1.5 min. The amplified PCR products were subsequently digested with a unique restriction enzyme, NcoI, which is present in all PCV-2 isolates but not in PCV-1 isolates (Fig. 2). The digested PCR products are separated on a 2% agarose gel for RFLP analysis.

Nucleotide sequence accession numbers. The complete genomic sequences of the 6 PCV-2 isolates reported in this paper have been deposited with the Genbank database

under the accession numbers; AF264038, AF264039, AF264040, AF264041, AF264042, AF264043.

RESULTS

Genetic characterization of PCV-2 isolates from pigs with PMWS in different geographic regions. To determine the extent of genetic heterogeneity among PCV-2 isolates, the complete genome of PCV-2 was amplified and sequenced from one case of PMWS in Canada (34464) and five cases of PMWS in the U.S.: two cases from Utah (26606, 26607), one from Missouri (40856), one from Iowa (40895), and one from Illinois (10489). The PMWS cases included in this study possessed clinical signs consistent with PMWS (Table 1) and were confirmed to be positive for PCV-2 antigen by IHC (data not shown). All six PMWS cases included in the study are negative for swine influenza virus, but four of the six cases are found positive for porcine reproductive and respiratory syndrome virus (PRRSV) antigen (Table 1).

The genomic DNA of PCV-1 isolates ranges from 1758 to 1760 bp in length. Sequence analyses of the complete genome of six PCV-2 isolates from this study showed that, like all other PCV-2 isolates, the complete genome of these six PCV-2 isolates is 1768 bp in length. All the PCV-2 isolates sequenced are closely related to each other, displaying 95 to 99% nucleotide sequence identity (Table 3). Two French PCV-2 isolates, AF055393 and AF055394, displayed the most sequence divergence from other PCV-2 isolates, ranging from 95 to 96% identity. Similarly, the four PCV-1 isolates sequenced thus far (Af071879, Y09921, U49186, AF012107) are closely related to each other and share 98 to 99% nucleotide sequence identity in the entire

genome (Table 3). Moreover, the nucleotide sequence identity between PCV-1 and PCV-2 is only about 75 to 77 % for the entire genome.

The open reading frame 2 (ORF2) of PCV is believed to code for the putative capsid protein (31, 32, 42). Sequence analysis indicated that the ORF2 of PCV-1 isolates encodes for a protein of 230 to 231 amino acid residues, whereas the ORF2 of PCV-2 isolates encodes for a protein of 233 amino acid residues (Fig. 3). Pairwise sequence comparisons revealed that the ORF2 of all PCV-2 isolates shared 91 to 100% nucleotide sequence and 90 to 100% amino acid sequence identity (Table 3). The two French isolates, AF055393 and AF055394, have only about 90 to 93% nucleotide sequence identity with other PCV-2 isolates (Table 3). The four PCV-1 isolates share 97 to 99% nucleotide sequence and 94 to 98% amino acid sequence identity in the ORF2. Between PCV-1 and PCV-2 isolates, there exists only 65 to 67% nucleotide sequence and 63 to 68% amino acid sequence identity in the ORF2 (Table 3). However, sequence analysis revealed that the N-terminal region of the ORF2 is very rich in basic amino acid residuals (arginine and lysine) and is highly conserved among PCV, both PCV-1 and PCV-2 isolates (Fig. 3).

Phylogenetic analysis of PCV-1 and PCV-2 isolates from different geographic regions worldwide. To gain a better understanding of the genetic relationship and evolution of PCV, phylogenetic analyses were performed based on the complete genomic sequences of 26 PCV isolates (both PCV-1 and PCV-2) worldwide, including the six North American PCV-2 isolates sequenced in this study (Fig. 4). These sequences were either published (18, 19, 31, 32, 33, 36, 37, 39, 41, 42, 54) or are

available in GenBank (Table 1). Phylogenetic analysis confirmed that two distinct genotypes of PCV exist: PCV-1 and PCV-2 (Fig. 4). All 22 PCV-2 isolates are clustered together and form one distinct branch. Similarly, all the four PCV-1 isolates are closely related and form another branch. Within the major genotype of PCV-2, a few minor branches were identified and some of these minor branches appear to be associated with geographic origins of the isolates. All the PCV-2 isolates from different geographic regions of the U.S. sequenced in this study are grouped closely with other U.S. and most of the Canadian PCV-2 isolates (Fig. 4). The Canadian isolate 34464 sequenced in this study is closely related to another Canadian isolate, 109399, but is less related to the U.S. and other Canadian isolates. Two other Canadian isolates, AF109398 and AF117753, form a distinguishable branch and are distantly related to other Canadian and U.S. isolates. An isolate of PCV-2 from Taiwan, AF166526, is clustered within the North American PCV-2 isolates but forms a single minor branch. The two French isolates of PCV-2, AF055393 and AF055394, are closely related to each other but diverge the most from North American PCV-2 isolates. Interestingly, a bovine isolate of circovirus is most closely related to the U.S. isolates of PCV-2.

Development of a PCR-RFLP assay to diagnose PCV-2 infection and to differentiate infections between PCV-1 and PCV-2. Based on the sequence alignment of all PCV-1 and PCV-2 isolates sequenced thus far, a set of consensus PCR primers was selected from two conserved regions of PCV genome to amplify a fragment of 243 bp for both PCV-1 and PCV-2 isolates (Fig. 5A). To test the

feasibility of these primers in amplifying both PCV-1 and PCV-2 isolates from clinical samples, DNA was extracted from tissue samples of the six PMWS cases in which the PCV-2 genomic sequences have been determined. DNA was also extracted from tissue samples of four additional cases of PMWS from Iowa (Table 1), and the PCV-2 sequence from these four cases of PMWS has not been determined. DNA extracted from the PK-15 cell line (ATCC CCL-33) was used as the source for PCV-1. DNA extracted from a sample of liver tissue collected from a specific-pathogen-free pig was used as a negative control. We were able to amplify an expected fragment of PCV genome from tissue samples of all 10 cases of PMWS as well as from the PCV-1 contaminated PK-15 cells. By utilizing a unique restriction enzyme site (Nco I) that is present only in the sequences of PCV-2 isolates (Fig. 2), a PCR-RFLP assay was developed to differentiate between infections with PCV-1 and PCV-2. After digestion of the PCR products with NcoI, the resulting RFLP patterns revealed that all products amplified from PCV-2 isolates produced two fragments of 168 bp and 75 bp each, whereas the PCR product amplified from PCV-1 produced only the undigested fragment of 243 bp (Fig. 5). DNA extracted from a sample mixed with both PCV-2 (case 40860) and PCV-1 (PK-15 cells) was also subjected to PCR amplification. After digestion with NcoI restriction enzyme, the PCR product amplified from the mixed sample produced 3 fragments of 243 bp, 168 bp and 75 bp, respectively (Fig. 5B). Thus, this PCR-RFLP assay is able to detect clinical samples with potential dual infection with PCV-1 and PCV-2.

DISCUSSION

PMWS is a new and unique disease of swine. Since the first recognition of the disease in 1991 (10, 20), PMWS has emerged to be an economically important global disease of swine (2, 3, 4, 6, 13, 18, 24, 26, 27, 33, 37, 39, 40, 44, 48, 49). The clinical and pathological presentations and etiology of PMWS are very complicated (6, 10, 16, 20, 21, 22, 46, 47). Many known swine pathogens such as porcine reproductive and respiratory syndrome virus (PRRSV), swine influenza virus, hemagglutinating encephalomyocarditis virus, porcine proliferative enteropathy, *M. hypopneumoniae*, *H. parasuis*, postweaning colibacillosis, etc. could cause postweaning wasting of pigs (21). However, increasing data indicates that PCV-2 is the causative agent of PMWS (2, 3, 4, 6, 13, 18, 37, 39). Experimental inoculation of conventional pigs with tissue homogenates from pigs with clinical PMWS produced PMWS-like lesions, and PCV-2 DNA and antibody to PCV-2 were detected in the inoculated pigs (7). Ellis et al (14) experimentally inoculated neonatal gnotobiotic piglets with filtered tissue culture materials and tissue homogenates from PMWS-affected pigs. The inoculated gnotobiotic piglets developed lesions typical of PMWS, but the study was complicated by the detection of porcine parvovirus (PPV) in inoculated piglets. In fact, coinfection by PPV and PCV in pigs with naturally acquired PMWS has been reported (16). It has also been shown that PCV-2 alone induced PMWS lesions in colostrum-deprived conventional pigs but concurrent infection with PPV increased the severity of the lesions (5, 25), suggesting that PMWS is a complex disease syndrome and multi-factors may be involved in the pathogenesis of PMWS. It has been suspected that

some of the clinical signs and pathological lesions attributable to PRRSV may actually be induced by PCV-2 as a result of PCV-2 infection or coinfection (15, 27). Synergism between a circovirus (CAV) and a reovirus was observed following dual infection of chickens by a natural route (34). In the present study, PCV-2 was readily detectable from pigs with PMWS in different regions of the North America, but PRRSV antigen was also detected in most of the PMWS cases (Table 1). Clearly, the etiological significance of PCV-2 in PMWS and its interrelationship with PRRSV, PPV or other agents need to be further studied.

The extent of genetic variation of PCV-2 isolated from different geographic regions of the U.S. is not known since only a few PCV-2 isolates from the U.S. have been genetically characterized (37, 39). In this study, we genetically characterized six North American isolates of PCV-2 (one Canadian and five U.S. isolates) from pigs with PMWS in different geographic regions. Sequence analysis of the complete genome and of the putative capsid gene ORF2 indicated that these six North American isolates of PCV-2 are closely related to other known PCV-2 isolates worldwide. The putative capsid gene (ORF2) of PCV is highly variable and displays as low as a 90% sequence identity among certain PCV-2 isolates. Despite the overall heterogeneic nature of ORF2, the N-terminal region of ORF2 among all PCVs is highly conserved and possesses a high percentage of basic amino acids, suggesting that the amino terminal region of the putative capsid protein may have DNA binding activity and may be in contact with the PCV DNA in the native virion (42). Phylogenetic analysis revealed that all PCV-2 isolates sequenced thus far form a major genotype, whereas all PCV-1 isolates are closely related and form another genotype. Based on the phylogenetic analysis, it is evident that both PCV-1 and

PCV-2 evolved from the same ancestor, but they may have undergone divergent evolution. Gibbs et al (17) analyzed the genomes of circoviruses and plant nanoviruses and showed that circoviruses most likely evolved from a plant nanovirus. It is believed that the plant nanovirus switched hosts to infect a vertebrate and then recombined with a vertebrate-infecting virus (17). Within the major genotype of PCV-2, several minor branches were also identified. The two French PCV-2 isolates (AF055393 and AF055394) diverge the most from all other PCV-2 isolates. The clinical significance of this divergence is not known. LeCann et al (29) reported that a PCV1-like virus was isolated from pigs with wasting disease in France, but they failed to experimentally reproduce the disease with this isolate. Phylogenetically, all the U.S. PCV-2 isolates sequenced thus far are closely related. However, genetic variation was observed among the Canadian PCV-2 isolates. Two of the Canadian isolates, AF109398 and AF117753, form a minor branch separating from other Canadian or U.S. isolates. Two other Canadian isolates, 34464 sequenced in this study and AF109399, also differ phylogenetically from the U.S. and other Canadian PCV-2 isolates. A PCV-2 isolate from Taiwan (AF116528) also forms a distinguishable minor branch. The origin of the bovine circovirus isolate is not known, but its close genetic relatedness with PCV-2 suggested that the bovine circovirus may be of swine origin, and that cross-species infection of PCV between bovine and swine is possible. These data suggest that, although the genome of PCV-2 is relatively stable in general, minor genetic differences do exist among PCV-2 isolates from different geographic regions. This observed difference might have important implications for the diagnosis of PCV-2 infection by

nucleic acid-based assays such as PCR. Genetic characterization of additional PCV-2 isolates from other geographic regions worldwide is warranted.

Since PCV-1 is nonpathogenic and widespread in pig population (6, 11, 12, 22, 23, 51, 52, 53), a test is needed to differentiate between infections with PCV-1 and PCV-2. In addition, since PCV antibody has been detected in humans (53), a major and growing concern is the inadvertent transmission of PCV from pig organs to human recipients during xenotransplantation. In xenotransplantation, there is zero tolerance for circovirus infection regardless of its pathogenic potential, since nonpathogenic PCV-1 may become pathogenic in immunocomprised xenograft recipients. Therefore, sensitive and easy-to-perform assays are needed to screen for both PCV-1 and PCV-2 infection in xenograft donor pigs. Several techniques such as PCR (19, 27, 28, 39, 40, 45), IHC (35, 39, 46), in situ hybridization (9, 35, 39, 46) are available for detecting PCV-2 infection; however, the ability of these tests to detect PCV-2 isolates from different geographic regions is not known. The data from this study suggest that PCV-2 isolates from different geographic regions vary to some extent in their genomic sequences. Thus, a universal and more sensitive PCR assay that can detect PCV isolates from various geographic regions is needed. Based on the genetic analyses of all PCV isolates, we developed a universal PCR-RFLP assay to diagnose PCV-2 infection and to differentiate between infections with PCV-1 and PCV-2 in pigs. This assay utilizes a pair of PCR primers selected from two conserved regions of PCV-1 and PCV-2 genome and a unique NcoI restriction enzyme site that exists only in PCV-2 isolates. The feasibility of this PCR-RFLP to detect PCV-2 and to differentiate between PCV-2 and PCV-1 was validated by using clinical samples of confirmed PMWS cases collected in different

geographic regions, and a sample intentionally mixed with both PCV-1 and PCV-2. Our results indicate that this PCR-RFLP is accurate and fast in diagnosing PCV-2 infection from PMWS cases of different geographic regions, in differentiating infections between PCV-1 and PCV-2 and in detecting dual infection with PCV-1 and PCV-2. This universal PCR-RFLP assay should help clinicians diagnose PMWS cases associated with PCV-2 infection in different geographic regions of the world, and should also be useful for screening xenograft donor pigs free of circovirus infection.

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REFERENCES

1. **Allan, G.M., F. McNeilly, J.P. Cassidy, G.A. Reilly, B. Adair, W.A. Ellis, and M.S. McNulty. 1995.** Pathogenesis of porcine circovirus; experimental infections of colostrum deprived piglets and examination of pig foetal material. Vet. Microbiol. **44**:49-64.
2. **Allan, G.M., B. Meehan, D. Todd, S. Kennedy, F. McNeilly, J. Ellis, E.G. Clark, J. Harding, E. Espuna, A. Botner, and C. Charreyre. 1998.** Novel porcine circoviruses from pigs with wasting disease syndromes. Vet. Rec. **142**:467-468.
3. **Allan, G.M., F. McNeilly, S. Kennedy, B. Daft, E.G. Clarke, J.A. Ellis, D.M. Haines, B.M. Meehan, and B.M. Adair. 1998**. Isolation of porcine circovirus-like viruses from pigs with a wasting disease in the USA and Europe. J. Vet. Diagn. Invest. **10**:3-10.
4. **Allan, G.M., F. McNeilly, B.M. Meehan, S. Kennedy, D.P. Mackie, J.A. Ellis, E.G. Clark, E. Espuna, N. Saubi, P. Riera, A. Botner, and C.E. Charreyre. 1999**. Isolation and characterisation of circoviruses from pigs with wasting syndromes in Spain, Denmark and Northern Ireland. Vet. Microbiol. **66**:115-23.
5. **Allan, G.M., S. Kennedy, F. McNeilly, J.C. Foster, J.A. Ellis, S.J. Krakowka, B.M. Meehan, and B.M. Adair. 1999**. Experimental reproduction of severe wasting disease by co-infection of pigs with porcine circovirus and porcine parvovirus. J. Comp. Pathol. **121**:1-11.
6. **Allan, G.M. and J.A. Ellis. 2000**. Porcine circoviruses: a review. J. Vet. Diagn. Invest. **12**:3-14.

7. **Balasch, M., J. Segales, C. Rosell, M. Domingo, A. Mankertz, A. Urniza, and J. Plana-Duran. 1999**. Experimental inoculation of conventional pigs with tissue homogenates from pigs with post-weaning multisystemic wasting syndrome. J. Comp. Pathol. **121**:139-148.

8. **Bassami, M.R., D. Berryman, G.E. Wilcox, and S.R. Raidal. 1998**. Psittacine beak and feather disease virus nucleotide sequence analysis and its relationship to porcine circovirus, plant circoviruses, and chicken anaemia virus. Virology **249**:453-459.

9. **Choi, C. and C. Chae. 1999**. In-situ hybridization for the detection of porcine circovirus in pigs with postweaning multisystemic wasting syndrome. J. Comp. Pathol. **121**:265-270.

10. **Clark, E.G. 1997.** Postweaning multisystemic wasting syndrome. Proceedings of American Association of Swine Practitioners. 499-501.

11. **Dulac, G.C. and A. Afshar. 1989**. Porcine circovirus antigens in PK-15 cell line (ATCC CCL-33) and evidence of antibodies to circovirus in Canadian pigs. Can. J. Vet. Res. **53**:431-433.

12. **Edwards, S. and J.J. Sands. 1994**. Evidence of circovirus infection in British pigs. Vet. Rec. **134**:680-1.

13. **Ellis, J., L. Hassard, E. Clark, J. Harding, G. Allan, P. Willson, J. Strakappe, K. Martin, F. McNeilly, B. Meehan, D. Todd, and D. Haines. 1998**. Isolation of circovirus from lesions of pigs with postweaning multisystemic wasting syndrome. Can. Vet. J. **39**:44-51.

14. **Ellis, J., S. Krakowka, M. Lairmore, D. Haines, A. Bratanich, E. Clark, G. Allan, C. Konoby, L. Hassard, B. Meehan, K. Martin, J. Harding, S. Kennedy, and F. McNeilly. 1999**. Reproduction of lesions of postweaning multisystemic wasting syndrome in gnotobiotic piglets. J. Vet. Diagn. Invest. **11**:3-14.

15. **Ellis, J.A. 1999**. "The clinical scope of porcine reproductive and respiratory syndrome virus infection has expanded since 1987": an alternative perspective. Vet. Pathol. **36**:262-264.

16. **Ellis, J.A., A. Bratanich, E.G. Clark, G. Allan, B. Meehan, D.M. Haines, J. Harding, K.H. West, S. Krakowka, C. Konoby, L. Hassard, K. Martin, and F. McNeilly. 2000**. Coinfection by porcine circoviruses and porcine parvovirus in pigs with naturally acquired postweaning multisystemic wasting syndrome. J. Vet. Diagn. Invest. **12**:21-27.

17. **Gibbs, M.J. and G.F. Weiller. 1999**. Evidence that a plant virus switched hosts to infect a vertebrate and then recombined with a vertebrate-infecting virus. Proc. Natl. Acad. Sci. U S A **96**:8022-8027.

18. **Hamel, A.L., L.L. Lin, and G.P. Nayar. 1998**. Nucleotide sequence of porcine circovirus associated with postweaning multisystemic wasting syndrome in pigs. J. Virol. **72**:5262-5267.

19. **Hamel, A.L., L.L. Lin, C. Sachvie, E. Grudeski, and G.P.Nayar. 2000**. PCR detection and characterization of type-2 porcine circovirus. Can. J. Vet. Res. **64**:44-52.

20. **Harding, J.C. 1997**. Postweaning multisystemic wasting syndrome (PMWS): preliminary epidemiology and clinical presentation. Proceedings of American Association of Swine Practitioners, 503.

21. **Harding, J.C. and E.G. Clark. 1997**. Recognizing and diagnosing postweaning multisystemic wasting syndrome (PMWS). Swine Health and Production. **5**:201-203.

22. **Hines, R.K. and P.D. Lukert. 1995**. Porcine circovirus: a serological survey of swine in the United States. Swine Health and Production. **3**:71-73.

23. **Hines, R.K., P.D. Lukert, D. Dau, and D. Case. 1995.** Some effects of porcine circovirus on performance. Swine Health and Production. **3**:251-255.

24. **Kennedy, S., G. Allan, F. McNeilly, B.M. Adair, A. Hughes, and P. Spillane. 1998.** Porcine circovirus infection in Northern Ireland. Vet. Rec. **142**:495-6.

25. **Kennedy, S., D. Moffett, F. McNeilly, B. Meehan, J. Ellis, S. Krakowka, and G.M. Allan. 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.

26. **Kiupel, M., G.W. Stevenson, S.K. Mittal, E.G. Clark, D.M. Haines. 1998.** Circovirus-like viral associated disease in weaned pigs in Indiana. Vet. Pathol. **35**:303-307.

27. **Larochelle, R., M. Morin, M. Antaya, and R. Magar. 1999.** Identification and incidence of porcine circovirus in routine field cases in Quebec as determined by PCR. Vet. Rec. **145**:140-142.

28. **Larochelle, R., M. Antaya, M. Morin, and R. Magar. 1999**. Typing of porcine circovirus in clinical specimens by multiplex PCR. J. Virol. Methods **80**:69-75.

29. **LeCann, P., E. Albina, F. Madec, R. Cariolet, and A. Jestin. 1997**. Piglet wasting disease. Vet. Rec. **141**:660.

30. **Lukert, P.D., G.F. de Boer, J.L. Dale, P. Keese, M.S. McNulty, J.W. Randles, and I. Tisher. 1995**. The Circoviridae. In: Murphy FA, Fauquet CM, Bishop DHL, Ghabrial SA, Jarvis AW, Martelli GP, Mayo MA, Summers MD. (eds). Virus Taxonomy: sixth report of the international committee on taxonomy of viruses. Pp166-168. Springer-Verlag, Wien New York.

31. **Mankertz, A., F. Persson, J. Mankertz, G. Blaess, and H.J. Buhk. 1997**. Mapping and characterization of the origin of DNA replication of porcine circovirus. J. Virol. **71**:2562-2566.

32. **Mankertz, J., H.J. Buhk, G. Blaess, and A. Mankertz. 1998**. Transcription analysis of porcine circovirus (PCV). Virus Genes **16**:267-276.

33. **Mankertz, A., M. Domingo, J.M. Folch, P. LeCann, A. Jestin, J. Segales, B. Chmielewicz, J. Plana-Duran, and D. Soike. 2000**. Characterisation of PCV-2 isolates from Spain, Germany and France. Virus Res. **66**:65-77.

34. **McNeilly, F., J.A. Smyth, B.M. Adair, and M.S. McNulty. 1995**. Synergism between chicken anemia virus (CAV) and avian reovirus following dual infection of 1-day-old chicks by a natural route. Avian Dis. **39**:532-537.

35. **McNeilly, F., S. Kennedy, D. Moffett, B.M. Meehan, J.C. Foster, E.G. Clarke, J.A. Ellis, D.M. Haines, B.M. Adair, and G.M. Allan. 1999.** A comparison of in situ hybridization and immunohistochemistry for the detection of a new porcine

circovirus in formalin-fixed tissues from pigs with post-weaning multisystemic wasting syndrome (PMWS). J. Virol. Methods **80**:123-128.

36. **Meehan, B.M., J.L. Creelan, M.S. McNulty, and D. Todd. 1997**. Sequence of porcine circovirus DNA: affinities with plant circoviruses. J. Gen. Virol. **78**:221-227.

37. **Meehan, B.M., F. McNeilly, D. Todd, S. Kennedy, V.A. Jewhurst, J.A. Ellis, L.E. Hassard, E.G. Clark, D.M. Haines, and G.M. Allan. 1998**. Characterization of novel circovirus DNAs associated with wasting syndromes in pigs. J. Gen. Virol. **79**:2171-2179.

38. **Miyata, H., H. Tsunoda, A. Kazi, A. Yamada, M.A. Khan, J. Murakami, T. Kamahora, K. Shiraki, and S. Hino. 1999**. Identification of a novel GC-rich 113-nucleotide region to complete the circular, single-stranded DNA genome of TT virus, the first human circovirus. J. Virol. **73**:3582-3586.

39. **Morozov, I., T. Sirinarumitr, S.D. Sorden, P.G. Halbur, M.K. Morgan, K.J. Yoon, and P.S. Paul. 1998.** Detection of a novel strain of porcine circovirus in pigs with postweaning multisystemic wasting syndrome. J. Clin. Microbiol. **36**:2535-2541.

40. **Nayar, G.P., A. Hamel, and L. Lin. 1997**. Detection and characterization of porcine circovirus associated with postweaning multisystemic wasting syndrome in pigs. Can. Vet. J. **38**:385-386.

41. **Nayar, G.P., A.L. Hamel, L. Lin, C. Sachvie, E. Grudeski, and G. Spearman. 1999**. Evidence for circovirus in cattle with respiratory disease and from aborted bovine fetuses. Can. Vet. J. **40**:277-278.

42. **Niagro, F.D., A.N. Forsthoefel, R.P. Lawther, L. Kamalanathan, B.W. Ritchie, K.S. Latimer, and P.D. Lukert. 1998**. Beak and feather disease virus and porcine circovirus genomes: intermediates between the geminiviruses and plant circoviruses. Arch. Virol. **143**:1723-1744.

43. **Nishizawa, T., H. Okamoto, K. Konishi, H. Yoshizawa, Y. Miyakawa, and M. Mayumi. 1997.** A novel DNA virus (TTV) associated with elevated transaminase levels in posttransfusion hepatitis of unknown etiology. Biochem. Biophys. Res. Commun. **241**:92-97.

44. **Onuki, A., K. Abe, K. Togashi, K. Kawashima, A. Taneichi, and H. Tsunemitsu. 1999**. Detection of porcine circovirus from lesions of a pig with wasting disease in Japan. J. Vet. Med. Sci. **61**:1119-1123.

45. **Ouardani M, Wilson L, Jette R, Montpetit C, Dea S. 1999**. Multiplex PCR for detection and typing of porcine circoviruses. J Clin Microbiol 37(12):3917-24

46. **Rosell, C., J. Segales, J. Plana-Duran, M. Balasch, G.M. Rodriguez-Arrioja, S. Kennedy, G.M. Allan, F. McNeilly, K.S. Latimer, and D. Domingo. 1999**. Pathological, immunohistochemical, and in-situ hybridization studies of natural cases of postweaning multisystemic wasting syndrome (PMWS) in pigs. J. Comp. Pathol. **120**:59-78.

47. **Rosell, C., J. Segales, J.A. Ramos-Vara, J.M. Folch, G.M. Rodriguez-Arrioja, C.O. Duran, M. Balasch, J. Plana-Duran, and M. Domingo. 2000**. Identification of porcine circovirus in tissues of pigs with porcine dermatitis and nephropathy syndrome. Vet. Rec. **146**:40-43.

48. **Segales, J., M. Sitjar, M. Domingo, S. Dee, M. Del Pozo, R. Noval, C. Sacristan, A. De las Heras, A. Ferro, and K.S. Latimer. 1997**. First report of postweaning multisystemic wasting syndrome in Spain. Vet. Rec. **141**:600-601.

49. **Spillane, P., S. Kennedy, B. Meehan, and G. Allan. 1998**. Porcine circovirus infection in the Republic of Ireland. Vet. Rec. **143**:511-512.

50. **Tischer, I., H. Gelderblom, W. Vettermann, and M.A. Koch. 1982**. A very small porcine virus with circular single-stranded DNA. Nature **295**:64-66.

51. **Tischer, I., W. Mields, D. Wolff, M. Vagt, and W. Griem. 1986.** Studies on epidemiology and pathogenicity of porcine circovirus. Arch. Virol. **91**:271-276.

52. **Tischer, I., L. Bode, D. Peters, S. Pociuli, and B. Germann. 1995**. Distribution of antibodies to porcine circovirus in swine populations of different breeding farms. Arch. Virol. **140**:737-743.

53. **Tischer, I., L. Bode, J. Apodaca, H. Timm, D. Peters, R. Rasch, S. Pociuli, and E. Gerike. 1995.** Presence of antibodies reacting with porcine circovirus in sera of humans, mice, and cattle. Arch. Virol. **140**:1427-1439.

54. **Todd, D., F.D. Niagro, B.W. Ritchie, W. Curran, G.M. Allan, P.D. Lukert, K.S. Latimer, W.L. Steffens, and M.S. McNulty. 1991.** Comparison of three animal viruses with circular single-stranded DNA genomes. Arch. Virol. **117**:129-135.

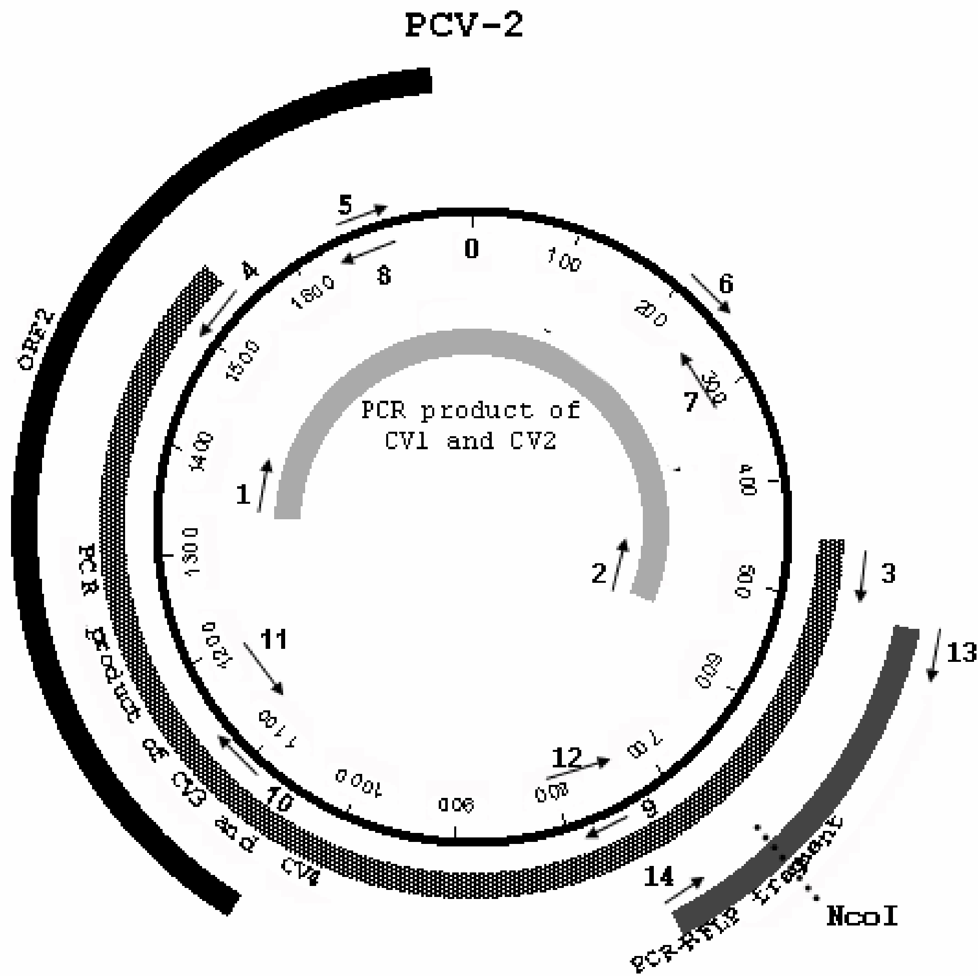

Fig. 1. Genome organization of PCV-2. The origin of replication (o), the putative capsid gene (ORF2), the PCR-RFLP fragment, and the two overlap PCR fragments used to determine the complete genome of PCV-2 are indicated in the circular map. The relative positions of the oligonucleotide primers used in this study are indicated by arrows with respective numbers: **1**: CV1; **2**: CV2; **3**: CV3; **4**: CV4; **5**: CV1-1; **6**: CV1-2; **7**: CV2-1; **8**: CV2-2; **9**: CV3-1; **10**: CV3-2; **11**: CV4-1; **12**: CV4-2; **13**: MCV1; **14**: MCV2. The sequences and designations of these primers are listed in Table 2.

Fig. 2

```
MCV 1>
26606     GCTGAACTTTTGAAAGTGAGCGGGAAAATGCAGAAGCGTGATTGGAAGACCAATGTACACGTCATTGTGGGGCCACCTGGGTGTGGTAAAAGCAAA
10489     ................................................................................................
26607     ................................................................................................
34464     ................................................................................................
40856     ................................................................................................
40895     ................................................................................................
AF027217  ................................................................................................
AF055391  ................................................................................................
AF055392  ................................................................................................
AF055393  ..................................................T.............................................
AF055394  ..................................................T.............................................
AF085695  ................................A..............A............T...................................
AF086834  ................................A..............A............T...................................
AF086835  ................................A..............A............T...................................
AF086836  ................................A..............A............T...................................
AF109397  ................................................................................................
AF109398  ..................................................G...................................C.........
AF109399  ................................................................................................
AF112862  ........................................................C.......................................
AF117753  ..................................................G...................................C.........
AF166528  ................................................................................................
AJ223185  ................................................................................................
AF012107  ..........................G......C................AGC............A.....C..G..C..T.....G..G...C.G
AF071879  ..........................G......C................AGC............A.....C..G..C..T.....G..G...C.G
U49186    ..........................G......C................AGC............A.....C..G..C..T.....G..G...C.G
Y09921    ..........................G......C................AGC............A.....C..G..C..T.....G..G...C.G
                                                                                ******NcoI site
26606     TGGGCTGCTAATTTTGCAGACCCGGAAACCACATACTGGAAACCACCTAGAAACAAGTGGTGGGATGGTTACCATGGTGAAGAAGTGGTTGTTATT
10489     ...............................................C................................................
26607     ................................................................................................
34464     ................................................................................................
40856     ................................................................................................
40895     ................................................................................................
AF027217  ................................................................................................
AF055391  ................................................................................................
AF055392  ................................................................................................
AF055393  ................................................................................................
AF055394  ................................................................................................
AF085695  ..................A..............................A...............................A..............
AF086834  ..................A..............................A...............................A..............
AF086835  ..................A..............................A...............................A..............
AF086836  ..................A..............................A...............................A..............
AF109397  ................................................................................................
AF109398  ................................................................................................
AF109399  ................................................................................................
AF112862  ................................................................................................
AF117753  ................................................................................................
AF166528  ................................................C...............................................
AJ223185  ................................................................................................
AF012107  .....CCG.........T..G..TAGGGA...C........G..TAG......T..............A..T.....A........T......T.G
AF071879  .....CCG.......A.T..G..TAGCGA...C........G..TAG......T..............A..T.....A........T......T.G
U49186    .....CCG.........T..G..TAGGGA...C........G..TAG......T..............A..T.....A........T......T.G
Y09921    .....CCG.........T..G..TAGCGA...C........G..TAG......T..............A..T.....A........T......T.G
                                <MCV 2
26606     GATGACTTTTATGGCTGGCTGCCGTGGGATGATCTACTGAGACTGTGTGA
10489     ..................................................
26607     ..................................................
34464     ..................................................
40856     ..................................................
40895     ..................................................
AF027217  ..................................................
AF055391  ..................................................
AF055392  ..................................................
AF055393  .......................C..........................
AF055394  .......................C..........................
AF085695  ..................................................
AF086834  ..................................................
AF086835  ..................................................
AF086836  ..................................................
AF109397  ..................................................
AF109398  ..............T...................................
AF109399  ..................................................
AF112862  ..................................................
AF117753  ..................................................
AF166528  ..............T...................................
AJ223185  ..................................................
AF012107  .....T............T.A..T..........................
AF071879  .....T............T.A..T..........................
U49186    .....T............T.A..T..........................
Y09921    .....T............T.A..T..........................
```

Fig. 2. Nucleotide sequence alignment of the region amplified in the PCR-RFLP assay. The regions in which the consensus PCR primers (MCV1 and MCV2) were chosen are underlined. The unique NcoI restriction enzyme site that is present in all PCV-2 isolates is indicated by asterisks (*). The sequence of the PCV-2 isolate 26606 from this study is shown on top, and only differences are indicated for other isolates. The sequences used in the alignment are cited in the text.

Fig. 3

```
                          *    *     *        *       ***  ** *     *  *     *   ***** ********    * **** ****
26606          MTYPRRRYRRRRHRPRSHLGQILRRRPWLLHP--RHRYRWRRKNGIFNTRLSRTFGYTVKRTTVTTPSWAVDMMRFKLDD
10489          MTYPRRRYRRRRHRPRSHLGQILRRRPWLVHP--RHRYRWRRKNGIFNTRLSRTFGYTVKATTVTTPSWAVDMMRFNIDD
26607          MTYPRRRYRRRRHRPRSHLGQILRRRPWLLHP--RHRYRWRRKNGIFNTRLSRTFGYTVKRTTVTTPSWAVDMMRFKLDD
34464          MTYPRRRFRRRRHRPRSHLGQILRRRPWLVHP--RHRYRWRRKNGIFNARLSRTFGYTVKATTVSTPSWAVDMLRFNLDD
40856          MTYPRRRYRRRRHRPRSHLGQILRRRPWLVHP--RHRYRWRRKNGIFNTRLSRTFGYTVKRTTVTTPSWAVDMMRFKLDD
40895          MTYPRRRYRRRRHRPRSHLGQILRRRPWLVHP--RHRYRWRRKNGIFNTRLSRTFGYTVKATTVRTPSWAVDMMRFNIDD
AF027217       MTYPRRRYRRRRHRPRSHLGQILRRRPWLVHP--RHRYRWRRKNGIFNTRLSRTFGYTVKATTVRTPSWAVDMMRFNIDD
AF055391       MTYPRRRYRRRRHRPRSHLGQILRRRPWLVHP--RHRYRWRRKNGIFNTRLSRTFGYTVKATTVRTPSWAVDMMRFNIDD
AF055392       MTYPRRRYRRRRHRPRSHLGQILRRRPWLVHP--RHRYRWRRKNGIFNTRLSRTFGYTVKRTTVTTPSWAVDMMRFKIDD
AF055393       MTYPRRRYRRRRHRPRSHLGQILRRRPWLVHP--RHRYRWRRKNGIFNTRLSRTFGYTVKRTTVRTPSWAVDMMRFNIND
AF055394       MTYPRRRYRRRRHRPRSHLGQILRRRPWLVHP--RHRYRWRRKNGIFNTRLSRTFGYTVKRTTVKTPSWAVDMMRFNIND
AF085695       MTYPRRRYRRRRHRPRSHLGQILRRRPWLVHP--RHRYRWRRKNGIFNTRLSRTFGYTVKRTTVTTPSWAVDMMRFKIDD
AF086834       MTYPRRRYRRRRHRPRSHLGQILRRRPWLVHP--RHRYRWRRKNGIFNTRLSRTFGYTVKATTVTTPSWAVDMMRFNIDD
AF086835       MTYPRRRYRRRRHRPRSHLGQILRRRPWLVHP--RHRYRWRRKNGIFNTRLSRTFGYTVKRTTVTTPSWAVDMMRFKIDD
AF086836       MTYPRRRYRRRRHRPRSHLGQILRRRPWLVHP--RHRYRWRRKNGIFNTRLSRTFGYTVKRTTVTTPSWAVDMMRFKIDD
AF109397       MTYPRRRYRRRRTRPRSHLGQILRRRPWLVHP--RHRYRWRRKNGIFNTRLSRTFGYTVKATTVRTPSWAVDMMRFNIDD
AF109398       MTYPRRRYRRRRTRPRSHLGQILRRRPWLVHP--RHRYRWRRKNGIFNTRLSRTFGYTVKATTVRTPSWAVDMLRFKIDD
AF109399       MTYPRRRFRRRRHRPRSHLGQILRRRPWLVHP--RHRYRWKRKNGIFNARLSRTFGYTVKATTVSTPSWAVDMLRFNLDD
AF112862       MTYPRRRYRRRRHRPRSHLGQILRRRPWLVHP--RHRYRWRRKNGIFNTRLSRTFGYTVKATTVTTPSWAVDMMRFNIDD
AF117753       MTFPRRRYRRRRHRPRSHLGQILRRRPWFLHP--RHRYRWRRKNGIFNTRLSRTFGYTVKATTVRTPSWAVDMLRFKIDD
AF166528       MTYPRRRFRRRRHRPRSHLGQILRRRPWLVHP--RHRYRWRRKNGIFNTRLSRTFGYTVKATTVRTPSWAVDMMRFNIND
AJ223185       MTYPRRRYRRRRHRPRSHLGQILRRRPWLVHP--RHRYRWRRKNGIFNTRLSRTFGYTVKATTVRTPSWAVDMMRFNIDD
AF012107       MTWPRRRYRRRRTRPRSHLGNILRRRPYLVHPAFRNRYRWRRKTGIFNSRLSREFVLTIRGGHSQ-PSWNVNELRFNIGQ
AF071879       MTWPRRRYRRRRTRPRSHLGNILRRRPYLAHPAFRNRYRWRRKTGIFNSRLSTELVLTIKGGYSQ-PSWNVNYLKFNIGQ
U49186         MTWPRRRYRRRRTRPRSHLGNILRRRPYLAHPAFRNRYRWRRKTGIFNSRLSTEFVLTIKGGYSQ-PSWNVNYLKFNIGQ
Y09921         MTWPRRRYRRRRTRPRSHLGNILRRRPYLAHPAFRNRYRWRRKTGIFNCRLSKEFVITIKGGYSQ-PSWHVNHLRFNIGQ

                 *   *   * ****  *      * * *   * **    ***       ***    *  ********      *          *   *
26606          FVPPGGGTNKISIPFEYYRIRKVKVEFWPCSPITQGDRGVGSTAVILDDNFVPKAKALTYDPYVNYSSRHTIPQPFSYHS
10489          FVPPGGGTNKISIPFEYYRIRKVKVEFWPCSPITQGDRGVGSTAVILDDNFVTKATALTYDPYVNYSSRHTIPQPFSYHS
26607          FVPPGGGTNKISIPFEYYRIRKVKVEFWPCSPITQGDRGVGSTAVILDDNFVPKATALTYDPYVNYSSRHTIPQPFSYHS
34464          FVPPGGGTNKISIPFEYYRIRKVKVEFWPCSPITQGDRGVGSTAVILDDNFVTKATALTYDPYVNYSSRHTIPQPFSYHS
40856          FVPPGGGTNKISIPFEYYRIRKVKVEFWPCSPITQGDRGVGSTAVILDDNFIPKANALTYDPYVNYSSRHTIPQPFSYHS
40895          FVPPGGGTNKISIPFEYYRIRKVKVEFWPCSPITQGDRGVGSTAVILDDNFVTKATALTYDPYVNYSSRHTIPQPFSYHS
AF027217       FVPPGGGTNKISIPFEYYRIRKVKVEFWPCSPITQGDRGVGSTAVILDDNFVTKATALTYDPYVNYSSRHTIPQPFSYHS
AF055391       FVPPGGGTNKISIPFEYYRIKKVKVEFWPCSPITQGDRGVGSTAVILDDNFVTKATALTYDPYVNYSSRHTIPQPFSYHS
AF055392       FVPPGGGTNKISIPFEYYRIRKVKVEFWPCSPITQGDRGVGSTAVILDDNFVTKATALTYDPYVNYSSRHTIPQPFSYHS
AF055393       FLPPGGGSNPRSVPFEYYRIRKVKVEFWPCSPITQGDRGVGSSAVILDDNFVTKATALTYDPYVNYSSRHTITQPFSYHS
AF055394       FLPPGGGSNPRSVPFEYYRIRKVKVEFWPCSPITQGDRGVGSSAVILDDNFVTKATALTYDPYVNYSSRHTITQPFSYHS
AF085695       FVPPGGGTNKISIPFEYYRIRKVKVEFWPCSPITQGDRGVGSTAVILDDNFVTKATALTYDPYVNYSSRHTIPQPFSYHS
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AF109398       FVPPGGGTNKISIPFEYYRIRKVKVEFWPCSPITQGDRGVGSSAVILDDNFVPKATALTYDPYVNYSSRHTITQPFSYHS
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AF112862       FVPPGGGTNKISVPFEYYRIRKVKVEFWPCSPITQGDRGVGSTAVILDDNFVTKATALTYDPYVNYSSRHTIPQPFSYHS
AF117753       FVPPGGGTNKISIPFEYYRIRKVKVEFWPCSPITQGDRGVGSSAVILDDNFFPKSTALTYDPYVNYSSRHTITQPFSYHS
AF166528       FVPPGGGTNKISIPFEYYRIRKVKVEFWPCSPITQGDRGVGSTAVILDDNFVTKATALTYDPYVNYSSRHTIPQPFSYHS
AJ223185       FVPPGGGTNKISIPFEYYRIRKVKVEFWPCSPITQGDRGVGSTAVILDDNFVTKATALTYDPYVNYSSRHTIPQPFSYHS
AF012107       FLPPSGGTNPLPLPFQYYRIRKAKYEFYPRDPITSNQRGVGSTVVILDANFVTPSTNLAYDPYINYSSRHTIRQPFTYHS
AF071879       FLPPSGGTNPLPLPFQYYRIRKAKYEFYPRDPITSNQRGVGSTVVILDANFVTPSTNLAYDPYINYSSRHTIRQPFTYHS
U49186         FLPPSGGTNPLPLPFQYYRIRKAKYEFYPRDPITSNQRGVGSTVVILDANFVTPSTNLAYDPYINYSSRHTIRQPFTYHS
Y09921         FLPPSGGTNPLPLPFQYYRIRKAKYEFYPRDPITSNERGVGSTVVILDANFVTPSTNLAYDPYINYSSRHTIRQPFTYHS

                 *      *  *   ** *     **     ** * **   *  *    * ** **** * ** *  *          *    *****
26606          RYFTPKPVLDSTIDYFQPNNKRNQLWLRLQTSRNVDHVGLGTAFENSKYDQDYNIRVTMYVQFREFNLKDPPLNP 233
10489          RYFTPKPVLDSTIDYFQPNNKRNQLWLRLQTSRNVDHVGLGTAFENSKYDQDYNIRVTMYVQFREFNLKDPPLKP 233
26607          RYFTPKPVLDSTIDYFQPNNKRNQLWLRLQTSRNVDHVGLGTAFENSKYDQDYNIRVTMYVQFREFNLKDPPLNP 233
34464          RYFTPKPVLDSTIDYFQPNNKRNQLWMRLQTSRNVDHVGLGTAFENSKYDQDYNIRVTMYVQFREFNLKDPPLKP 233
40856          RYFTPKPVLDSTIDYFQPNNKRNQLWLRLQTSRNVDHVGLGTAFENSKYDQDYNIRVTMYVQFREFNLKDPPLNP 233
40895          RYFTPKPVLDSTIDYFQPNNKRNQLWMRLQTSRNVDHVGLGTAFENSIYDQDYNIRVTMYVQFREFNLKDPPLKP 233
AF027217       RYFTPKPVLDSTIDYFQPNNKRTQLWLRLQTSRNVDHVGLGTAFENSIYDQDYNIRVTMYVQFREFNLKDPPLKP 233
AF055391       RYFTPKPVLDSTIDYFQPNNKRNQLWLRLQTSRNVDHVGLGTAFENSIYDQDYNIRVTMYVQFREFNLKDPPLKP 233
AF055392       RYFTPKPVLDSTIDYFQPNNKRNQLWLRLQTSGNVDHVGLGAAFENSKYDQDYNIRVTMYVQFREFNLKDPPLKP 233
AF055393       RYFTPKPVLDSTIDYFQPNNKRNQLWLRLQTAGNVDHVGLGTAFENSIYDQEYNIRVTMYVQFREFNLKDPPLNP 233
AF055394       RYFTPKPVLDSTIDYFQPNNKRNQLWLRLQTTGNVDHVGLGTAFENSIYDQEYNIRVTMYVQFREFNLKDPPLNP 233
AF085695       RYFTPKPVLDSTIDYFQPNNKRNQLWLRLQTSGNVDHVGLGTAFENSKYDQDYNIRVTMYVQFREFNLKDPPLEP 233
AF086834       RYFTPKPVLDSTIDYFQPNNKRNQLWLRLQTSRNVDHVGLGTAFENSKYDQDYNIRVTMYVQFREFNLKDPPLKP 233
AF086835       RYFTPKPVLDSTIDYFQPNNKRNQLWLRLQTSGNVDHVGLGTAFENSKYDQDYNIRVTMYVQFREFNLKDPPLKP 233
AF086836       RYFTPKPVLDSTIDYFQPNNKRNQLWLRLQTSGNVDHVGLGTAFENSKYDQDYNIRVTMYVQFREFNLKDPPLEP 233
AF109397       RXFTPKPVLDSTIDYFQPNNKRNQLWLRLQTSRNVDHVGLGTAFENSKYDQDYNIRVTMYVQFREFNLKDPPLKP 233
AF109398       RXFTPKPVLDGTIDYFQPNNKRNQLWLRLQTTGNVDHVGLGTAFENSIYDQDYNIRVTMYVQFREFNLKDPPLKP 233
AF109399       RYFTPKPVLDSTIDYFQPNNKRNQLWMRLQTSRNVDHVGLGTAFENSKYDQDYNIRVTMYVQFREFNLKDPPLKP 233
AF112862       RXFTPKPVLDRTIDYFQPNNKRNQLWLRLQTSRNVDHVGLGTAFENSKYDQDYNIRVTMYVQFREFNLKDPPLKP 233
AF117753       RXFTPKPVLDSTIDYFQPNNKRNQLWLRLQTTGNVDHVGLGTAFENSIYDQDYNIRVTMYVQFREFNLKDPPLKP 233
AF166528       RYFTPKPVLDSTIDYFQPNNKRNQLWLRLQTSANVDHVGLGTAFENSKYDQDYNIRVTMYVQFREFNLKDPPLKP 233
AJ223185       RYFTPKPVLDSTIDYFQPNNKRNQLWLRLQTSRNVDHVGLGTAFENSKYDQDYNIRVTMYVQFREFNLKDPPLKP 233
AF012107       RYFTPKPELDQTIDWFQPNNKRNQLWLHLNTHTNVEHTGLGYALQNATTAQNYVVRLTIYVQFREFILKDPL    231
AF071879       RYFTPKPELDQTIDWFHPNNKRNQLWLHLNTHTNVEHTGLGYALQNAATAQNYVVRLTIYVQFREFILKDL     230
U49186         RYFTPKPELDQTIDWFHPNNKRNQLWLHLNTHTNVEHTGLGYALQNAATAQNYVVRLTIYVQFREFILKDPL    231
Y09921         RYFTPKPELDQTIEWFHPNNKRNQLWLHLNTHTNVEHTGLGYALQNAATAQNYVVRLTIYVQFREFILKDPL    231
```

Fig. 3. Amino acid sequence alignment of the putative capsid protein (ORF2) of PCV-1 and PCV-2 isolates sequenced thus far. Deletions are indicated by (-). Amino acid sequence differences are indicated with asterisks (*) on top of the alignment. The sequences used in the alignment are cited in the text.

Fig. 4

Fig. 4. A phylogenetic tree based on the complete genomic nucleotide sequences of all PCV isolates. The tree was constructed with the aid of the PAUP program. Branch-and-bound searching and midpoint rooting options were used to produce a consensus tree. The scale bar representing the numbers of character state changes is shown. Branch lengths are proportional to the numbers of character state changes.

Fig. 5

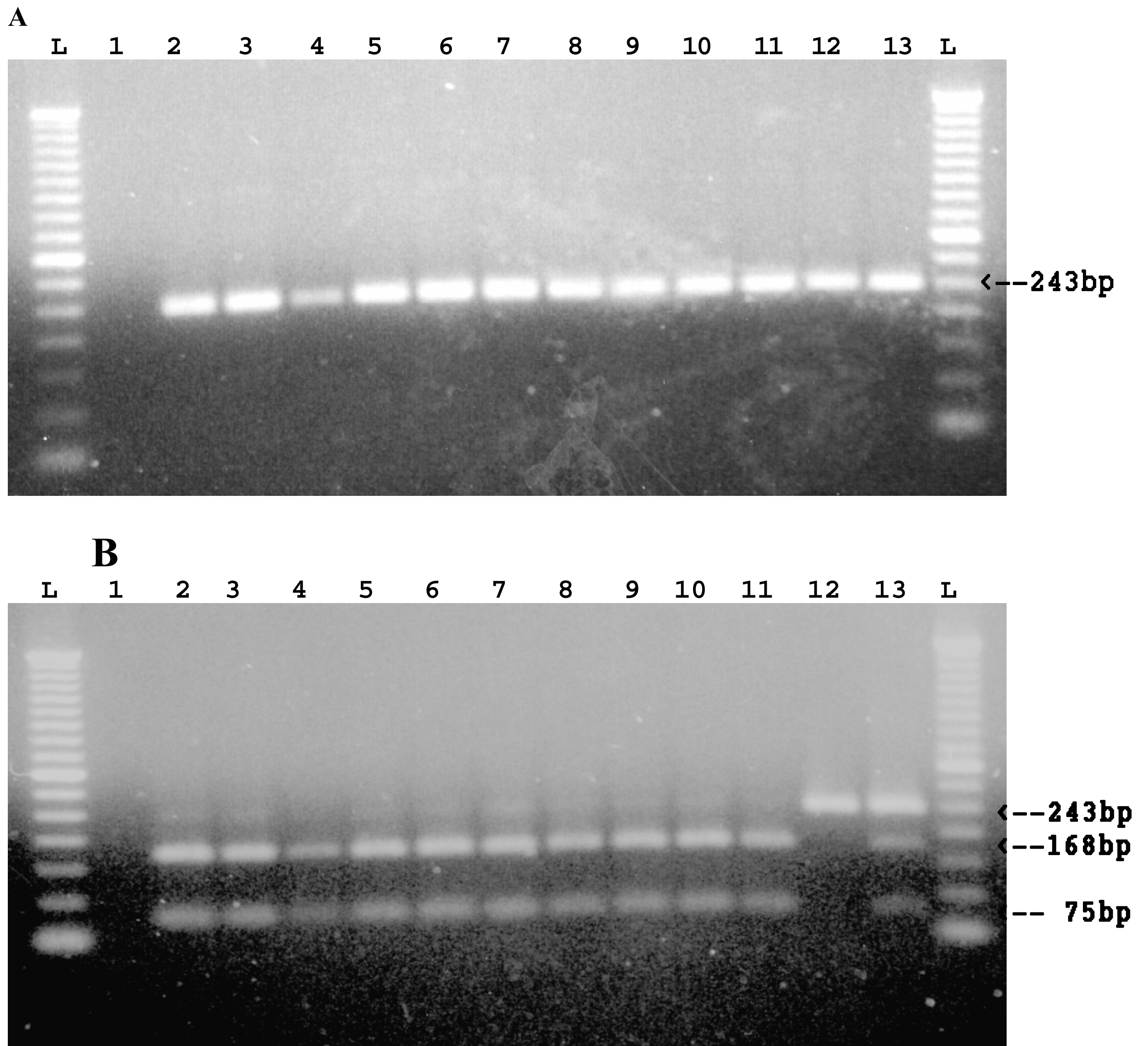

Fig. 5. Detection and differentiation of PCV infections by a PCR-RFLP assay. Panel A shows the results of PCR amplification of a 243 bp fragment from tissue samples containing PCV isolates (both PCV-1 and PCV-2) but not from a negative control liver tissue sample (lane 1). Panel B shows the results of RFLP analysis of the PCR products. L: 50 bp DNA ladder; Lane 1: a sample of liver tissue from a control SPF pig; Lanes 2 to 11: tissue samples from 10 PMWS cases; Lane 12: PK15 cells containing PCV-1; Lane 13: a sample containing both PCV-1 and PCV-2. The expected PCR fragment (Panel A) and three RFLP fragments, 243 bp, 168 bp and 75 bp, respectively (Panel B) are indicated with arrows.

Table 1. Porcine circovirus isolates used in this study as well as reported previously

Type	ID	Geographic location	IHC[a] PRRSV	IHC PCV	ISH[b] PCV	Clinical signs[c]	Histopathological lesions[d]	References
PCV-2	26606[e]	UT	+	ND	+	Resp	Pneumonia, lymphoid depletion, enteritis	this study
	26607[e]	UT	+	ND	+	Resp, diarrhea	Pneumonia, enteritis, hepatitis, nephritis	this study
	40856[e]	MO	+	+	ND	Resp, wasting	Pneumonia, lymphoid depletion, hepatitis, nephritis	this study
	40895[e]	IA	-	+	ND	Resp, wasting	Pneumonia, lymphoid depletion	this study
	34464[e]	Canada	+	+	ND	Resp	Pneumonia	this study
	10489[e]	IL	-	+	ND	Resp, wasting	Pneumonia, lymphoid depletion	this study
	38835	IA	+	+	ND	Resp	Pneumonia, lymphoid depletion	this study
	36688	IA	+	+	ND	Resp, wasting, dermatitis	Pneumonia, lymphoid depletion, nephritis, dermatitis	this study
	40860	IA	+	+	ND	Resp	Pneumonia	this study
	40887	IA	+	+	ND	Resp	Pneumonia	this study
	AF055391	CA						37
	AF027217	CA						19
	AF109397	France[f]						29,GenBank
	AJ223185	IA						39
	AF055394	France						37
	AF085695	Canada						GenBank
	AF086836	Canada						GenBank
	AF086835	Canada						GenBank
	AF086834	Canada						GenBank
	AF112862	Canada						GenBank
	AF166528	Taiwan						GenBank
	AF109399	Canada						GenBank
	AF109398	Canada						GenBank
	AF117753	Canada						GenBank
	AF055393	France						37
	AF055392	Canada						37
PCV-1	AF071879	PK15 cell						42
	Y09921	Germany						31
	U49186	Ireland						36
	AF012107	France						31

[a]IHC: immunohistochemistry; [b]ISH: in situ hybridization

[c]Clinical signs: Resp=respiratory disease, wasting=anorexia and weight loss

[d]Histopathological lesions: Pneumonia=interstitial pneumonia

[e]PCV-2 isolates sequenced in this study; [f]Bovine isolate

Table 2. Oligonucleotide primers used in the study

ID	Primer Sequence	Application	Position[b]
CV 1	**5'-AGGGCTGTGGCCTTTGTTAC-3'**	**PCR, Seq[a]**	**1336-1356**
CV 2	**5'-TCTTCCAATCACGCTTCTGC-3'**	**PCR, Seq**	**536-556**
CV 3	**5'-TGGTGACCGTTGCAGAGCAG-3'**	**PCR, Seq**	**453-475**
CV 4	**5'-TGGGCGGTGGACATGATGAG-3'**	**PCR, Seq**	**1523-1544**
CV 1-1	**5'-GAGGATCTGGCCAAGATGGCTG-3'**	**Seq**	**1674-1695**
CV 1-2	**5'-AGGACGAACACCTCACCTCCAG-3'**	**Seq**	**213-234**
CV 2-1	**5'-GCAGCGGGCACCCAAATACCAC-3'**	**Seq**	**279-300**
CV 2-2	**5'-ACGTATCCAAGGAGGCGTTACC-3'**	**Seq**	**1718-1739**
CV 3-1	**5'-AGACTAAAGGTGGAACTGTACC-3'**	**Seq**	**770-791**
CV 3-2	**5'-TTGTACATACATGGTTACACGG-3'**	**Seq**	**1083-1104**
CV 4-1	**5'-TGTGGACCACGTAGGCCTCGGC-3'**	**Seq**	**1146-1167**
CV 4-2	**5'-TGGTAATCAGAATACTGCGGGC-3'**	**Seq**	**799-820**
MCV 1	**5'-GCTGAACTTTTGAAAGTGAGCGGG-3'**	**PCR-RFLP**	**508-517**
MCV 2	**5'-TCACACAGTCTCAGTAGATCATCCCA-3'**	**PCR-RFLP**	**725-750**

[a]Seq: primers used for DNA sequencing to determine the complete genome of PCV-2

[b]The relative positions of these oligonucleotide primers are indicated in Figure

Table 3. Pairwise comparison of the complete genomic and putative capsid gene (ORF2) sequences of porcine circovirus type-1 and type-2

	26606	10489	26607	40895	34464	40856	AF085695	AF086834	AF086835	AF086836	AF109398	AF109399	AF112862	AF117753	AF027217	AF055391	AF055392	AF055393	AF055394	AF109397	AF166528	AJ223185	**AF012107**	**AF071879**	**U49186**	**YO9921**
26606		98/96	99/99	98/95	96/95	99/98	98/97	98/96	98/97	98/97	93/93	94/93	97/95	93/93	98/95	98/95	98/96	92/92	92/92	97/95	95/95	98/96	66/66	65/64	65/65	65/65
10489	99[a]		98/97[b]	99/98	97/97	98/96	98/98	99/100	99/98	98/98	94/94	95/95	98/98	93/93	99/98	99/98	98/98	92/93	92/93	99/98	96/98	99/99	67/68	66/66	66/66	66/66
26607	99	99		98/96	96/95	99/98	98/97	98/97	99/97	98/97	93/93	94/93	97/95	93/93	98/96	98/96	98/97	92/92	92/92	98/96	95/95	98/96	66/66	65/64	66/65	66/65
40895	99	99	99		97/97	98/95	98/96	99/98	98/97	98/96	94/95	95/95	98/96	93/93	99/99	99/99	98/96	92/93	92/93	99/98	96/97	99/99	66/67	66/65	66/66	66/66
34464	98	98	98	98		96/95	96/95	97/97	97/96	96/95	94/93	97/97	96/95	93/92	97/96	97/96	96/95	92/91	92/91	97/96	96/96	97/97	66/67	65/65	65/66	65/66
40856	99	99	99	98	98		98/97	98/96	98/97	98/97	93/93	94/93	97/95	92/92	98/95	98/95	98/96	92/92	92/92	97/95	95/95	98/96	66/66	65/63	65/64	65/64
AF085695	98	98	98	98	97	98		99/98	99/99	100/100	94/94	94/93	97/96	93/93	98/96	98/96	99/99	92/93	92/93	98/96	96/96	98/97	66/67	66/65	66/66	66/66
AF086834	98	98	98	98	97	98	99		99/98	99/98	94/94	95/95	98/98	93/93	99/98	99/98	99/98	92/93	93/93	99/98	96/98	99/99	67/68	66/66	66/66	66/66
AF086835	98	98	98	98	97	98	99	99		99/99	94/95	94/93	98/96	93/93	98/97	98/97	99/99	93/93	93/93	98/97	96/97	98/98	67/67	66/65	66/66	66/66
AF086836	98	98	98	98	97	98	99	98	99		94/94	94/93	97/96	93/93	98/96	98/96	99/99	92/93	92/93	98/96	96/96	98/97	66/67	66/65	66/66	66/66
AF109398	96	96	96	96	96	96	95	95	95	95		93/92	94/94	97/96	94/95	94/95	94/94	93/93	93/93	95/96	93/94	94/95	67/67	66/65	66/66	66/66
AF109399	97	97	97	97	98	97	96	96	96	96	96		94/93	93/91	95/94	95/94	94/93	91/90	91/90	95/94	94/94	95/95	66/65	65/63	65/63	65/63
AF112862	98	99	98	99	98	98	98	98	98	97	96	97		93/92	98/96	98/96	98/96	92/92	92/92	98/97	95/96	98/97	67/67	66/65	66/66	66/66
AF117753	96	96	96	96	96	95	95	95	95	95	97	96	96		93/93	93/93	93/93	91/91	92/91	93/93	93/93	93/93	66/66	65/64	65/65	65/65
AF027217	99	99	99	99	98	99	98	98	98	98	96	97	99	96		99/99	98/96	93/93	93/93	99/98	96/97	99/99	66/67	66/65	66/66	66/66
AF055391	99	99	99	99	98	99	98	98	98	98	96	97	99	96	99		98/96	93/93	93/93	99/98	96/97	99/99	66/67	66/65	66/66	66/66
AF055392	99	99	99	99	98	99	99	98	98	98	96	97	99	96	99	99		93/93	93/93	98/96	96/96	98/97	66/67	66/65	66/66	66/66
AF055393	95	95	95	95	95	95	95	95	95	95	95	95	95	95	96	96	96		99/99	92/93	93/93	92/93	67/68	66/66	66/66	66/66
AF055394	95	96	95	96	95	95	95	95	95	95	95	95	95	95	96	96	96	99		92/92	93/93	92/93	67/68	66/66	66/66	66/66
AF109397	99	99	99	99	98	99	98	98	98	98	97	97	99	96	99	99	99	95	95		96/97	99/99	67/68	66/66	66/66	66/66
AF166528	97	97	97	97	97	97	96	97	96	97	96	96	97	95	97	97	97	96	96	97		96/98	66/67	65/65	65/66	65/66
AJ223185	99	99	99	99	98	98	98	98	98	98	96	97	99	96	99	99	99	95	95	99	97		66/68	66/66	66/66	66/66
AF012107	76	77	76	76	76	76	76	76	76	76	76	76	76	76	76	76	76	77	77	77	76	76		97/95	97/96	97/94
AF071879	76	76	76	76	76	76	76	75	76	76	76	76	76	75	76	76	76	76	76	76	76	76	98		99/98	98/95
U49186	76	76	76	76	76	76	76	76	76	76	76	76	76	75	76	76	76	76	77	77	76	76	98	99		98/96
YO9921	76	76	76	76	76	76	76	76	76	76	76	76	76	75	76	76	76	76	76	76	76	76	98	99	99	

[a]The values in the table are percentage identity of amino acid or nucleotide sequences. The nucleotide sequence comparisons of the complete genomes are presented in the lower left half. The four PCV-1 isolates (AF012107, AF071879, U49186, YO9921) are highlighted with bold face.

[b]The putative capsid (ORF2) gene is shown at the upper right: nucleotide/amino acid.

Chapter 3

The Cloned Genomic DNA of the Type-2 Porcine Circovirus Is Infectious When Injected Directly Into the Liver and Lymph Nodes of Pigs: Characterization of Clinical Disease, Virus Distribution and Pathologic Lesions

Fenaux M, P. G. Halbur, G. Haqshenas, R. Royer, P. Thomas, P. Nawagitgul, M. Gill, T. E. Toth, and X. J. Meng. *2002. J. Virol. 76: 541-551.*

ABSTRACT

Infection of animals with a molecular viral clone is critical to study the genetic determinants of viral replication and virulence in the host. Type-2 porcine circovirus (PCV2) has been incriminated as the cause of postweaning multisystemic wasting syndrome (PMWS), an emerging disease in pigs. We report here for the first time the construction and use of an infectious molecular DNA clone of PCV2 to characterize the disease and pathological lesions associated with PCV2 infection by direct in vivo transfection of pigs with the molecular clone. The PCV2 molecular clone was generated by ligating two copies of the complete PCV2 genome in tandem into the pSK vector, and was shown to be infectious in vitro when transfected into PK-15 cells. Forty specific-pathogen-free pigs at four weeks of age were randomly assigned to four groups of 10 each. Group 1 pigs served as uninoculated controls. Pigs in group 2 were each inoculated intranasally with about 1.9 x 10^5 $TCID_{50}$ of a homogeneous PCV2 live virus stock derived from the molecular clone. Pigs in group 3 were each injected

intrahepatically with 200 µg of the cloned PCV2 plasmid DNA, and pigs in group 4 were each injected into the superficial iliac lymph nodes with 200 µg of the cloned PCV2 plasmid DNA. Animals injected with the cloned PCV2 plasmid DNA developed infection resembling that induced by intranasal inoculation with PCV2 live virus stock. Seroconversion to PCV2-specific antibody was detected in the majority of pigs from the three inoculated groups at 35 days postinoculation (DPI). Viremia, beginning at 14 DPI and lasting 2 to 4 weeks, was detected in the majority of the pigs from all three inoculated groups. There were no remarkable clinical signs of PMWS in control or any of the inoculated pigs. Gross lesions in pigs of the 3 inoculated groups were similar and characterized by systematically enlarged tan-colored lymph nodes and lungs that failed to collapse. Histopathological lesions and PCV2-specific antigen were detected in numerous tissues and organs including brain, lung, heart, kidney, tonsil, lymph nodes, spleen, ileum and liver of infected pigs. This study more definitively characterizes the clinical course and pathological lesions exclusively attributable to PCV2 infection. The data from this study indicate that the cloned PCV2 genomic DNA may replace infectious virus for future PCV2 pathogenesis and immunization studies. The data also suggest that PCV-2, although essential for development of PMWS, may require other factors or agents to induce the full spectrum of clinical signs and lesions associated with advanced cases of PMWS.

INTRODUCTION

Porcine circovirus (PCV) was originally isolated as a cell culture contaminant of a porcine kidney cell line (PK-15) (56, 60). PCV is a small non-enveloped virus that contains a single stranded circular DNA genome of about 1.76 kb. PCV is classified in the family of *Circoviridae*, which consists of three other animal circoviruses: chicken anemia virus (CAV), psittacine beak and feather disease virus (PBFDV) and the recently discovered columbid circovirus (CoCV) from pigeons, and three plant circoviruses (banana bunchy top virus, coconut foliar decay virus, and subterranean clover stunt virus) (10, 35, 37, 38, 39, 61). Members of the three previously recognized animal circoviruses (PCV, CAV, and PBFDV) do not share nucleotide sequence homology or antigenic determinants with each other (10, 61). The genome of the newly identified CoCV shared about 40% nucleotide sequence identity with that of PCV (37). Recently, a novel human virus with a circular genome, designated as TT virus (TTV), was identified from individuals associated with post-transfusion hepatitis (40, 45) and a human TTV-like mini virus (TLMV) was also identified from normal blood donors (12, 55). The genomic organization of both human TTV and TLMV is similar to that of the CAV (12, 40, 55). Although antibodies to PCV were found in various animal species including humans, mice, cattle and pigs (15, 16, 25, 26, 44, 58, 59), little is known regarding the pathogenesis of PCV in these animal species. Experimental infection of pigs with the PK15 cells-derived PCV did not produce clinical disease and thus, this virus is not considered to be pathogenic to pigs (2, 57). The nonpathogenic PCV derived from the contaminated PK-15 cell line was designated PCV1.

Postweaning multisystemic wasting syndrome (PMWS) is an emerging disease in pigs first described in 1991 (25). PMWS primarily affects pigs between 5-18 weeks of age. Clinical PMWS signs include progressive weight loss, dyspnea, tachypnea, anemia, diarrhea, and jaundice. Mortality rate may vary from 1-2% up to 30% in complicated cases. Microscopic lesions characteristic of PMWS include granulomatous interstitial pneumonia, lymphadenopathy, hepatitis, and nephritis (9, 25). PMWS has now been recognized in pigs in Canada, the United States (3, 5, 9, 17, 24, 30, 33, 39, 41), many European countries (5, 9, 16, 28, 36, 48, 53, 62) and some countries in Asia (13, 46), and potentially has serious economic impact on the swine industry worldwide.

The causative agent of PMWS is believed to be a pathogenic strain of PCV designated as PCV2 (3, 5, 7, 9, 17, 24, 39, 41). The complete genomic sequence of the PMWS-associated PCV2 has been determined (20, 24, 35, 38, 39, 41). Sequence analyses revealed that the PMWS-associated PCV2 shared only about 75% nucleotide sequence identity with the non-pathogenic PCV1. Experimental reproduction of clinical PMWS in gnotobiotic pigs and conventional pigs with tissue homogenates from pigs with naturally occurring PMWS and with cell culture propagated PCV-2 produced mixed results. Clinical PMWS was reproduced in gnotobiotic pigs and colostrum-deprived and caesarian-derived pigs co-infected with PCV2 and porcine parvovirus (PPV) (8, 32), and in PCV-2 inoculated gnotobiotic pigs when their immune system was activated by keyhole hemocyanin in incomplete Freund's adjuvant (31). However, clinical PMWS was not reproduced in gnotobiotic pigs infected with PCV2 alone (4, 6, 8, 11, 18, 29, 31, 32, 47). The virus inocula used in these studies

were either homogenates of tissues from pigs with naturally occurring PMWS, or virus propagated in PK-15 cell cultures (4, 6, 8, 11, 18, 29, 31, 32, 47). Since tissue homogenates may contain other common swine agents such as PPV and porcine reproductive and respiratory syndrome virus (PRRSV) (4, 8, 9, 19, 48), and since the ATCC PK-15 cell line used for PCV2 propagation was persistently infected with PCV1 (15), the clinical disease and pathological lesions reproduced in those studies may not be solely attributable to PCV2 infection (4, 6, 8, 9, 19). Therefore, it will be advantageous to construct an infectious clone of PCV2 so that a biologically pure and homogeneous infectious virus stock can be generated for pathogenesis studies.

We report here for the first time that a molecular DNA clone of PCV2 is infectious when injected directly into the liver and lymph nodes of pigs. The course of clinical disease, virus distribution and pathological lesions associated with PCV2 infection were more definitively characterized by using this molecular DNA clone and a biologically pure and homogeneous infectious PCV2 virus stock derived from the molecular DNA clone.

MATERIALS AND METHODS

Source of PCV2. The PCV2 isolate used in this study was from a spleen tissue sample of a pig with naturally occurring PMWS (PCV-2 # 40895) (20). Immunohistochemical staining (IHC) with PCV2 specific antibody confirmed the presence of PCV2 antigen in the tissue (data not shown). The spleen tissues were stored at -80ºC until use.

Generation of a PK-15 cell line free of PCV1 contamination by end-point dilution. The PK-15 cell line purchased from the ATCC was persistently infected with PCV1 (15). Since only a subpopulation of PK-15 cells was persistently infected (15), we therefore attempted to generate a PK-15 cell line that is free of PCV1 contamination by end-point dilution. PK-15 cells were grown in MEM with Earle's salts and L-glutamine (Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) and 1X antibiotic (Life Technologies, Inc.). Confluent cell monolayers were trypsinized, and the cells were then counted and serially diluted to an end point with one cell per 0.2 ml. The end point dilution was plated in 96-well plates and allowed to grow into a monolayer starting from a single cell. Cells from each well were tested for PCV1 DNA using a PCR-RFLP assay capable of detecting and differentiating PCV1 and PCV2 (20). PK-15 cells from wells that were tested negative for PCV1 by the PCR-RFLP assay were subsequently expanded. The PCV-1 free PK-15 cell line used in this study was sub-cultured five additional passages and was found negative for PCV1 DNA by PCR at each passage.

Construction of a PCV2 molecular DNA clone. A pair of PCR primers was designed according to the published sequence of the PCV2 isolate 40895 (20): forward primer F-PCVSAC2 (5'-GAACCGCGGGCTGGCTGAACTTTTGAAAGT-3') and reverse primer R-PCVSAC2 (5'-GCACCGCGGAAATTTCTGACAAACGTTACA-3'). This pair of primers amplifies the complete genome of PCV2 with an overlapping region containing the unique Sac II restriction enzyme site (Fig. 1). Briefly, DNA was extracted using the QIAamp DNA Minikit (Qiagen, Inc., Valencia, CA) from a spleen tissue sample of a pig

with naturally occurring PMWS (isolate 40895) (20). The extracted DNA was amplified by PCR with AmpliTaq Gold polymerase (Perkin-Elmer, Norwalk, CT). The PCR reaction consisted of an initial enzyme activation step at 95ºC for 9 min, followed by 35 cycles of denaturation at 94ºC for 1 min, annealing at 48ºC for 1 min, extension at 72ºC for 3 min, and a final extension at 72ºC for 7 min. The PCR product of expected size was separated by gel electrophoresis and purified with the glassmilk procedure with a Geneclean Kit (Bio 101, Inc., La Jolla, CA).

To construct a molecular DNA clone containing a tandem dimmer of PCV2 genome, the PCR product containing the complete PCV2 genome was first ligated into the advanTAge plasmid vector (Clontech, Palo Alto, CA). *E. coli* DH5α competent cells were transformed. The recombinant plasmids were verified by restriction enzyme digestion. The full length PCV-2 genomic DNA was excised from the advanTAge vector by digestion with *Sac*II restriction enzyme. The digested PCV2 genomic DNA was ligated with T4 DNA ligase at 37°C for only 10 min, which favors the production of tandem dimmers. The tandem dimmers were subsequently cloned into pBluescript SK vector (pSK) (Stratagene, La Jolla, CA) (Fig. 1). Recombinant plasmids containing tandem dimmers of PCV2 genome (referred to as PCV2 molecular DNA clone) were confirmed by PCR, restriction enzyme digestion, and DNA sequencing. The DNA concentration of the recombinant plasmids was determined spectrophotometricaly.

In vitro transfection with the PCV2 molecular DNA clone and generation of a biologically pure and homogenous PCV2 infectious virus stock. To test the infectivity of the molecular DNA clone in vitro, PK-15 cells free of PCV1 contamination were

grown in 8-well LabTek chamber slides. When the PK-15 cells reached about 85% confluency, cells were transfected with the molecular DNA clone using Lipofectamine Plus Reagents according to the protocol supplied by the manufacturer (Life Technologies, Inc). Mock-transfected cells with empty pSK vector were included as controls. Three days after transfection, the cells were fixed with a solution containing 80% acetone and 20% methanol at 4º C for 20 min., and an immunofluorescence assay using a PCV2-specific rabbit polyclonal antisera was performed to determine the in vitro infectivity of the molecular DNA clone (see below).

To generate a biologically pure and homogeneous PCV2 infectious virus stock for the animal inoculation experiment, PK-15 cells free of PCV1 contamination were cultivated in T-25 culture flasks and transfected with the PCV2 molecular DNA clone. Briefly, PK-15 cells were grown to about 85% confluency in T-25 flasks. The cells were washed once with sterile PBS buffer before transfection. For each transfection reaction in a T-25 flask, 12 µg of the PCV2 plasmid DNA was mixed with 16 µl of Plus Reagent in 0.35 ml of MEM media. A flask of mock-transfected cells with empty pSK vector was included as the negative control. After incubation at room temperature for 15 min., 50 µl of Lipofectamine Reagent diluted in 0.35 ml of MEM media was added to the mixture and incubated at room temperature for another 15 min. The transfection mixture was then added to a T-25 flask of PK-15 cells containing 2.5 ml of fresh MEM. After incubation at 37º C for 3 hrs, the media was replaced with fresh MEM media containing 2 % FBS and 1 X antibiotics. The transfected cells were harvested at 3 days post transfection and stored at -80° C until use. The infectious titer of the virus stock was determined by IFA (see below).

Virus titration by immunofluorescence assay (IFA). To determine the infectious titer of the homogenous PCV2 virus stock, PK-15 cells were cultivated on 8-well LabTek chamber slides. The virus stock was serially diluted 10-fold in MEM, and each dilution was inoculated onto 10 wells of the monolayers of the PK-15 cells growing on the LabTek chamber slides. Wells of non-inoculated cells were included as controls. The infected cells were fixed at 3 days post inoculation with a solution containing 80% acetone and 20% methanol at 4°C for 20 min. After washing the cells with PBS buffer, the infected cells were incubated with a 1:1,000 diluted PCV2-specific rabbit polyclonal antibody (50) at 37°C for 1 hr. The cells were then washed three times with PBS buffer, and incubated with a secondary FITC-labeled goat anti-rabbit Ig G (Kirkegaard & Perry Laboratories Inc, Gaithersburg, MD) at 37°C for 45 min. After washing the slides three times with PBS buffer, and the slides were mounted with fluoromount-G, coversliped and examined under a fluorescence microscope. The 50% tissue culture infectious dose per ml ($TCID_{50}$/ml) was calculated. Initially, we transfected cells with a plasmid construct containing a single copy of PCV2 genome but the infectious PCV2 titer from the single genome construct is much lower than the one containing the tandem genome (data not shown). Therefore, the plasmid construct containing the dimeric form of PCV2 genome was used for the in vitro and in vivo transfection experiments in this study.

In vivo transfection of pigs with the PCV2 molecular DNA clone and experimental inoculation of pigs with the homogeneous PCV2 infectious virus stock. Forty specific-pathogen-free (SPF) swine of 4 weeks of age were randomly assigned into 4

rooms of 10 animals each. Prior to inoculation, the SPF pigs were tested for antibodies to PCV, PRRSV, PPV and swine hepatitis E virus. Pigs in group 1 were uninoculated and served as negative controls. Pigs in group 2 were each inoculated intranasally with about 1.9×10^5 $TCID_{50}$ of the PCV2 infectious virus stock derived from the PCV2 molecular DNA clone. Pigs in group 3 received direct intrahepatic injection of the recombinant plasmid DNA of the PCV2 molecular clone. Each pig was injected with a total of 200 μg of recombinant plasmid DNA, through an ultrasound-guided technique, into 6 different sites of the liver. Pigs in group 4 were each injected with a total of 200 μg of the recombinant PCV2 plasmid DNA into the superficial iliac lymph nodes, and each lymph node received two separate injections. The animals were monitored daily for clinical signs of disease. Serum samples were collected from each animal at 0, 7, 14, 21, 28, 35 day post inoculation (DPI). At 21 DPI, five pigs were randomly selected from each group and necropsied. The remaining five animals in each group were necropsied at 35 DPI. Various tissues and organs were collected during necropsy and processed for histological examination and immunohistochemical staining (see below).

Clinical evaluation. Pigs were weighed on 0 DPI and at the time of necropsy. Rectal temperatures and clinical respiratory disease scores, ranging from 0 to 6 (0 = normal, 6 = severe) (23), were recorded every other day from 0 to 35 DPI. Clinical observations including evidence of central nervous system disease, liver disease (icterus), musculoskeletal disease, and changes in body condition, were also recorded daily. Clinical evaluation was performed by a team of 2 people.

Gross pathology and histopathology. Five pigs from each group were randomly selected for necropsies at 21 and 35 DPI. The necropsy team was blinded to infection status of the pigs at necropsy. Complete necropsies were performed on all pigs. An estimated percentage of the lung with grossly visible pneumonia was recorded for each pig based on a previously described scoring system (23). The scoring system is based on the approximate volume that each lung lobe contributes to the entire lung: the right cranial lobe, right middle lobe, cranial part of the left cranial lobe, and the caudal part of the left cranial lobe each contribute 10% of the total lung volume, the accessory lobe contributes 5%, and the right and left caudal lobes each contribute 27.5%. Other lesions such as enlargement of lymph nodes were noted separately. Sections for histopathologic examination were taken from nasal turbinate, lungs (seven sections) (23), heart, brain, lymph nodes (tracheobronchial, iliac, mesenteric, subinguinal), tonsil, thymus, liver, gall bladder, spleen, joints, small intestine, colon, pancreas, and kidney. The tissues were examined in a blinded fashion and given a subjective score for severity of lung, lymph node, and liver lesions. Lung scores ranged from 0 (normal) to 3 (severe lymphohistiocytic interstitial pneumonia). Liver scores ranged from 0 (normal) to 3 (severe lymphohistiocytic hepatitis). Lymph node scores were for an estimated amount of lymphoid depletion of follicles ranging from 0 (normal or no lymphoid depletion) to 3 (severe lymphoid depletion and histiocytic replacement of follicles).

Serology. Blood was collected on arrival at 11 to 12 days of age, and from all pigs at 0, 7, 14, 21, 28, and 35 DPIs. Serum antibodies to PRRSV were assayed using Herd Check

PRRSV ELISA (IDEXX Laboratories, Westbrook, MA). Serum antibodies to PPV were detected by a hemagglutination inhibition (HI) assay (27). Serum antibodies to PCV2 were detected by a modified indirect ELISA based on the recombinant ORF2 protein of PCV2 (42). Briefly, a partially purified PCV2 antigen was prepared from Hi Five cells (Invitrogen, Carlsbad, CA) infected with recombinant baculovirus containing the major capsid ORF2 protein of PCV2 (43). Cell lysates of Hi Five cells infected with wild-type baculovirus were prepared similarly and served as negative control antigen. The Immulon 2 HB polystyrene microtiter plates (Dynex Technologies Inc, Chantilly, VA) were coated with optimal concentrations of positive and negative antigens at 4°C for 36 hrs. One hundred μl of each serum sample diluted 1:100 in 5% milk diluent (Kirkegaard & Perry Laboratories, Inc) was added into each well. The serum samples were tested in quadruplicate: 2 wells for negative control antigen and 2 parallel wells for PCV2 antigen. Positive control and negative control sera were included in each plate. The sera were incubated at 37°C for 30 min. and then washed 5 times with 0.1 M PBS buffer containing 0.1% Tween-20. A peroxidase-labeled secondary anti-swine IgG (Sigma Co, St. Louis, MO) was incubated at 37°C for 30 min. The plates were washed again and incubated with 2,2'-azino-di-(3-ethylbenzthiazoline-6-sulfonate) (Kirkegaard & Perry Laboratories Inc) at 37°C for 15 min. for color development. The optical density (OD) was read at 405 nm. The corrected OD of each tested and control sera was calculated by subtraction of mean OD value of the wells containing negative antigen from that of the parallel wells containing PCV2 antigen. The data was normalized by dividing the corrected OD value of a tested serum sample (S) with that of the positive control serum (P) and reported as

S/P ratios. The samples with S/P ratios ≤0.12, 0.12 to 0.2, and >0.2 were considered as negative, equivocal and positive, respectively.

PCR-RFLP analyses. To measure PCV2 viremia in pigs transfected with PCV2 molecular DNA clone and in pigs infected with PCV2 infectious virus stock, serum samples collected at different DPIs were tested for the presence of PCV2 DNA by a previously described PCR-RFLP assay (20). Viral DNA was extracted from 50 µl of each serum sample using the DNAzol® reagent according to the protocol supplied by the manufacturer (Molecular Research Center, Cincinnati, OH). The extracted DNA was resuspended in DNase-, RNase-, and proteinase-free water and tested for PCV2 DNA by PCR-RFLP (20). PCR products from selected animals were sequenced to verify the origin of the virus infecting pigs.

Immunohistochemistry (IHC). IHC detection of PCV2-specific antigen was performed on all tissues collected during necropsies at DPIs 21 and 35. A rabbit polyclonal PCV2-specific antiserum was used for the IHC, and the procedures have been previously described (50).

RESULTS

Generation of a PK-15 cell line free of PCV1 contamination. Four cell lines that are negative for PCV1 contamination were produced by the end-point dilution of the persistently infected PK-15 cells from ATCC. The cell lines remained negative for PCV1 by PCR after 5 additional passages. One of the cell lines was subsequently expanded and

was shown to be able to support PCV2 replication when the cells were transfected with the PCV2 molecular DNA clone (Fig. 2) and infected with PCV2 virus (data not shown). The cloned cell s were further used for the in vitro transfection of PCV2 molecular DNA clone to generate a biologically pure PCV2 infectious virus stock for the animal inoculation experiment.

Construction of an infectious PCV2 molecular DNA clone. The complete genome of the PCV2 (isolate 40895) was amplified by PCR. Two copies of the complete PCV2 genome were ligated in tandem into the pSK vector to produce the PCV2 molecular DNA clone (Fig. 1). The infectivity of the PCV2 molecular DNA clone was determined by in vitro transfection of the PK-15 cells. IFA with PCV2 specific antibody confirmed that the molecular DNA clone is infectious in vitro and that about 10-15% of the PK-15 cells were transfected. PCV2-specific antigen was visualized by IFA in the nucleus, and to a lesser degree cytoplasm of the transfected cells (Fig. 2). The cells mock-transfected with the empty pSK vector remained negative for PCV-2 antigen.

Generation of a biologically pure and homogenous PCV2 infectious virus stock. The lack of a biologically pure form of PCV2 infectious virus stock has impeded the understanding of PCV2 pathogenesis and the etiological role of PCV2 in PMWS. In this study, we generated a biologically pure PCV2 infectious virus stock by transfection of PK-15 cells with the PCV2 molecular DNA clone. PCV2 virions produced by in vitro transfection were infectious, as the transfected cell lysates were successfully used to infect PK-15 cells. Thus, the PCV2 molecular DNA clone is capable of producing

infectious PCV2 virions when transfected in vitro. The infectious titer of the homogenous PCV2 virus stock prepared from transfected cells was determined to be 1 x $10^{4.5}$ $TCID_{50}$/ml, and this virus stock was used to inoculate pigs in group 2. Lysates of cells mock-transfected with the empty pSK vector were unable to infect PK-15 cells.

The PCV2 molecular DNA clone is infectious when injected directly into the liver and superficial iliac lymph nodes of SPF pigs. Serum samples were collected from all control and inoculated animals at 0, 7, 14, 21, 28, and 35 DPIs and assayed for PCV2 viremia by detection of PCV2 DNA (20). PCV2 DNA was not detected in the group 1 uninoculated control pigs at any DPI (Table 1). Viremia was detected in 7/10 pigs from group 2 at 14 DPI and 8/10 by 35 DPI (Table 1). Viremia lasted only a few weeks as the PCV2 DNA was not detectable at 28 DPI and 35 DPI in all 5 remaining pigs from group 2. In group 3 pigs that were intrahepatically injected with PCV2 molecular DNA clone, 8/10 pigs were viremic at 14 DPI, and 9/10 pigs had had detectable viremia by 35 DPI (Table 1). Group 4 pigs were injected with PCV2 molecular DNA clone into the lymph nodes. Two of 10 pigs at 14 DPI and 8 of 10 pigs at 21 DPI from group 4 were viremic (Table 1). PCR products amplified from selected animals were sequenced. The sequence of the PCR products amplified from selected animals was identical to the corresponding region of the PCV2 molecular DNA clone (data not shown).

All inoculated pigs from groups 2, 3 and 4 were negative for PCV2 antibodies at 0 DPI. Two pigs in the uninoculated control group 1 had detectable PCV2 maternal antibody at 0 DPI. The maternal antibody in these two piglets waned by 7 DPI (Table 2). No seroconversion to PCV2 antibody was detected in any of the 10 uninoculated control

pigs. In group 2 pigs intranasally inoculated with PCV2 infectious virus, 1 piglet seroconverted to PCV2 antibody at 21 DPI. By 35 DPI, 4 of the 5 remaining group 2 pigs had seroconverted (Table 2). Seroconversion in transfected animals from groups 3 and 4 first appeared at 28 DPI. By 35 DPI, 5 of 5 remaining pigs from group 3 and 3 of 5 remaining pigs from group 4 had seroconverted to PCV2 antibody (Table 2).
PPV antibodies were tested at 3 and 21 DPI for all pigs, and at 35 DPI for the remaining pigs. As expected, maternal antibodies to the ubiquitous swine agent PPV were detected in the SPF piglets. The PPV HI antibody titers in all piglets but one decreased significantly from 3 DPI (an average titer of 1:2,665) to 21 DPI (an average titer of 1:246), indicating the antibody detected in these piglets was passively derived. One piglet had a slightly increased PPV HI titer from 1:32 at 3 DPI to 1:64 at 21 DPI, which is likely due to testing variation. Serum samples collected from all pigs at 0, 21, and 35 DPI were further tested for PPV DNA with a published PCR assay (51). No PPV viremia was detected from any pigs at any DPI, further indicating the pigs were not infected by PPV.

Clinical evaluation. None of the control and inoculated pigs showed obvious signs of disease resembling those of clinical PMWS. There was no difference in weight gain or mean rectal temperatures between any of the four groups (data not shown). The group 1 control pigs remained normal throughout the study. There was mild transient respiratory disease observed in the majority of the pigs in PCV2 DNA-transfected and PCV2 virus-infected groups from 8 to 14 DPI. This was characterized by mild dyspnea (clinical respiratory scores of 1 to 2) of one-to-two days duration in individual pigs and 5-6 days duration for the group.

Gross lesions. There were no gross lesions observed in the control pigs at necropsy. Pigs in the three inoculated groups had gross lesions limited to the lungs and lymph nodes (Table 3). The lesions were similar among pigs in the PCV2 plasmid DNA-transfected and PCV2 virus-infected groups. Lungs failed to collapse and had random, multifocal, moderately well-demarcated areas of tan-to-purple consolidation involving 0-2% of the lung (Fig. 3) at 21 DPI, and 0-13% of the lung at 35 DPI. Lymph nodes were systemically enlarged 2 to 5 times normal size, firm, and tan (Fig. 3) at both 21 and 35 DPI in most of the pigs from all three PCV2-inoculated groups.

Microscopic lesions. Microscopic examination revealed no lesions in any tissues of the control pigs except for the livers. Eight of ten control pigs had very mild multifocal lymphoplasmacytic inflammation predominately in the periportal regions of the liver as is commonly observed in normal pigs and considered normal background (22).

Pigs from the two PCV2 plasmid DNA-transfected groups (intrahepatic and intralymphoid) and the PCV2 virus-infected group (intranasal) had similar lesions in brain, lung, heart, kidney, lymphoid tissues (tonsil, lymph nodes, spleen), ileum, and liver (Table 4). Brain lesions were observed in 23/30 of the pigs from the 3 inoculated groups and characterized as mild-to-moderate multifocal lymphoplasmacytic meningoencephalitis with perivascular cuffing and gliosis. Lung lesions were observed in 28/30 PCV2-inoculated pigs and characterized as mild-to-moderate peribronchiolar lymphoplasmacytic and histiocytic bronchointerstitial pneumonia (Fig. 3C). One pig from the PCV2 virus-infected group 2 necropsied at 21 DPI, and one pig each from the

two PCV2 plasmid DNA-transfected groups necropsied at 35 DPI had ulcerative and proliferative bronchiolitis with fibroplasia and granulomatous inflammation in the lamina propria and peribronchiolar regions of bronchi. Mild multifocal lymphoplasmacytic myocarditis was also observed in 18/30 PCV2-inoculated pigs. Mild-to-moderate multifocal lymphoplasmacytic interstitial nephritis was observed in 14/30 of the PCV2-inoculated pigs. No lesions were observed in the thymuses. Mild-to-moderate lymphoid depletion (Fig. 4B) and histiocytic replacement of follicles was observed in the tonsil of 8/30, in the spleen of 7/30, and in the lymph nodes of 26/30 of the PCV2-inoculated pigs. Moderate granulomatous lymphadenitis with giant cells (Fig. 4C) was observed at 21 DPI in three pigs inoculated intranasally with PCV2 virus, and in one pig at 35 DPI in each of the PCV2 plasmid DNA-transfected groups. Mild lymphoplasmacytic and histiocytic enterocolitis was observed in 3/5 pigs in the PCV2 virus-infected group, in 3/5 pigs in the PCV2 plasmid DNA intrahepatically-transfected group, and 1/5 pigs in the PCV2 plasmid DNA intralymphoid-transfected group at 35 DPI. One pig in each of the PCV2 plasmid DNA-transfected groups had mild lymphoid depletion with histiocytic replacement and low numbers of giant cells in the Peyer's patches. Mild-to-moderate lymphoplasmacytic hepatitis was observed in 29/30 of the three PCV2-inoculated pigs. Low numbers of widely scattered individually necrotic hepatocytes surrounded by lymphohistiocytic inflammation was observed in one pig in each of the PCV2 plasmid DNA-transfected groups at 21 DPI. Lesions in other tissues were unremarkable.

Microscopic lesions in the lung, liver and lymph nodes were scored according to published scoring systems (Table 4) (22-23). There were no acceptable scoring systems for other tissues and organs. The average scores of lesions in lung and lymph nodes in

pigs of the three PCV2-inoculated groups were statistically different from those in group 1 control pigs. The average scores of the liver lesions in pigs of the three PCV2-inoculated groups are not statistically different from those of control pigs.

Detection and tissue distribution of PCV2 antigen. IHC staining of PCV2 antigen was done on brain, lungs, turbinate, heart, kidneys, tonsil, lymph nodes, spleen, thymus, ileum, liver, gall bladder, and pancreas of all pigs necropsied at 21 and 35 DPI. All tissues from the control pigs were negative for PCV2 antigen. Tissue distribution of PCV2 antigen in the three PCV2-inoculated groups was similar (Table 5). In the brain, the PCV2 antigen was found predominately in mononuclear cells, fibroblast-like cells, and endothelial cells in the meninges and choroid plexus and less often in endothelial cells and perivascular mononuclear cells in the cerebrum and cerebellum. In the lungs, PCV2 antigen was detected within alveolar and septal macrophages and in fibroblast-like cells in the lamina propria of airways (Fig. 3D). In the heart, PCV2 antigen was detected in widely scattered macrophages and endothelial cells. In kidneys, PCV2 antigen was detected within tubular epithelial cells and mononuclear cells in the interstitium. In the lymphoid tissues (lymph nodes, spleen, tonsil, and Peyer's patches), PCV2 antigen was detected primarily within macrophages and dendritic-like cells and giant cells within follicles (Fig. 4D). PCV2 antigen was also detected within macrophages in the lamina propria of the small intestine. In the liver, PCV2 antigen was detected within mononuclear cells and Kupffer cells. PCV2 antigen was not detected in turbinate, thymus, or gall bladder.

DISCUSSION

PMWS is a complex disease syndrome in swine and multiple factors may be involved in the clinical presentation of PMWS. Increasing data indicate that PCV2 is the causative agent of PMWS (3, 5, 7, 9, 17, 24, 39, 41). However, the difficulty in producing a biologically pure form of PCV2 due to the presence of other common swine agents in the tissue homogenates of diseased pigs has impeded a definitive characterization of the clinical disease and pathological lesions solely attributable to PCV2 infection.

We report here for the first time that the cloned PCV2 genomic DNA is infectious when directly injected into the livers and superficial iliac lymph nodes of SPF pigs. Animals directly injected with the cloned PCV2 plasmid DNA developed an infection and disease resembling that induced by infection via intranasal route of inoculation with a homogenous PCV2 infectious virus stock. It is known that PCV2 replicates in the lymph nodes, lungs and liver during natural infection (9, 25, 29, 31, 62). It will be interesting to know if other routes of injection of the cloned PCV2 plasmid DNA such as intramuscularly can also initiate an infection. By using this PCV2 molecular DNA clone, the clinical disease, pathological lesions and virus distribution exclusively attributable to PCV2 infection were more definitively characterized. Viremia, beginning at 14 DPI and lasting about 2-4 weeks, was detected in the majority of the PCV2-inoculated animals. Similarly, the majority of inoculated pigs necropsied at 35 DPI seroconverted to PCV2-antibodies. PCV2 antigen was detected in various tissues and organs in inoculated pigs. Gross lesions were limited to the lungs and lymph nodes, and were characterized by systematically enlarged tan colored lymph nodes and lungs that failed to collapse and

mild multifocal tan-colored foci of consolidation. Histopathological lesions in multiple tissues and organs similar to those of PMWS were reproduced with the PCV2 molecular DNA clone as well as with the infectious virus prepared in vitro from the molecular DNA clone. However, we failed to reproduce characteristic clinical PMWS with the cloned PCV2 plasmid DNA or with a biologically pure PCV2 infectious virus stock. PCV2 is clearly responsible for the PMWS-like histopathological lesions reproduced in this study, but whether or not PCV2 is the sole cause of clinical PMWS remains questionable. Clinical PMWS was reproduced only in gnotobiotic pigs co-infected with PCV2 and PPV (8), and in PCV-2 inoculated gnotobiotic pigs when their immune system was activated by keyhole limpet hemocyanin in incomplete Freund's adjuvant (31). Clearly, more studies are needed to determine the etiological significance of PCV2 in clinical PMWS and its interrelationship with PRRSV, PPV, other infectious swine agents and immune stimulants.

Will et al. (63) first demonstrated the feasibility of using a cloned hepatitis B virus DNA to infect chimpanzees by direct *in vivo* injection. This approach has since been used to study viral replication and pathogenesis of several other viruses (14, 21, 34, 49, 52, 54, 63, 64). The construction of an infectious PCV2 molecular DNA clone, and the demonstration of infection by direct injection of cloned PCV2 plasmid DNA into the liver and lymph nodes of pigs in this study, should be very advantageous for future PCV2 studies. This *in vivo* transfection system will enable us to study the structural and functional relationship of PCV2 genes using recombinant plasmids constructed in vitro to test different regions or genes of PCV2 for their roles in virus replication and pathogenesis in the host. The replication and pathogenesis of PCV2 can be studied in

vivo without having to produce infectious virus stocks by propagating PCV2 in cell cultures. This is advantageous as serial cell culture passages may select for viral variants. Another advantage of using cloned PCV2 genomic DNA, instead of live virus, for animal studies is its relative ease for quantitation of the inoculation dose. The amount of the cloned PCV2 DNA used for animal inoculation can be easily determined by a spectrophotometer, whereas the dose of live PCV2 virus requires infectivity titration in cell cultures and confirmation of infection by IFA. Direct injection of animals with cloned PCV2 plasmid DNA eliminates the problems associated with the presence of other indigenous swine agents in tissue homogenate inocula in animal studies. From the vaccine development point of view, the relatively easy storage and stability of DNA, and the economy of large-scale recombinant PCV2 plasmid DNA production should provide an attractive means of delivering genetically engineered, attenuated PCV2 vaccines to pigs. However, the intrahepatic and intralymphoid routes of inoculation used in this study is not practical for vaccine delivery and thus, future studies are warranted to determine if pigs can be infected by intramuscular (I.M) route of injection with the PCV2 DNA clone. The I.M. and intradermal routes have been successfully used to infect animals with cloned genomic DNA of other viruses (34, 52, 64).

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REFERENCES

1. **Allan, G.M., D.P. Mackie, J. McNair, B.M. Adair, and M.S. McNulty.** 1994. Production, preliminary characterization and applications of monoclonal antibodies to porcine circovirus. Vet. Immunol. Immunopathol. **43**:357-371.
2. **Allan, G.M., F. McNeilly, J.P. Cassidy, G.A. Reilly, B. Adair, W.A. Ellis, and M.S. McNulty.** 1995. Pathogenesis of porcine circovirus; experimental infections of colostrum deprived piglets and examination of pig fetal material. Vet. Microbiol. **44**:49-64.
3. **Allan, G.M., B. Meehan, D. Todd, S. Kennedy, F. McNeilly, J. Ellis, E.G. Clark, J. Harding, E. Espuna, A. Botner, and C. Charreyre.** 1998. Novel porcine circoviruses from pigs with wasting disease syndromes. Vet. Rec. **142**:467-468.
4. **Allan, G.M., F. McNeilly, J. Ellis, S Krakowka, B. Meeham, I. McNair, I. Walker, and S. Kennedy.** 2000. Experimental infection of colostrums deprived piglets with porcine circovirus 2 (PCV2) and porcine reproductive and respiratory syndrome virus (PRRSV) potentiates PCV2 replication. Arch. Virol. **145**:2421-2429.
5. **Allan, G.M., F. McNeilly, S. Kennedy, B. Daft, E.G. Clarke, J.A. Ellis, D.M. Haines, B.M. Meehan, and B.M. Adair.** 1998. Isolation of porcine circovirus-like viruses from pigs with a wasting disease in the USA and Europe. J. Vet. Diagn. Invest. **10**:3-10.
6. **Allan, G.M., F. McNeilly, B.M. Meehan, J.A. Ellis, T.J. Conner, I. McNair, S. Krakowka, and S. Kennedy.** 2000. A sequential study of experimental

infection of pigs with porcine circovirus and porcine parvovirus: immunostaining of cryostat sections and virus isolation. J. Vet. Med. **B47**:81-94.

7. **Allan, G.M., F. McNeilly, B.M. Meehan, S. Kennedy, D.P. Mackie, J.A. Ellis, E.G. Clark, E. Espuna, N. Saubi, P. Riera, A. Botner, and C.E. Charreyre.** 1999. Isolation and characterization of circoviruses from pigs with wasting syndromes in Spain, Denmark and Northern Ireland. Vet. Microbiol. **66**:115-23.
8. **Allan, G.M., S. Kennedy, F. McNeilly, J.C. Foster, J.A. Ellis, S.J. Krakowka, B.M. Meehan, and B.M. Adair.** 1999. Experimental reproduction of severe wasting disease by co-infection of pigs with porcine circovirus and porcine parvovirus. J. Comp. Pathol. **121**:1-11.
9. **Allan, G.M. and J.A. Ellis.** 2000. Porcine circoviruses: a review. J. Vet. Diagn. Invest. **12**:3-14.
10. **Bassami, M.R., D. Berryman, G.E. Wilcox, and S.R. Raidal.** 1998. Psittacine beak and feather disease virus nucleotide sequence analysis and its relationship to porcine circovirus, plant circoviruses, and chicken anemia virus. Virology **249**:453-459.
11. **Balasch, M., J. Segales, C. Rosell, M. Domingo, A. Mankertz, A. Urniza, and J. Plana-Duran.** 1999. Experimental inoculation of conventional pigs with tissue homogenates from pigs with post-weaning multisystemic wasting syndrome. J. Comp. Pathol. **121**:139-148.
12. **Biagini, P., P. Gallian, H. Attoui, M. Touinssi, J-F. Cantaloube, P. de Micco, and X. de Lamballerie.** 2001**.** Genetic analysis of full-length genomes and

subgenomic sequences of TT virus-like mini virus human isolates. J. Gen. Virol. **82**: 379-383.

13. **Choi, C., C. Chae, and E.G. Clark.** 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.
14. **Dubensky, T.W., B.A. Cambell, and L.P. Villarreal.** 1984. Direct transfection of viral and plasmid DNA into the liver or spleen of mice. Proc. Natl. Acad. Sci. USA **81**:7529-7533.
15. **Dulac, G.C. and A. Afshar.** 1989. Porcine circovirus antigens in PK-15 cell line (ATCC CCL-33) and evidence of antibodies to circovirus in Canadian pigs. Can. J. Vet. Res. **53**:431-433.
16. **Edwards, S. and J.J. Sands.** 1994. Evidence of circovirus infection in British pigs. Vet. Rec. **134**:680-1.
17. **Ellis, J., L. Hassard, E. Clark, J. Harding, G. Allan, P. Willson, J. Strakappe, K. Martin, F. McNeilly, B. Meehan, D. Todd, and D. Haines.** 1998. Isolation of circovirus from lesions of pigs with postweaning multisystemic wasting syndrome. Can. Vet. J. **39**:44-51.
18. **Ellis, J., S. Krakowka, M. Lairmore, D. Haines, A. Bratanich, E. Clark, G. Allan, C. Konoby, L. Hassard, B. Meehan, K. Martin, J. Harding, S. Kennedy, and F. McNeilly.** 1999. Reproduction of lesions of postweaning multisystemic wasting syndrome in gnotobiotic piglets. J. Vet. Diagn. Invest. **11**:3-14.

19. **Ellis, J.A., A. Bratanich, E.G. Clark, G. Allan, B. Meehan, D.M. Haines, J. Harding, K.H. West, S. Krakowka, C. Konoby, L. Hassard, K. Martin, and F. McNeilly.** 2000. Coinfection by porcine circoviruses and porcine parvovirus in pigs with naturally acquired postweaning multisystemic wasting syndrome. J. Vet. Diagn. Invest. **12**:21-27.

20. **Fenaux M, P.G. Halbur, M. Gill, T.E. Toth, and X.J. Meng.** 2000**.** Genetic characterization of type 2 porcine circovirus (PCV-2) from pigs with postweaning multisystemic wasting syndrome in different geographic regions of North America and development of a differential PCR-restriction fragment length polymorphism assay to detect and differentiate between infections with PCV-1 and PCV-2. J. Clin. Microbiol. **38**: 2494-503.

21. **Girones, R., P.J. Cote, W.E. Hornbuckle, B.C. Tennant, J.L. Gerin, R.H. Purcell, and R.H. Miller.** 1989**.** Complete nucleotide sequence of a molecular clone of woodchuck hepatitis virus that is infectious in the natural host. Proc. Natl. Acad. Sci. USA. **86**:1846-1849.

22. **Halbur P.G., C. Kasorndorkbua, C. Gilbert, D. Guenette, M.B. Potters, R.H. Purcell, S.U. Emerson, T.E. Toth, and X.J. Meng.** 2001**.** Comparative pathogenesis of infection of pigs with hepatitis E viruses recovered from a pig and a human. J. Clin. Microbiol. **39**:918-923.

23. **Halbur P.G., P.S. Paul, M.L. Frey, J. Landgraf, K. Eernisse, X.J. Meng, M.A. Lum, J.J. Andrews, and J.A. Rathje.** 1995. Comparison of the pathogenicity of two U.S. porcine reproductive and respiratory syndrome virus isolates with that of the Lelystad virus. Vet. Pathol. **32**:648-660.

24. **Hamel, A.L., L.L. Lin, and G.P. Nayar.** 1998. Nucleotide sequence of porcine circovirus associated with postweaning multisystemic wasting syndrome in pigs. J. Virol. **72**:5262-5267.

25. **Harding, J.C. and E.G. Clark.** 1997. Recognizing and diagnosing postweaning multisystemic wasting syndrome (PMWS). Swine Health and Production. **5**:201-203.

26. **Hines, R.K. and P.D. Lukert.** 1995. Porcine circovirus: a serological survey of swine in the United States. Swine Health and Production. **3**:71-73.

27. **Joo H.S., C.R. Donaldson-Wood, and R.H. Johnson.** 1976**.** A standardized haemagglutination inhibition test for porcine parvovirus antibody. Aust. Vet. J. **52**:422-4.

28. **Kennedy, S., G. Allan, F. McNeilly, B.M. Adair, A. Hughes, and P. Spillane.** 1998. Porcine circovirus infection in Northern Ireland. Vet. Rec. **142**:495-6.

29. **Kennedy, S., D. Moffett, F. McNeilly, B. Meehan, J. Ellis, S. Krakowka, and G.M. Allan.** 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.

30. **Kiupel, M., G.W. Stevenson, S.K. Mittal, E.G. Clark, and D.M. Haines.** 1998. Circovirus-like viral associated disease in weaned pigs in Indiana. Vet. Pathol. **35**:303-307.

31. **Krakowka, S., J.A. Ellis, F. McNeilly, S. Ringler, D.M. Rings, and G. Allen.** 2001. Activation of the immune system is the pivotal event in the production of

wasting disease in pigs infected with porcine circovirus-2 (PCV-2). Vet. Pathol. **38**:31-42.

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

33. **Larochelle, R., M. Morin, M. Antaya, and R. Magar.** 1999. Identification and incidence of porcine circovirus in routine field cases in Quebec as determined by PCR. Vet. Rec. **145**:140-142.

34. **Letvin, N.L., C.I. Lord, N.W. King, and M.S. Wyand.** 1991. Risks of handling HIV. Nature **349**:573.

35. **Mankertz, J., H.J. Buhk, G. Blaess, and A. Mankertz.** 1998. Transcription analysis of porcine circovirus (PCV). Virus Genes **16**:267-276.

36. **Mankertz, A., M. Domingo, J.M. Folch, P. LeCann, A. Jestin, J. Segales, B. Chmielewicz, J. Plana-Duran, and D. Soike.** 2000. Characterization of PCV-2 isolates from Spain, Germany and France. Virus Res. **66**:65-77.

37. **Mankertz A., K. Hattermann, B. Ehlers, and D. Soike.** 2000**.** Cloning and sequencing of columbid circovirus (CoCV), a new circovirus from pigeons. Arch. Virol. **145**:2469-2479.

38. **Meehan, B.M., J.L. Creelan, M.S. McNulty, and D. Todd.** 1997. Sequence of porcine circovirus DNA: affinities with plant circoviruses. J. Gen. Virol. **78**:221-227.

39. **Meehan, B.M., F. McNeilly, D. Todd, S. Kennedy, V.A. Jewhurst, J.A. Ellis, L.E. Hassard, E.G. Clark, D.M. Haines, and G.M. Allan.** 1998. Characterization of novel circovirus DNAs associated with wasting syndromes in pigs. J. Gen. Virol. **79**:2171-2179.

40. **Miyata, H., H. Tsunoda, A. Kazi, A. Yamada, M.A. Khan, J. Murakami, T. Kamahora, K. Shiraki, and S. Hino.** 1999. Identification of a novel GC-rich 113-nucleotide region to complete the circular, single-stranded DNA genome of TT virus, the first human circovirus. J. Virol. **73**:3582-3586.

41. **Morozov, I., T. Sirinarumitr, S.D. Sorden, P.G. Halbur, M.K. Morgan, K.J. Yoon, and P.S. Paul.** 1998. Detection of a novel strain of porcine circovirus in pigs with postweaning multisystemic wasting syndrome. J. Clin. Microbiol. **36**:2535-2541.

42. **Nawagitgul, P., P.A. Harms, I. Morozov, B.J. Thacker, S.D. Sorden, C. Lekcharoensuk, and P.S. Paul.** 2001. Modified indirect porcine circovirus (PCV) type 2-based and recombinant capsid protein (ORF2)-based ELISA for the detection of antibodies to PCV. Clin. Diagn. Lab. Immunol. Submitted.

43. **Nawagitgul, P., I. Morozov, S.R. Bolin, P.A. Harms, S.D. Sorden, and P.S. Paul.** 2000. Open reading frame 2 of porcine circovirus type 2 encodes a major capsid protein. J. Gen. Virol. **81**:2281-2287.

44. **Nayar, G.P., A.L. Hamel, L. Lin, C. Sachvie, E. Grudeski, and G. Spearman.** 1999. Evidence for circovirus in cattle with respiratory disease and from aborted bovine fetuses. Can. Vet. J. **40**:277-278.

45. **Nishizawa, T., H. Okamoto, K. Konishi, H. Yoshizawa, Y. Miyakawa, and M. Mayumi.** 1997. A novel DNA virus (TTV) associated with elevated transaminase levels in posttransfusion hepatitis of unknown etiology. Biochem. Biophys. Res. Commun. **241**:92-97.

46. **Onuki, A., K. Abe, K. Togashi, K. Kawashima, A. Taneichi, and H. Tsunemitsu.** 1999. Detection of porcine circovirus from lesions of a pig with wasting disease in Japan. J. Vet. Med. Sci. **61**:1119-1123.

47. **Pogranichnyy, R.M., K-J. Yoon, P.A. Harms, S.L. Swenson, J.J. Zimmerman, and S.D. Sorden.** 2000. Characterization of immune response of young pigs to porcine circovirus type 2 infection. Viral. Immunol. **13**:143-153.

48. **Rosell, C., J. Segales, J.A. Ramos-Vara, J.M. Folch, G.M. Rodriguez-Arrioja, C.O. Duran, M. Balasch, J. Plana-Duran, and M. Domingo.** 2000. Identification of porcine circovirus in tissues of pigs with porcine dermatitis and nephropathy syndrome. Vet. Rec. **146**:40-43.

49. **Seeger, C., D. Ganem, and H.E. Varmus.** 1984. The cloned genome of ground squirrel hepatitis virus is infectious in the animal. Proc. Natl. Acad. Sci. USA. **81**:5849-5852.

50. **Sorden S.D., P.A. Harms, P. Nawagitgul, D. Cavanaugh, and P.S. Paul.** 1999. Development of a polyclonal-antibody-based immunohistochemical method for the detection of type 2 porcine circovirus in formalin-fixed, paraffin-embedded tissue. J. Vet. Diagn. Invest. **11**:528-530.

51. **Soucie, J.M., D.D. Erdman, B.L. Anderson, T.J. Torok, M. El-Jamil, E. Barnhart, M. Tepper, H.N. Burrill, A.M. Pickett, and W.L. Mengeling.** 2000.

Investigation of porcine parvovirus among persons with hemophilia receiving Hyate: C porcine factor VIII concentrate. Transfusion **40**:708-711.

52. **Sparger, E.E., H. Louie, A.M. Ziomeck, and P.A. Luciw.** 1997. Infection of cats by injection with DNA of feline immunodeficiency virus molecular clone. Virology **238**:157-160

53. **Spillane, P., S. Kennedy, B. Meehan, and G. Allan.** 1998. Porcine circovirus infection in the Republic of Ireland. Vet. Rec. **143**:511-512**.**

54. **Sprengel, R., H.E.Varmus, and D. Ganem.** 1987. Homologous recombination between hepadnaviral genomes following in vivo DNA transfection: implications for studies of viral infectivity. Virology **159**:454-456.

55. **Takahashi, K., Y. Iwasa, M. Hijikata, and S. Mishiro.** 2000. Identification of a new human DNA virus (TTV-like mini virus, TLMV) intermediately related to TT virus and chicken anemia virus. Arch. Virol. **145**:979-93.

56. **Tischer, I., H. Gelderblom, W. Vettermann, and M.A. Koch.** 1982. A very small porcine virus with circular single-stranded DNA. Nature **295**:64-66.

57. **Tischer, I., W. Mields, D. Wolff, M. Vagt, and W. Griem.** 1986**.** Studies on epidemiology and pathogenicity of porcine circovirus. Arch. Virol. **91**:271-276.

58. **Tischer, I., L. Bode, D. Peters, S. Pociuli, and B. Germann.** 1995. Distribution of antibodies to porcine circovirus in swine populations of different breeding farms. Arch. Virol. **140**:737-743.

59. **Tischer, I., L. Bode, J. Apodaca, H. Timm, D. Peters, R. Rasch, S. Pociuli, and E. Gerike.** 1995. Presence of antibodies reacting with porcine circovirus in sera of humans, mice, and cattle. Arch. Virol. **140**:1427-1439.

60. **Tischer, I., R. Rasch, and G. Tochtermann.** 1974. Characterization of papovavirus and picornavirus-like particles in permanent pig kidney cell lines. Zentralbl. Bakteriol. Hyg. A **226**:153-167.

61. **Todd, D., F.D. Niagro, B.W. Ritchie, W. Curran, G.M. Allan, P.D. Lukert, K.S. Latimer, W.L. Steffens, and M.S. McNulty.** 1991. Comparison of three animal viruses with circular single-stranded DNA genomes. Arch. Virol. **117**:129-135.

62. **Wellenberg G.J., S. Pesh, F.W. Berndsen, P.J.G.M. Steverink, W. Hunneman, T.J.K. Van der Vorst, N.H.M.T. Peperkamp, V.F. Ohlinger, R. Schippers, J.T. Van Oirschot, and M.F. de Jong.** 2000. Isolation and characterization of porcine circovirus type 2 from pigs showing signs of post-weaning multisystemic wasting syndrome in the Netherlands. Vet. Quart. **22**:167-72.

63. **Will, H., R. Cattaneo, H-G. Koch, G. Darai, H. Schaller, H. Schellekens, P.M.C.A. van Eerd, and F. Deinhardt.** 1982. Cloned HBV DNA causes hepatitis in chimpanzees. Nature **299**:740-742.

64. **Willems, L., D. Portetelle, P Kerkhofs, G. Chen, A. Burney, M Mammerickx, and R. Kettmann.** 1992. In vivo transfection of bovine leukemia provirus into sheep. Virology **189**:775-777.

Fig. 1

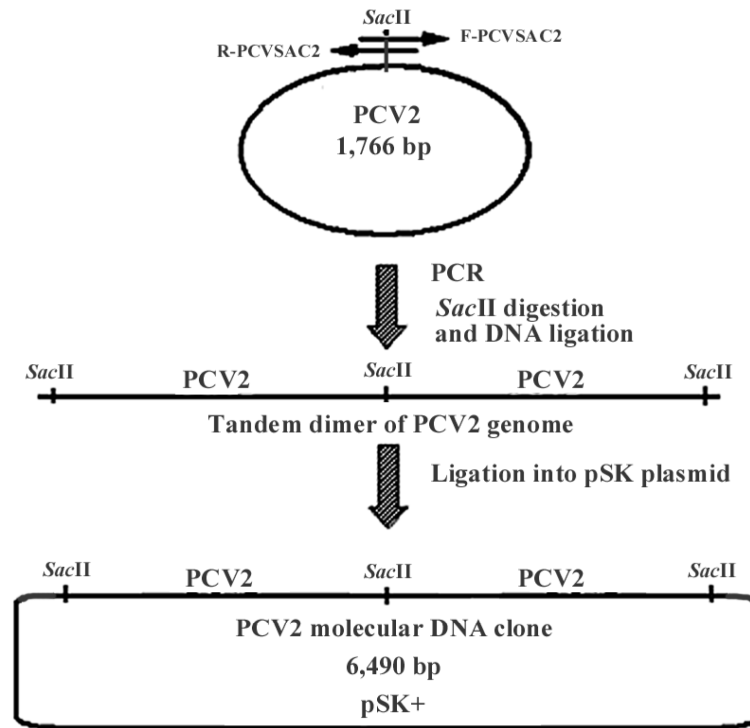

Fig. 1. Construction of an infectious PCV2 molecular DNA clone. The relative positions of the primer pair used to amplify the complete PCV2 genome are indicated by the arrows (reverse primer PCVSAC2, forward primer PCVSAC2). The PCV2 genomic DNA amplified by PCR is digested with Sac II restriction enzyme, and purified. The purified and Sac II-digested genomic DNA was ligated to form concatemers. Ligated concatemers were separated by gel electrophoresis, the tandem genome dimmer of PCV2 was purified and cloned into pSK vector that is pre-digested with Sac II enzyme to produce a molecular PCV2 DNA clone.

Fig. 2

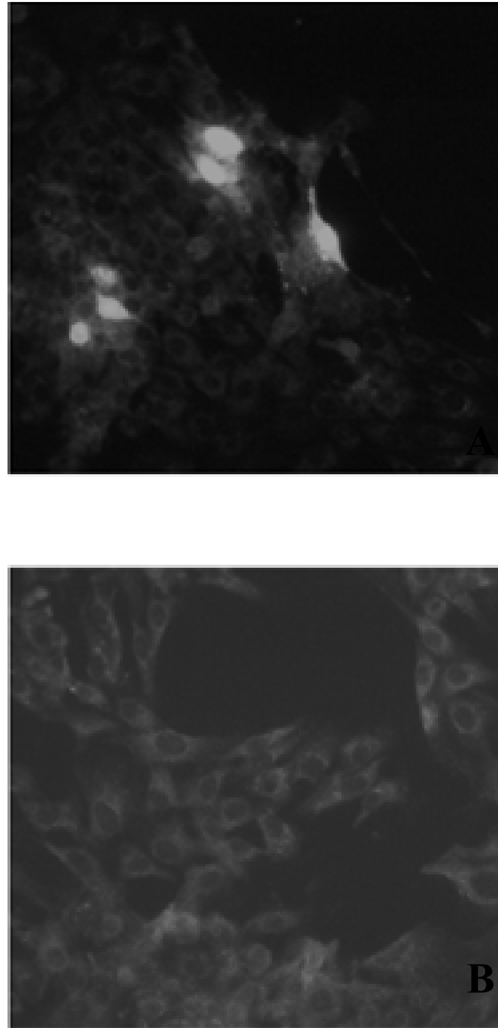

Fig. 2. The cloned PCV2 plasmid DNA is infectious when transfected in vitro in PK-15 cells. (A). Detection of PCV2 antigen by IFA in PK-15 cells transfected with the cloned PCV2 plasmid DNA. Intense immunolabeling of PCV2 antigen was visualized in the nucleus, and to a lesser degree, cytoplasm of the transfected cells. (B): Mock-transfected PK-15 cells.

Fig. 3

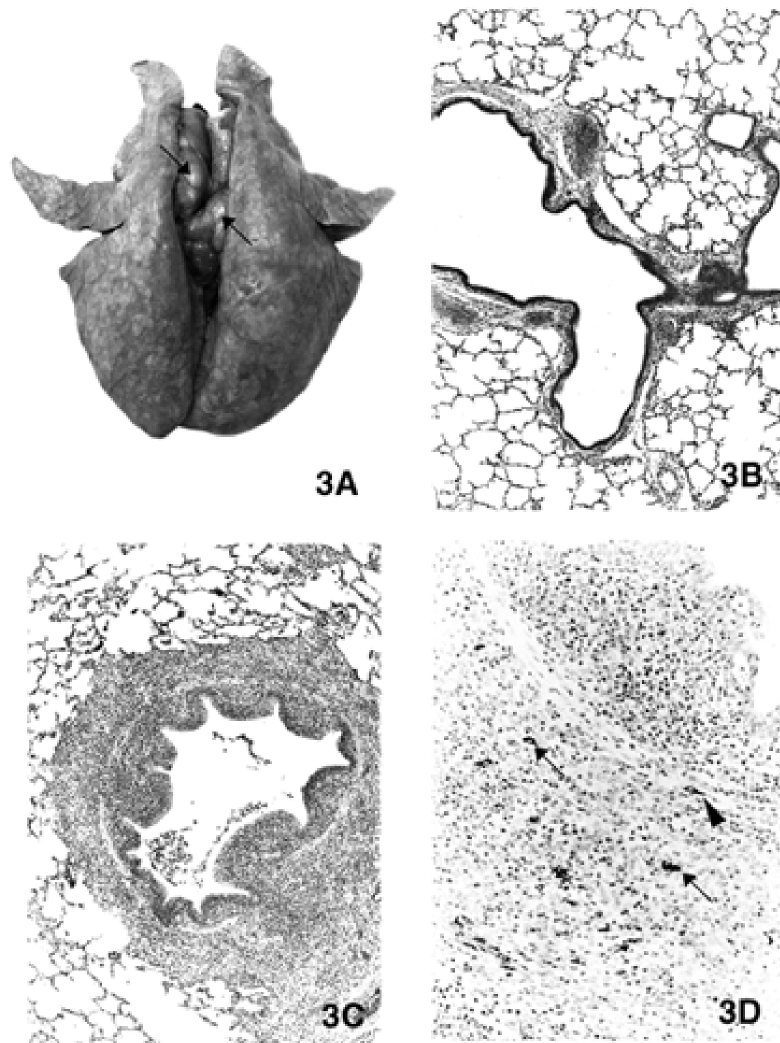

Fig. 3. A. Lung from a pig inoculated by intralymphoid route with PCV-2 DNA at 21 DPI. The lungs were rubbery, failed to collapse, and were mottled tan-red. Tracheobronchial lymph nodes were markedly enlarged and tan (arrows).

B. Microscopic section of a normal lung from a control pig (25 X).

C. Microscopic section of the lung from the pig in figure 3A. Note the peribronchiolar lymphohistiocytic inflammation and mild necrotizing bronchiolitis (25 X).

D. Immunohistochemical staining of the lung in figure 3A. Note the PCV-2 antigen in macrophages (arrows) and fibroblast-like cells (arrow heads) around airways (64 X).

Fig. 4

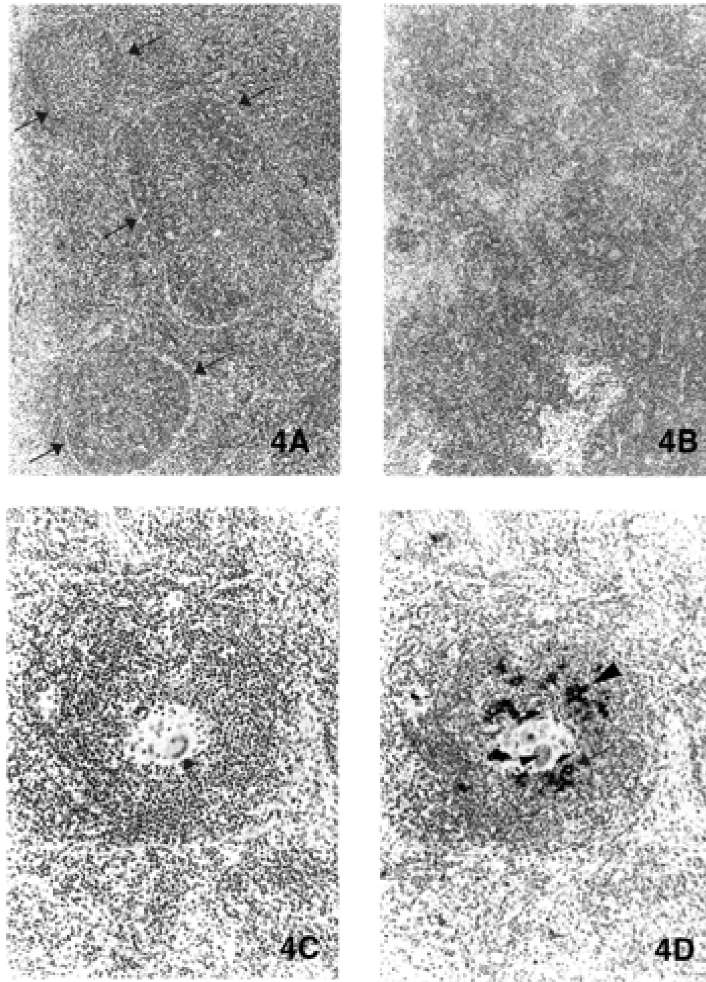

Fig. 4. A. Normal lymph node from a control pig. Note the well-defined lymphoid follicles (arrows) (25 X).

B..Microscopic section of the tracheobronchial lymph node from the pig in figure 3A inoculated 21 days previously by intralymphoid route with cloned PCV2 genomic DNA. Lymphoid follicles are poorly defined, there is mild-to-moderate lymphoid depletion, and mild multifocal granulomatous inflammation (25 X).

C. Same lymph node as figure 4B. Note the poorly defined follicle with macrophages and giant cells (arrow) replacing follicular lymphocytes (64 X).

D. Same lymph node as figure 4B. Immunohistochemical detection of PCV-2 antigen in macrophages (arrows) and giant cells (small arrowheads), and dendritic-like cells (large arrowheads) in the follicles (64 X).

Table 1. Detection of viremia (PCV2 DNA) by PCR in sera of inoculated and control pigs

Group	Inocula	Route of Inoculation	Days postinoculation						
			0	7	14	21	28	35	Total
1	None		0/10[a]	0/10	0/10	0/10	0/5	0/5	0/10
2	PCV2 live virus[b]	Intranasal	0/10	0/10	7/10	5/10	0/5	0/5	8/10
3	PCV2 DNA[c]	Intrahepatic	0/10	0/10	8/10	6/10	3/5	3/5	9/10
4	PCV2 DNA[c]	Intralymphoid	0/10	0/10	2/10	8/10	2/5	0/5	8/10

[a]10 pigs in each group, number positive/number tested.

[b]A biologically pure and homogeneous PCV2 virus stock generated by transfection of PK-15 cells with PCV2 molecular DNA clone.

[c]Cloned PCV2 genomic DNA in pSK plasmid.

Table 2. Seroconversion to PCV2 specific antibodies in pigs inoculated with PCV2 live virus or directly injected with cloned PCV2 plasmid DNA

Group	Inocula	Route of Inoculation	Days postinoculation					
			0	7	14	21	28	35
1	None		2/10[a]	0/10	0/10	0/10	0/5	0/5
2	PCV2 live virus[b]	Intranasal	0/10	0/10	0/10	1/10	1/5	4/5
3	PCV2 DNA[c]	Intrahepatic	0/10	0/10	0/10	0/10	1/5	5/5
4	PCV2 DNA[c]	Intralymphoid	0/10	0/10	0/10	0/10	1/5	3/5

[a]PCV2 antibody was measured with an ELISA, number positive/number tested.

[b]A biologically pure and homogeneous PCV2 virus stock generated by transfection of PK-15 cells with PCV2 molecular DNA clone.

[c]Cloned PCV2 genomic DNA in pSK plasmid.

Table 3. Gross lesions of lung and lymph nodes in control and PCV2-inoculated pigs

Group	Inocula	Route of Inoculation	21 DPI		35DPI	
			Lymph nodes	Lung	Lymph nodes	Lung
1	None		0/5[a]	0/5	0/5	0/5
2	PCV2 live virus[b]	Intranasal	5/5	1/5(0-1)[c]	5/5	4/5(0-5)
3	PCV2 DNA[d]	Intrahepatic	2/5	2/5(0-2)	5/5	2/5(0-13)
4	PCV2 DNA[d]	Intralymphoid	4/5	5/5(0-1)	3/5	1/5(0-9)

[a]Five pigs from each group were necropsied at 21 DPI, and the remaining 5 pigs were necropsied at 35 DPI. Number positive / number tested.

[b]A biologically pure and homogeneous PCV2 virus stock generated by transfection of PK-15 cells with PCV2 molecular DNA clone.

[c] Number with lesions / number tested (range of the estimated percent of the lung affected by grossly visible pneumonia lesions, 0-100%)

[d]Cloned PCV2 genomic DNA in pSK plasmid.

Table 4. Distribution of histopathological lesions in control and PCV2-inoculated pigs

Group	Inocula	Route of Inoculation	DPI[a]	Lung[b]	Liver[c]	LN[d]	Spleen	Thymus	Ileum	Brain	Heart	Kidney	Tonsil
1	None		21	0/5(0.0)	4/5(0.8)	0/5(0.0)	0/5	0/5	0/5	0/5	0/5	0/5	0/5
			35	0/5(0.0)	4/5(0.8)	0/5(0.0)	0/5	0/5	0/5	0/5	0/5	0/5	0/5
2	PCV2 virus	Intranasal	21	5/5(1.6)	5/5(1.2)	3/5(1.2)	1/5	0/5	0/5	4/5	3/5	1/5	0/5
			35	3/5(0.6)	4/5(1.0)	4/5(0.8)	3/5	0/5	3/5	4/5	0/5	1/5	3/5
3	PCV2 DNA	Intrahepatic	21	5/5(1.0)	5/5(1.0)	5/5(1.0)	1/5	0/5	0/5	5/5	4/5	1/5	0/5
			35	5/5(1.2)	5/5(1.0)	4/5(1.0)	2/5	0/5	3/5	3/5	4/5	5/5	3/5
4	PCV2 DNA	Intralymphoid	21	5/5(1.2)	5/5(1.0)	5/5(0.8)	0/5	0/5	0/5	4/5	4/5	3/5	0/5
			35	5/5(1.0)	5/5(1.2)	5/5(1.4)	0/5	0/5	1/5	3/5	3/5	3/5	2/5

[a]Days postinoculation (DPI): 5 animals from each group were necropsied at 21 DPI and the remaining 5 animals from each group were necropsied at 35 DPI

[b]Number positive/number tested (Average histological lung score: 0=normal, 1=mild interstitial pneumonia, 2=moderate, 3=severe)

[c]Number positive/number tested (Average histological liver score: 0=normal, 1=mild hepatitis, 2=moderate, 3=severe)

[d]Number positive/number tested (Average histological lymphoid (LN) depletion score: 0=normal, 1=mild, 2=moderate, 3=severe)

Table 5. Detection and distribution of PCV2-specific antigen by immunohistochemistry in control and PCV2-inoculated pigs

Group	**Inocula**	**Route of Inoculation**	DPI[a]	Lung	Liver	LN	Spleen	Thymus	Ileum	Brain	Heart	Kidney	Tonsil
1	None		21	0/5[b]	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
			35	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
2	PCV2 virus	Intranasal	21	4/5	5/5	5/5	3/5	0/5	3/5	3/5	1/5	1/5	2/5
			35	1/5	2/5	3/5	2/5	0/5	0/5	2/5	0/5	0/5	0/5
3	PCV2 DNA	Intrahepatic	21	5/5	5/5	5/5	5/5	0/5	0/5	5/5	1/5	0/5	2/5
			35	4/5	4/5	3/5	4/5	0/5	3/5	4/5	2/5	2/5	3/5
4	PCV2 DNA	Intralymphoid	21	4/5	4/5	5/5	4/5	0/5	3/5	3/5	0/5	0/5	3/5
			35	3/5	4/5	5/5	4/5	0/5	2/5	3/5	1/5	0/5	4/5

[a]Days postinoculation (DPI): 5 animals from each group were necropsied at 21 DPI and the remaining 5 animals from each group were necropsied at 35 DPI

[b]Number positive/number tested.

Chapter 4

Immunogenicity and Pathogenicity of the Chimeric Infectious DNA Clones between Pathogenic Type 2 Porcine Circovirus (PCV2) and Non-Pathogenic PCV1 in Weaning Pigs

Fenaux M., T. Opriessnig, P.G. Halbur, and X.J. Meng. 2003. J. Virol. 77:11232-11243.

ABSTRACT

Porcine circovirus type 2 (PCV2) is the primary causative agent of postweaning multisystemic wasting syndrome (PMWS), whereas the ubiquitous porcine circovirus type 1 (PCV1) is non-pathogenic to pigs. We report here the construction and characterization of two chimeric infectious DNA clones between PCV1 and PCV2. The chimeric PCV1-2 clone contains the PCV2 capsid gene cloned in the backbone of the non-pathogenic PCV1. A reciprocal chimeric PCV2-1 DNA clone was also constructed by replacing the PCV2 capsid gene with that of PCV1 in the backbone of the PCV2. The PCV1, PCV2, chimeric PCV1-2 and PCV2-1 DNA clones were all shown to be infectious in PK-15 cells and their growth characteristics *in vitro* were determined and compared. To evaluate the immunogenicity and pathogenicity of the chimeric infectious DNA clones, forty specific-pathogen-free (SPF) pigs were randomly assigned into five groups of eight pigs each. Group 1 pigs received phosphate-buffered saline as the negative control. Group 2 pigs were each injected into the superficial inguinal lymph nodes with 200 μg of the PCV1 infectious DNA clone. Group 3 pigs were each similarly injected with 200 μg of the PCV2 infectious DNA clone, group 4 pigs each with 200 μg

of the chimeric PCV1-2 infectious DNA clone, and group 5 pigs each with 200 µg of the reciprocal chimeric PCV2-1 infectious DNA clone. As expected, seroconversion to antibodies against PCV2 capsid antigen were detected in group 3 pigs and in group 4 pigs. Group 2 and 5 pigs all seroconverted to PCV1 antibody. Gross and microscopic lesions in various tissues of animals inoculated with the PCV2 infectious DNA clone were significantly more severe than those found in pigs inoculated with PCV1, chimeric PCV1-2, and reciprocal chimeric PCV2-1 infectious DNA clones. These data indicated that the chimeric PCV1-2 virus with the immunogenic ORF2 capsid gene of the pathogenic PCV2 cloned into the non-pathogenic PCV1 genomic backbone induces a specific antibody response to the pathogenic PCV2 capsid antigen but is attenuated in pigs. Future studies are warranted to evaluate the usefulness of the chimeric PCV1-2 infectious DNA clone as a genetically engineered live-attenuated vaccine against PCV2 infection and PMWS.

INTRODUCTION

Porcine circovirus (PCV) was first discovered as a non-cytopathic contaminant of the porcine kidney cell culture PK-15 (64, 68). PCV is a small icosahedral non-enveloped virus with a single stranded circular DNA genome of about 1.76 kb. PCV genome contains at least two potentially functional ORFs: ORF 1 (930 bp) encodes the Rep protein involved in viral replication and ORF 2 (690 bp) encodes the immunogenic capsid protein (16, 24, 40, 50). PCV belongs to the *Circoviridae* family along with other animal circoviruses such as psittacine beak and feather disease virus, chicken anemia virus (CAV) (12), and columbid circovirus (CoCV) (42). There are also three plant circoviruses, banana bunchy top virus, coconut foliar decay virus, and subterranean clover stunt virus (12, 43). Recently, three novel human circoviruses have been discovered, including transfusion transmitted virus (TTV), SEN virus (SENV), and TTV-like minivirus (14, 45, 46, 52, 63, 69, 72). Although antibodies to PCV have been found in many animal species including humans, mice, cattle, and pigs (1, 18, 19, 31, 44, 51, 66, 67), little is known regarding the pathogenesis of PCV in these animal species (57, 67). The PK-15 derived PCV did not produce clinical disease in experimentally inoculated pigs, and thus, the virus is considered to be non-pathogenic (3, 65), and was designated PCV1.

Postweaning multisystemic wasting syndrome (PMWS) is an emerging disease in pigs first described in 1991 (28). PMWS primarily affects pigs between 5 to 18 weeks of age. Clinical PMWS signs include progressive weight loss, dyspnea, tachypnea, anemia, diarrhea, and jaundice. Mortality rate may vary from 1% to 2% and up to 40% in some complicated cases in the U.K (48). Microscopic lesions characteristic of PMWS lesions

include granulomatous interstitial pneumonia, lymphodenopathy, hepatitis and nephritis (4, 8, 11, 28). PMWS has now been recognized in pigs in Canada, the United States (5, 20, 27, 31, 35, 39, 47), most European countries (5, 11, 19, 33, 37, 41, 59, 62, 70), and some countries in Asia (17, 53). PMWS potentially has a serious economic impact on the swine industry worldwide.

The primary causative agent of PMWS is a pathogenic strain of PCV, designated PCV2 (2, 5, 9, 11, 20, 22, 23, 24, 44, 47). The complete genomic sequence of the PMWS-associated PCV2 has been determined (24, 27, 40). Sequence analyses revealed that PMWS-associated PCV2 shares about 75% nucleotide sequence identity with the nonpathogenic PCV1. The pathogenic PCV2 shares very similar genomic organization with the non-pathogenic PCV1. The ORF2 gene of both PCV1 and PCV2 encodes for the major immunogenic capsid protein (16, 49, 50). Initial attempts to reproduce clinical PMWS in conventional pigs by PCV2 inoculation were unsuccessful (13, 23, 34). Recently, clinical PMWS was reproduced in cesarean derived/colostrum deprived pigs (CD/CD) and in SPF pigs inoculated with PCV2 alone (29, 38). Clinical PMWS was also reproduced in conventional pigs co-infected with PCV2 and either porcine parvovirus (PPV) or porcine reproductive and respiratory syndrome virus (PRRSV) (54, 58). In addition, PMWS was reproduced in PCV2 inoculated gnotobiotic pigs when their immune system was activated by keyhole limpet hemocyanin in incomplete Freund's adjuvant (15, 36). Two recent field studies by Allan et al. (7) and Kyriakis et al. (37) tested the effect of immuno-modulation by *Mycoplasma hyopneumoniae* vaccine on the development of PMWS in endemic herds, and showed a significant decrease in PMWS cases in unvaccinated groups compared to the vaccinated animals. However, a controlled

laboratory study by Opriessnig et al. (55) was able to induce significantly longer length of viremia and more severe lymphoid lesions but was not able to reproduce clinical PMWS in *Mycoplasma hyopneumoniae* and *Actinobacillus pleuropneumonia* vaccinated and PCV2 inoculated specific-pathogen-free (SPF) piglets. Taken together, PCV2 is generally considered to be the primary, but not the sole, causative agent of PMWS.

We have previously shown that the cloned genomic DNA of PCV2 is infectious when directly injected into the liver and lymph nodes of pigs (23) and the infectious PCV2 DNA clone produced pathological lesions characteristic of PMWS in SPF pigs (23). Here in this study, we report for the first time, that chimeric DNA clones between PCV1 and PCV2 are infectious when transfected into PK-15 cells and expressed respective capsid antigen, and are also infectious when injected directly into the superficial inguinal lymph nodes of SPF piglets. The immunogenicity and pathogenicity of the chimeric infectious DNA clones were characterized in pigs.

MATERIALS AND METHODS

Cell and virus. The PK-15 cell line used in this study (ATCC, CCl-33) was free of PCV1 contamination, as generated by endpoint dilutions (23). The PCV1 virus used in the study originated from the contaminated ATCC PK-15 cell line (65, 68). The PCV2 virus used in the study was originally isolated from a spleen tissue sample of a pig with naturally occurring PMWS (23, 24). The PCV2 infectious DNA clone was described previously (23).

Construction of the non-pathogenic PCV1 infectious DNA clone. The procedure used for the construction of the PCV1 infectious DNA clone is very similar to that for PCV2 (23). Briefly, the primers KPNPCV1.U and KPNPCV1.L (Table 1) were designed based on the published sequence of PCV1 (43) to amplify the complete PCV1 genome, as a 1758 bp PCR product, with an overlapping region containing the unique *Kpn*I restriction enzyme site. The PCV1 DNA was extracted from PK-15 cells (ATCC, CCL-33) persistently infected with PCV1, using the QIAmp DNA minikit (Qiagen, Inc.,Valencia, CA). The extracted DNA was amplified by PCR using Amplitaq Gold Polymerase (Perkin-Elmer, Norwalk, CT). The PCR cycles consisted of an initial step of 95°C for 10 min., followed by 35 cycles of denaturation at 94°C for 1 min., annealing at 48°C for 1 min., extension at 72°C for 2 min., and a final extension at 72°C for 7 min. The PCR product of expected size was separated by gel electrophoresis and purified using a Geneclean Kit (BIO 101, Inc., La Jolla, CA), digested by the *Kpn*I restriction enzyme and cloned into pBluescript SK (pSK) vector (Stratagene, La Jolla, CA). *Escherichia coli* DH5α competent cells were used for transformation. Recombinant plasmids containing the full-length PCV1 genome were isolated with a Qiagen plasmid mini kit (Qiagen, Valencia, CA) and were verified by restriction enzyme digestion. The full-length PCV1 genome was excised from the pSK vector by *Kpn*I digestion, and dimmerized as described previously for PCV2 infectious DNA clone (23) to produce the PCV1 infectious DNA clone. Based on our previous data (23), the dimmerized tandem DNA clone is more efficient to transfect cells and produce infectious virions than the clone containing a single copy of viral genome.

Construction of a chimeric PCV1-2 infectious DNA clone. The ORF2 gene of both PCV1 and PCV2 encodes the immunogenic viral capsid protein (50). To construct a chimeric PCV1-2 DNA clone, the ORF2 capsid gene of PCV1 was replaced with that of the pathogenic PCV2 in the genome backbone of PCV1 (Fig. 1). Briefly, two pairs of PCR primers were designed: the first pair, Psi I-5 and Acl I-6, amplify the PCV2 ORF2 gene, a fragment of 693 bp, and introduced flanking *Psi* I and *Acl* I restriction enzyme sites by point mutations. The PCR reaction for the amplification of PCV2 ORF2 consisted of an initial step at 95°C for 9 min., followed by 38 cycles of denaturation at 95°C for 1 min., annealing at 48°C for 1 min., extension at 72°C for 1 min., and a final extension at 72°C for 7 min. The second PCR primer pair, Hpa I-2 and Nar I-3, amplified the pSK vector and the PCV1 genome without the PCV1 ORF2 (pSK-PCV1 ΔORF2), a fragment of 4023 bp, by using the PCV1 infectious DNA clone as the PCR template, and introduced flanking restriction enzymes sites *Hpa* I and *Nar* I by point mutations. The PCR reaction consisted of an initial step at 95°C for 9 min., followed by 38 cycles of denaturation at 95°C for 1 min., annealing at 50°C for 1 min., extension at 72°C for 3.5 min., and a final extension at 72°C for 7 min. The pSK-PCV1 ΔORF2 PCR product was digested by the *Hpa* I and *Nar* I to produce a sticky end and a blunt end complementary to the PCV2 ORF2 PCR product digested by *Acl* I and *Psi* I restriction enzymes. Once the two PCR products were digested and ligated, all the PCR introduced point mutations used to facilitate cloning steps were removed in the resulting chimeric DNA clone. *Escherichia coli* DH5α competent cells were transformed. The recombinant plasmids containing the chimeric PCV1-2 DNA clone were isolated and were verified by restriction enzyme digestion and partial DNA sequencing. The full-length chimeric

PCV1-2 genome was excised from the recombinant plasmid by *Kpn*I digestion, and dimmerized (23) to produce the PCV1-2 chimeric infectious DNA clone (Fig. 1).

Construction of a reciprocal chimeric PCV2-1 infectious DNA clone. To construct a reciprocal PCV2-1 chimeric DNA clone, the ORF2 capsid gene of PCV2 was replaced by that of the non-pathogenic PCV1 in the genome backbone of the pathogenic PCV2 (Fig. 1). Briefly, two PCR primer pairs were designed: the first pair, Bgl-II-ORF2 and SpH-I-ORF2, amplified the PCV1 ORF2 gene, a fragment at 690 bp, and introduces flanking *Bgl* II and *Sph* I restriction enzyme sites by point mutations. The second PCR primer pair, Bgl-II-PCV2 and SpH-I-PCV2, amplified the pSK vector and the PCV2 genome without the ORF2 gene (pSK-PCV2 ΔORF2), a fragment at 4030 bp, by using the PCV2 infectious DNA clone as the PCR template, and introduced flanking restriction enzymes sites *Bgl* II and *Sph* I by point mutations. The pSK-PCV2 ΔORF2 product and the PCV1 ORF2 PCR product were digested by *Bgl* II and *Sph* I restriction enzymes to produce complementary sticky and blunt ends and subsequently ligated together. After transformation into *E. Coli* cells, the authentic recombinant plasmids were isolated and confirmed by enzyme digestion and partial DNA sequencing. The full-length reciprocal chimeric PCV2-1 genome was excised from the recombinant plasmid by *Sac* II digestion, and dimmerized (23) to produce the reciprocal chimeric PCV2-1 infectious DNA clone.

***In vitro* transfection of PK-15 cells with PCV1, PCV2, chimeric PCV1-2, and reciprocal chimeric PCV2-1 DNA clones.** We have already demonstrated the infectivity of PCV2 DNA clone both *in vitro* and *in vivo* (23). To test the infectivity of the PCV1

and two chimeric clones *in vitro*, PK-15 cells free of PCV1 contamination (23) were grown in 8-well LabTek chambers slides (Nalge Nunc Intl. Denmark). When the PK-15 cells reached about 80% confluency, cells were transfected with PCV1, PCV2 (23), PCV1-2, and PCV2-1 DNA clones, respectively, using the Lipofectamine Plus Reagent according to the protocols supplied by the manufacturer (Life Technologies, Inc.). Mock-transfected cells with pSK+ vector alone were included as controls. Three days after transfection, the cells were fixed with a solution containing 80% acetone and 20% methanol at 4°C for 20 min. Evidence of viral capsid protein expression was confirmed in cells transfected with the PCV1 and PCV2-1 DNA clones by indirect immunofluorescence assay (IFA) using monoclonal antibody against PCV1 ORF2 capsid gene (1). Briefly, the fixed cells were washed with phosphate-buffered saline (PBS) and incubated with a diluted PCV1 monoclonal antibody at 37°C for 1 hour. The cells were then washed three times with PBS buffer and incubated with fluorescein isothiocyanate (FITC) labeled goat anti-mouse immunoglobulin G (Kirkegaard& Perry Laboratories, Inc., Gaithersburg, Md.) at 37°C for 45 min. After washing three times with PBS buffer, the slides were mounted with fluoromount-G, coverslipped, and examined under a fluorescence microscope. The infectivity of cells transfected with the PCV2 and the chimeric PCV1-2 DNA clones were confirmed by IFA using antibody specific for PCV2, as previously described (23).

Transfection and subsequent serial passages of PCV1, PCV2, PCV1-2 and PCV2-1 infectious DNA clones in PK-15 cells. In order to compare transfection efficiency of each PCV DNA clone *in vitro* and to determine the viability of subsequent progeny

viruses, four synchronized PK-15 cell cultures in T-25 flasks were transfected each with 44 μg of PCV1, PCV2, PCV1-2 or PCV2-1 DNA clone, respectively. The transfected cell cultures were grown to confluency and then serially passaged 5 times. At each passage, 1 ml of the suspended cells was harvested, frozen and thawed three times. The virus titers at each passage were determined according to the Kärber method. Briefly, serial 10-fold dilutions of the samples were used to inoculate PK-15 cells grown on 8 well LabTek chamber slides. After three days incubation, the infected PK-15 cells were fixed, and the virus titers were determined by IFA using PCV1 or PCV2 specific antibodies according to the same IFA protocols described above.

One step growth curves of PCV1, PCV2, PCV1-2 and PCV2-1 viruses in PK-15 cells. PCV1-free PK-15 cells were grown on four 12-well plates. Each plate was infected with PCV1, PCV2, PCV1-2 and PCV2-1 live virus, respectively at 0.1 multiplicity of infection (MOI) per well. After 1 hour incubation, the viral inoculum was removed and the cell mono-layer was washed 5 times each with 2 ml of PBS buffer. Maintenance media (2% bovine calf serum and 1X antibiotics) was subsequently added to each well and the infected cells were continuously incubated at 37°C. Every 12 hours, the media and cells from one well of each inoculation group were harvested and frozen down at -80ºC until virus titration. The PCV1, PCV2, PCV1-2 and PCV2-1 virus infectious titers at different time points were determined by IFA specific for detecting PCV1 or PCV2 as described above using the Kärber method.

Experimental inoculation of pigs with PCV1, PCV2, chimeric PCV1-2 and reciprocal chimeric PCV2-1 DNA clones. Forty SPF pigs of 3-4 weeks of age were randomly assigned into five rooms of 8 animals each. Pregnant sows, which the piglets used in this study were derived from, were negative for antibodies to PCV, PRRSV, PPV, or swine hepatits E virus (23, 32). In addition, pre-inoculation serum samples were tested for all piglets by PCR for the presence of PCV1 and PCV2 nucleic acids to confirm that the pigs used in the study are not naturally infected by either virus (56). The PCV1, PCV2, PCV1-2 and PCV2-1 infectious DNA clones were all inoculated by direct injection of the cloned plasmid DNA into the superficial inguinal lymph nodes of pigs as described previously (23). Pigs in group 1 received PBS buffer as negative control. Group 2 animals were each injected with 200 μg of infectious PCV1 DNA clone. Group 3 pigs were each injected with 200 μg of infectious PCV2 DNA clone. Group 4 animals were each injected with 200 μg of chimeric PCV1-2 infectious DNA clone. Group 5 pigs were each injected with 200 μg of the reciprocal chimeric PCV2-1 infectious DNA clone. All animals were monitored daily for clinical signs of disease. Serum samples were collected from each animal at -2, 7, 14, 21, 28, 35, 42, 49 days post-inoculation (DPI). At 21 DPI, four randomly selected animals from each group were necropsied. The remaining four animals in each group were necropsied at 49 DPI.

Clinical evaluation. Pigs were weighed on 0 DPI and at the time of necropsies. Rectal temperatures and clinical respiratory scores, ranging from 0 to 6 (0 = normal; 6 = severe) (25), were recorded every other day from 0 to 49 DPI. Clinical observations, including evidence of central nervous system disease, liver disease (icterus), musculoskeletal

disease, and changes in body condition, were also recorded daily. All clinical evaluations were performed by a team of two people.

Gross pathology and histopathology. Four pigs from each group were necropsied at 21 and 49 DPI, respectively (only 3 pigs from group 4 were necropsied at 21 DPI because one pig died shortly after inoculation). The necropsy team was blinded to infection status of the pigs at necropsy. Complete necropsies were performed on all pigs. An estimated percentage of the lung with grossly visible pneumonia was recorded for each pig based on a previously described scoring system (25). Other lesions such as enlargement of lymph nodes (ranging from 0 = normal to 3 = three times of normal size) were scored separately. Sections for histopathologic examination were taken from nasal turbinate, lungs (seven sections) (25), heart, brain, lymph nodes (tracheobronchial, iliac, mesenteric, subiliac, superficial inguinal), tonsil, thymus, liver, gall bladder, spleen, joints, small intestine, colon, pancreas, and kidney. The tissues were examined in a blinded fashion and given a subjective score for severity of lung, lymph node, and liver lesions (25). Lung scores ranged from 0 (normal) to 3 (severe lymphohistiocytic interstitial pneumonia). Liver scores ranged from 0 (normal) to 3 (severe lymphohistiocytic hepatitis). Lymph node scores were for an estimated amount of lymphoid depletion of follicles ranging from 0 (normal or no lymphoid depletion) to 3 (severe lymphoid depletion and histiocytic replacement of follicles).

Serology. Blood was collected from all pigs at -2, 7, 14, 21, 28, 35, 42, and 49 DPIs. Serum antibodies to PRRSV were assayed using Herd Check PRRSV ELISA (IDEXX

Laboratories, Westbrook, MA). Serum antibodies to PPV were detected by a hemagglutination inhibition (HI) assay (32). Serum antibodies to PCV2 were detected by a modified indirect ELISA based on the recombinant ORF2 capsid protein of PCV2 (49). Serum antibodies to PCV1 were detected by an indirect immunofluorescence assay (IFA). Briefly, PK-15 cells infected with PCV1 were grown on eight-well LabTek chamber slides. When the infected PK-15 cells reach about 95-100% confluency, the cells were fixed with a solution containing 80% acetone and 20% methanol at 4°C for 20 min. The fixed cells were washed once with PBS buffer. One hundred microliters of 1:10 diluted pig serum sample in PBS was added to the chambers, and incubated for 1 hour at 37°C. The cells were then washed three times with PBS and incubated for 45 min. at 37°C with FITC-labeled goat anti-swine secondary antibody. The slides were subsequently washed three times with PBS, mounted with fluoromount-G, cover-slipped and examined under a fluorescent microscope. For the positive control, PCV1 infected cells were incubated with a diluted PCV1 specific monoclonal antibody, followed by an incubation with FITC-labeled goat anti-mouse IgG (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.). For the negative control, PCV1 infected cells were incubated with 1:10 diluted negative swine serum free of PCV1 or PCV2 antibody, followed by an incubation with FITC-labeled goat anti-swine IgG (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.).

PCR. To detect PCV1, PCV2, chimeric PCV1-2 and reciprocal chimeric PCV2-1 viremia in sera from inoculated pigs, serum samples collected at different DPIs were tested by PCR (Fig. 1). Briefly, viral DNA was extracted from 100 μl of each serum sample using

DNAzol reagent according to the manufacture's protocol (Molecular Research Center, Cincinnati, OH). The extracted DNA was resuspended in DNase, RNase, and proteinase-free water. To amplify clone-specific genomic sequences of PCV1, PCV2, chimeric PCV1-2 and reciprocal chimeric PCV2-1, two sets of nested PCR primer pairs were designed (Table 1). The first set of nested primers was designed based on published PCV1 sequences. Primers Gen.PCV1 and Orf.PCV1 amplified a 400 bp fragment in the presence of the PCV1 genome. The nested primers, nested.Gen.PCV1 and nested.Orf.PCV1, amplified a 220 bp fragment.

To detect PCV2 viremia, PCV2 primer pair Gen.PCV2 and Orf.PCV2 amplified a 900 bp fragment in the presence of PCV2 in the first round of PCR. Primers nested.Gen.PCV2 and nested.Orf.PCV2 amplified a 600 bp fragment in the nested PCR.

To detect chimeric PCV1-2 viremia, the first round of PCR reaction employed the PCV1-specific primer Gen.PCV1 and the PCV2 ORF2-specific primer Orf.PCV2 to amplify a chimeric PCV1-2 fragment of 580 bp. For the nested PCR, PCV1-specific primer nested.Gen.PCV1 and the PCV2 ORF2-specific primer nested.Orf.PCV2 were used to amplify a chimeric PCV1-2 fragment of 370 bp.

To detect reciprocal chimeric PCV2-1 viremia, the first round of PCR employed the PCV2-specific primer Gen.PCV2 and the PCV1 ORF2-specific primer Orf.PCV1 to amplify a chimeric PCV2-1 fragment of 700 bp. For the nested PCR, the PCV2-specific primer nested.Gen.PCV2 and the PCV1 ORF2-specific primer nested.Orf.PCV1 were used to amplify a 460 bp chimeric PCV2-1 fragment.

All PCR parameters were essentially the same, consisting of 38 cycles of denaturation at 94°C for 1 min, annealing at 45°C for 1 min, and extension at 72°C for

1.5 min. The serum samples from negative control pigs were tested by a PCR-RFLP diagnostic assay, which can detect and differentiate both PCV1 and PCV2 as described previously (23).

Sequencing. PCR products from selected animals in each inoculation group, except for the PCV2-1 group, were sequenced to confirm the origin of the infecting virus by comparing the sequences recovered from infected pigs to the sequences of the respective infectious DNA clone by MacVector software (Oxford Molecular Ltd., Beaverton, Oregon).

Immunohistochemistry (IHC). IHC detection of PCV2-specific antigen was performed on lymph nodes collected during necropsies at 21 and 49 DPIs. A rabbit polyclonal antiserum against PCV2 was used for the IHC, according to the procedures described previously (60). The amount of PCV2 antigen distributed in the lymph nodes was scored in a blinded fashion by assigning a score of 0, if no signal, to 3 for a strong positive signal. The mean scores of inoculation groups were determined and statistically analyzed. No IHC detection was performed on PCV1 and PCV2-1 inoculated pig lymph nodes due to insufficient amount of the gift PCV1 monoclonal antibody.

RESULTS

The PCV1 clone, PCV1-2 and PCV2-1 chimeric clones are infectious when transfected into PK-15 cells and express expected viral capsid antigen. Two identical copies of the complete PCV1 genome were ligated in tandem into the pSK vector to

produce the PCV1 DNA clone (Fig. 1). The infectivity of the PCV1 DNA clone was determined by *in vitro* transfection of PK-15 cells. IFA using monoclonal antibody against PCV1 ORF2 gene product confirmed that the PCV1 DNA clone is infectious and that about 10-20% of the transfected PK-15 cells were positive for PCV1 capsid antigen in the nucleus of transfected cells (Fig. 2C).

The infectious PCV2 DNA clone was constructed by ligating two tandem copies of PCV2 genome into the pSK+ vector (23, Fig. 1). The PCV2 DNA clone was shown to be infectious *in vitro* and *in vivo* in a previous study (23, Fig. 2F).

The chimeric PCV1-2 DNA clone had the ORF2 capsid gene of PCV1 replaced by that of the pathogenic PCV2 in the backbone of the nonpathogenic PCV1 genome. The infectivity of the PCV1-2 chimeric DNA clone was determined by *in vitro* transfection of PK-15 cells. Therefore, the chimeric PCV1-2 DNA clone should, if infectious *in vitro*, replicate and produce the ORF2 capsid antigen of PCV2. IFA using antibodies against PCV2 confirmed that the PCV1-2 DNA clone is infectious and that about10-20% of the transfected PK-15 cells were positive for PCV2 antigen within the nucleus of infected cells (Fig. 2H).

The reciprocal chimeric PCV2-1 DNA clone had the ORF2 capsid gene of PCV2 replaced by that of the nonpathogenic PCV1 in the backbone of the pathogenic PCV2 genome. Therefore, the reciprocal chimeric PCV2-1 DNA clone should replicate and express PCV1 ORF2 capsid antigen in transfected PK-15 cells, if it is infectious. IFA using PCV1 ORF2-specific monoclonal antibody showed that the PCV2-1 chimeric DNA clone is infectious and that about 10-20% of the transfected PK-15 cells expressed PCV1 ORF2 antigen in the nucleus of transfected cells (Fig. 2I).

In vitro characterization of PCV1, PCV2, PCV1-2 and PCV2-1 infectious DNA clones. To confirm the viability of the progeny viruses after initial transfection and to compare the *in vitro* replication levels of the 4 viruses, four synchronized PK-15 cell cultures were transfected each with 44 μg of PCV1, PCV2, PCV1-2 or PCV2-1 infectious DNA clone, respectively. The infectious titers of the initial transfected cell cultures for all four infectious DNA clones range from $10^{4.33}$ to 10^{5} $TCID_{50}$ /ml (Fig. 3), indicating that PCV1, PCV2, PCV1-2 and PCV2-1 infectious DNA clones all gave rise to similar number of progeny viruses after initial transfection. The infectious virus titers fluctuated within one log during 5 serial passages (Fig. 3)

To further determine the growth characteristics of the 4 viruses, a one-step growth curve was performed simultaneously for each virus. Samples were collected on twelve-hour intervals from cells infected by either PCV1, PCV2, PCV1-2 or PCV2-1 virus stock at a MOI of 0.1. The infectious titers for each virus at different time points were determined by IFA (Fig. 4). The initial titers after infection at 12 hours post-inoculation (h.p.i.) are about $10^{1.5}$ $TCID_{50}$ /ml for all 4 viruses. The infectious titers increase from 12 hr to 96 hr for all 4 viruses. By 96 h.p.i, PCV1 has a titer of $10^{4.33}$ $TCID_{50}$ /ml, while PCV2, PCV1-2 and PCV2-1 infectious titers range from $10^{2.66}$ to $10^{2.33}$ $TCID_{50}$ /ml.

Immunogenicity of PCV1, PCV2, the chimeric PCV1-2 and PCV2-1 infectious DNA clones in pigs. Serum samples collected from all control and inoculated animals at -2, 7, 14, 21, 28, 35, 42, and 49 DPIs were assayed for PCV1, PCV2, PCV1-2, and PCV2-1 viremia by PCR detection of clone-specific DNA sequences, for anti-PCV1 antibody by

IFA, and for anti-PCV2 ORF2 antibody by ELISA. Prior to inoculation at –2 DPI, animals from all five groups were tested negative by PCR for both PCV1 and PCV2 nucleic acid.

Negative control animals were negative for both PCV1 and PCV2 viremia throughout the study (Table 2). Five pigs in the uninoculated control group had detectable PCV2 maternal antibody at -2 DPI and 2 pigs had detectable PCV1 maternal antibodies on 7 DPI (Table 3). The maternal antibodies to both PCV1 and PCV2 in these piglets waned by 21 DPI. No seroconversion to either PCV1 or PCV2 was detected in any of the 8 uninoculated control pigs throughout the study.

In the PCV1 inoculated group, viremia was first detected in an inoculated pig at 7 DPI (Table 2), and was last detected at 35 DPI. Five out of 8 animals inoculated with PCV1 infectious DNA clone were positive for PCV1 viremia. Average length of continuous PCV1 viremia was 0.625 weeks. By 21 DPI, all animals in the PCV1 inoculated group had seroconverted to PCV1 and remained positive to PCV1 antibodies until the end of the study at 49 DPI.

We have previously shown that the PCV2 DNA clone is infectious in pigs (23). In the PCV2 DNA clone inoculated group, PCV2 viremia was first detected at 7 DPI (Table 2). By 21 DPI, all PCV2 inoculated group 3 animals were positive for PCV2 viremia. The average length of PCV2 viremia was 2.12 weeks. Two pigs in the PCV2 inoculated group had detectable low levels of maternal PCV2 antibodies (OD < 0.4) at 7 DPI (Table 3), and the maternal antibodies in these piglets waned by 14 DPI. Seroconversion to PCV2, assayed by a PCV2-specific ELISA, was first detected at 35 DPI. By 42 DPI, all pigs inoculated with PCV2 infectious DNA clone had seroconverted to PCV2.

In group 4 pigs inoculated with PCV1-2 chimeric infectious DNA clone, viremia specific for the chimeric PCV1-2 virus was first detected at 14 DPI (Table 2). Four out of 7 inoculated animals became viremic to PCV1-2 between 14 DPI and 42 DPI. The average length of chimeric PCV1-2 viremia was 1 week. One pig had detectable low levels of maternal PCV2 antibodies at 7 and 14 DPI (OD < 0.4), but the maternal antibody waned by 21 DPI (Table 3). Seroconversion to PCV2 ORF2-specific antibody first occurred at 28 DPI. By 49 DPI, all pigs inoculated with chimeric PCV1-2 infectious DNA clone had seroconverted to PCV2 ORF2-specific antibody.

In pigs inoculated with the reciprocal chimeric PCV2-1 DNA clone, viral DNA specific for PCV2-1 chimeric virus was not detected in serum samples (Table 2). However, by 21 DPI, all animals in group 5 seroconverted to PCV1 antibody.

PCR products amplified from selected pigs in group 2, 3, and 4 were sequenced and confirmed to be the authentic respective infectious DNA clones used in the inoculation in each group (data not shown). No PCR product from group 5 animals injected with the PCV2-1 reciprocal chimeric clone was sequenced since we were unable to detect viremia.

Pathogenicity of PCV1, PCV2, chimeric PCV1-2, and reciprocal chimeric PCV2-1 infectious DNA clones in pigs.

Clinical evaluation. As expected, none of the control or inoculated pigs showed signs of PMWS (wasting, pneumonia, icterus). There were no differences in weight gain or mean rectal temperatures between any of the groups (data not shown). One of the pigs from PCV1-2 inoculated group 4 was found dead the morning following inoculation. No

pathogenic agents were detected and lesions consistent with infectious disease were lacking and it was concluded that the death was not associated with inoculation procedure or the chimeric PCV1-2 virus.

Gross lesions. Pigs in the four inoculated groups 2-5 had variable degrees of gross lesions limited to the lymph nodes (Table 4). Lymph nodes of animals from the uninoculated control group 1 were normal at both 21 and 49 DPIs (Table 4). In PCV1 inoculated group 2 pigs, lymph nodes were grossly normal at 21 DPI, however, mild to moderate enlargement of all lymph nodes was detected at 49 DPI. All PCV2 inoculated group 3 pigs had enlarged tan lymph nodes two to five times the normal size, at both 21 and 49 DPIs. Lymph nodes from chimeric PCV1-2 inoculated animals were mildly to moderately enlarged and tan colored at both 21 and 49 DPIs in 5 out of 7 pigs (Table 4). In group 5 pigs, inoculated with the PCV2-1 clone, 1 out of 8 animals had mildly enlarged and tan lymph nodes at 21 DPI (Table 4). The average scores of gross lesions of the lymph nodes in pigs inoculated with chimeric PCV1-2 clone were not statistically different from those in groups 1, 2, and 5, but were significantly different ($p<0.05$) from those of the pathogenic PCV2 inoculated group 3 pigs at 21 DPI. Average lymph node gross lesion scores on 49 DPI from the PCV1, PCV2, and PCV1-2 inoculated animals were not significantly different from each other, but were all statistically different ($p<0.05$) from the average gross lesion scores of group 1 and 5 pigs (Table 4).

Microscopic lesions. No microscopic lesions were detected in either uninoculated control group 1 pigs or PCV1 inoculated group 2 pigs at any DPI (Table 5). Microscopic lung lesions characterized as mild peribronchiolar lymphoplasmacytic and histiocytic bronchointerstitial pneumonia (Table 5), were observed in 1 out 8 of the PCV2 inoculated

pigs. In the PCV1-2 and PCV2-1 inoculated animals, no microscopic lesions were observed in the lungs. No lesions were observed in the thymuses of any inoculated pigs (Table 5). Mild multifocal lymphoplasmacytic myocarditis was observed in 2 of 8 pigs in the PCV2 inoculated group (Table 5). Heart tissues from PCV1-2 and PCV2-1 inoculated animals were free of microscopic lesions. Mild multifocal lymphoplasmacytic interstitial nephritis was observed in 4 out of 8 pigs in PCV2 inoculated group, in 2 out of 7 PCV1-2 inoculated pigs and in 1 out of 8 PCV2-1 inoculated pigs (Table 5). Mild-to-moderate lymphoid depletion and histiocytic replacement of follicles was observed in the tonsil in 5 out of 8 pigs, in the spleen in 3 out of 8 pigs, and in the lymph nodes in 8 out of 8 pigs in the PCV2-inoculated group. In the chimeric PCV1-2 inoculated animals, mild lymphoid depletion and histiocytic replacement of follicles was observed in the lymph nodes of 2 out of 7 pigs but was not detected in either the spleen or tonsils. No lymphoid depletion and histiocytic replacement of follicles was observed in the lymph nodes, spleen or tonsils of the reciprocal chimeric PCV2-1 inoculated animals (Table 5). Mild-to-moderate lymphoplasmacytic hepatitis was observed in 7 out of the 8 PCV2-inoculated pigs. Mild lymphoplasmacytic hepatitis was observed in 2 out of the 7 chimeric PCV1-2 inoculated pigs. No lymphoplasmacytic hepatitis was observed in reciprocal chimeric PCV2-1 inoculated pigs (Table 5). Lesions in other tissues were unremarkable, except for one animal inoculated with PCV2 that showed mild microscopic lesions in the brain.

Microscopic lesions in the lung, liver, and lymph nodes were scored according to a published scoring system (25). Mean scores of lesions in lymph nodes in pigs from the chimeric PCV1-2 inoculated group 4 were similar to those from group 1, 2, and 5 but

were significantly different ($p<0.05$) from those of the pathogenic PCV2 inoculated group 3 pigs, at both 21 and 49 DPIs (Table 5). Mean microscopic liver lesion scores from the chimeric PCV1-2 inoculated group at 21 DPI were significantly different ($p<0.05$) from those of PCV2 inoculated group 3 animals but were similar to those of group 1, 2, and 5 pigs at 21 DPI. At 49 DPI, the mean microscopic liver scores from group 4 chimeric PCV1-2 inoculated pigs were not statistically different from those of group 1, 2, 3, and 5 pigs.

Detection of PCV antigen. IHC staining of PCV2 specific antigen was performed on lymph nodes of all pigs necropsied at 21 and 49 DPIs. As expected, lymph nodes from the uninoculated control, PCV1 and PCV2-1 inoculated pigs were negative for PCV2 antigen. PCV2 antigen was detected in lymphoid tissues of 4 out of 4 animals in the PCV2 inoculated group at 21 DPI and in 3 out of 4 animals at 49 DPI. PCV2 antigen was also detected in lymphoid tissue of 1 out of 4 pigs from the chimeric PCV1-2 inoculated group at 49 DPI, but no PCV2 antigen was detected in lymphoid tissues of PCV1-2 inoculated animals at 21 DPI (Table 6). At 21 DPI, the mean scores for the estimated amount of PCV2 antigen in PCV1-2 inoculated animals was significantly different ($p<0.05$) from that in PCV2-inoculated pigs but were not significantly different from that in the control group 1. At 49 DPI, the mean IHC score of the PCV1-2 inoculated animals was not significantly different from that of the PCV2 inoculated group 3 or the control group 1.

DISCUSSION

PMWS has become a major global swine disease. It has become a serious economic problem for the swine industry, and there is an urgent need to develop a vaccine against PCV2, the primary causative agent of PMWS. We report here, for the first time, the construction and characterization of a chimeric PCV1-2 infectious DNA clone, and demonstrated that the chimeric PCV1-2 infectious clone induces an immune response specific to the pathogenic PCV2 immunogenic ORF2 capsid protein while it is attenuated in SPF pigs. Animals inoculated via intralymphoid injections with the chimeric PCV1-2 infectious DNA clone developed a mild infection resembling that of PCV1 inoculated animals while seroconverting to antibody to the ORF2 capsid protein of the pathogenic PCV2. The average length of viremia observed in PCV1 and chimeric PCV1-2 inoculated animals was shorter, 0.625 weeks and 1 week respectively, than that in pathogenic PCV2 inoculated animals which was about 2.12 weeks. The lack of detectable chimeric PCV1-2 viremia in sera or viral antigen in lymphoid tissues in some of the inoculated animals did not affect seroconversion to antibody against PCV2 ORF2 capsid protein as all pigs in PCV1-2 inoculated group 4 pigs had seroconverted. The results suggested that, even though the chimeric PCV1-2 viremia and viral antigen were undetectable in some inoculated animals, chimeric PCV1-2 virus was still able to induce antibody response against PCV2 ORF2 capsid protein. Similarly, none of the PCV2-1 inoculated pigs had detectable viremia but they all seroconverted to anti-PCV1 capsid antibody by 49 DPI. The lack of viremia or viral antigen in some PCV1-2 inoculated pigs and the lack of viremia in PCV2-1 inoculated pigs could be due to a lower replication level of the PCV1-2 and PCV2-1 chimeric viruses in pigs. The one-step growth curve

results indicated that PCV1-2 and PCV2-1 chimeric viruses had a similar *in vitro* growth pattern, comparable to that of the wild-type PCV2. Thus, PCV2-1 and PCV1-2 are viable infectious DNA clones capable of producing infectious virions *in vitro*. We found that PCV1 replicated more efficiently than PCV2, PCV1-2 and PCV2-1 in PK-15 cells. This is likely due to the fact that the PCV1 isolate used in this study originated from the PK-15 cell line, and thus it may have already adapted to replicate in the PK-15 cells. After the initial transfection which produced similar infectious virions for all 4 viruses, the serially passaged PCV1, PCV2, PCV1-2 and PCV2-1 viruses replicate well in PK-15 cells although the two chimeric viruses replicate slightly less efficient *in vitro*.

The presence of low levels of maternal antibodies in some pigs did not seem to have any confounding influences on the onset of viremia or seroconversion. Since only a few animals in each experimental group had low levels of maternal antibodies at the time of inoculation, we were unable to make a definitive assessment as to whether or not low levels of maternal antibodies could prevent infection. However, a recent study by Harms et al. (30) showed that a PCV2 maternal antibody titer of 0.6 OD_{405} or above is required to prevent PCV2 replication. The highest maternal antibody titer detected in this study was 0.4 OD_{405} in only one pig, and all other pigs with maternal antibodies had very low titers (<0.4 OD_{405}) at the time of inoculation.

It is known that PCV2 replicates in the lymph nodes, lung and liver, and that one of the major pathogenic effects is the impairment of the immune system by degradation of the lymphoid structures (10, 11, 21, 28, 34, 70). Gross lesions affecting the lymph nodes in both the non-pathogenic PCV1 and the chimeric PCV1-2 inoculated pigs were mild and limited to only a few pigs, whereas the pathogenic PCV2 inoculated pigs all had

moderate-to-severe enlargement and discoloration of lymph nodes (Table 4). The gross lymph node lesions found in PCV1 inoculated pigs at 49 DPI are likely non-specific, which is sometimes seen in pigs, and are not induced by PCV1 since no microscopic lesions in the lymph nodes were found in these same animals at 49 DPI. Statistical analysis revealed that the scores of the gross lesions in the lymph nodes of the chimeric PCV1-2 inoculated animals were similar to those in non-pathogenic PCV1 inoculated pigs. At 21 DPI, PCV2 inoculated pigs had gross lesions that were significantly ($p<0.05$) more severe than those of the PCV1 and of the chimeric PCV1-2 inoculated pigs. Microscopically, at both 21 and 49 DPIs, the chimeric PCV1-2 inoculated animals had significantly ($p<0.05$) fewer and less severe microscopic lesions than the PCV2 inoculated animals. The microscopic lesion scores in lymph nodes of the chimeric PCV1-2 inoculated pigs were similar to those of the non-pathogenic PCV1, the reciprocal chimeric PCV2-1, and the uninoculated control animals. IHC detection of PCV2 antigen within the lymph nodes from inoculated animals showed that the mean score of PCV2 antigen in PCV2 inoculated animals was significantly more intense ($p<0.05$) than that from PCV1-2 inoculated animals, suggesting that during PCV1-2 replication *in vivo*, there is a reduced level of antigen presence within the lymphoid tissues, the main target tissue of pathogenic PCV2. Mild-to-moderate lesions were found in multiple tissues of pathogenic PCV2 inoculated animals including lung, liver, lymphoid, spleen, brain, heart, kidney, and tonsil tissue. However, in the chimeric PCV1-2 inoculated animals, mild to moderate microscopic lesions were limited only to liver, lymph nodes and kidney tissues (Table 5). The microscopic liver lesion score at 21 DPI in chimeric PCV1-2 inoculated pigs was significantly less severe ($p<0.05$) then that of PCV2 inoculated animals.

However, at 49 DPI the microscopic liver lesion scores in both PCV2 and PCV1-2 inoculated animals were not significantly different (p>0.05). It is important to note, however, that the microscopic liver lesion score of chimeric PCV1-2 inoculated animals at 49 DPI is also not significantly different (p>0.05) from the scores of PCV1, PCV2-1 inoculated pigs and of the negative control pigs (Table 5). Our recent studies showed that the pathological lesions in PCV2 infected pigs generally peaks at about 21 to 28 DPI (54, 55), and this could explain why there was no significant difference in liver lesion scores at 49 DPI.

Our previous study showed that the infectious PCV2 DNA clone induced pathological lesions characteristic of PMWS but not clinical PMWS (23). It is generally believed that PCV2 is the primary, but not the sole, pathogenic agent responsible for the onset of clinical PMWS. In most experimental models to date, clinical PMWS was only reproduced in SPF pigs co-infected with PCV2 and either PPV or PRRSV (54, 58), or in PCV2-inoculated gnotobiotic pigs when their immune system was activated by keyhole limpet hemocyanin in incomplete Freund's adjuvant (36) or administration of bacterins (6). In cases of a PRRSV/PCV2 co-infection, the PMWS characteristic pathological signs such as lymphoid depletion, granulomatous inflammation, and necrotizing hepatitis, were induced by PCV2 and not by PRRSV (29). Field studies continue to indicate a reduction of PMWS cases when piglets in PMWS endemic herds with PPV circulation are vaccinated against PPV (26). However, under laboratory conditions, upon challenge with PCV2, PPV vaccination of SPF pigs failed to decrease the occurrence of PMWS lesions compared to unvaccinated animals (54). Therefore, vaccinations against PPV and possibly PRRSV, and thus limiting the infections caused by them, may not prevent the

onset of PMWS in PCV2 infected pigs and a vaccine against PCV2 and PMWS is needed.

Availability of the PCV2, PCV1, chimeric PCV1-2, and reciprocal chimeric PCV2-1 infectious DNA clones enables us to study the structural and functional relationships of PCV genes. In this study, we swapped the immunogenic ORF2 capsid gene between the pathogenic PCV2 and the non-pathogenic PCV1, and showed that the chimeric PCV1-2 infectious DNA clone replicated and expressed the immunogenic ORF2 capsid antigen of PCV2 *in vitro* and *in vivo*, and induced specific antibody response against PCV2 ORF2 but retained the non-pathogenic nature of PCV1. Therefore, the chimeric PCV1-2 infectious DNA clone developed in this study could serve as a useful vaccine candidate against PCV2 infection and PMWS. The chimeric PCV1-2 infectious DNA clone has the ability to induce a strong immune response against PCV2 while inducing only a limited infection with mild pathologic lesions, low levels of viremia and low or non-detectable viral antigen in lymphoid tissues similar to that of the nonpathogenic PCV1. The average ELISA OD values at 49 DPI are 0.511 (with a 0.2 as the cutoff) for the remaining 4 seropositive pigs in chimeric PCV1-2 group, and 0.807 for the remaining 4 seropositive pigs in the PCV2 group, and 0.046 for the remaining 4 seronegative pigs in the negative control group. Therefore, the chimeric PCV1-2 infectious DNA clone induced a relative high anti-PCV2 antibody response that is comparable to that of PCV2. Future studies are warranted to determine if the PCV2 ORF2-specific antibody response induced by the chimeric PCV1-2 infectious DNA clone can protect pigs against challenge with wild-type pathogenic PCV2. The relatively easy storage and stability of cloned DNA and the economy of large-scale chimeric PCV1-2

DNA clone production should provide an attractive means of delivering a live infectious viral DNA vaccine to pigs. However, the intra-lymphoid route of inoculation used in this study is not practical for vaccine delivery and thus, future studies will determine if pigs can be infected by alternate routes such as the intra-muscular injection of the chimeric PCV1-2 infectious DNA clone. The intra-muscular and intra-dermal routes of inoculation have been successful in other studies using viral infectious DNA clones (61, 71).

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REFERENCES

1. **Allan, G.M., D.P. Mackie, J. McNair, B.M. Adair, and M.S. McNulty.** 1994. Production, preliminary characterization and applications of monoclonal antibodies to porcine circovirus. Vet. Immunol. Immunopathol. **43**:357-371.
2. **Allan, G.M., B. Meehan, D. Todd, S. Kennedy, F. McNeilly, J. Ellis, E.G. Clark, J. Harding, E. Espuna, A. Botner, and C. Charreyre.** 1998. Novel porcine circoviruses from pigs with wasting disease syndromes. Vet. Rec. **142**:467-468.
3. **Allan, G.M., F. McNeilly, J.P. Cassidy, G.A. Reilly, B. Adair, W.A. Ellis, and M.S. McNulty.** 1995. Pathogenesis of porcine circovirus; experimental infections of colostrum deprived piglets and examination of pig foetal material. Vet. Microbiol. **44**:49-64.
4. **Allan, G.M., F. McNeilly, J. Ellis, S Krakowka, B. Meeham, I. McNair, I. Walker, and S. Kennedy.** 2000. Experimental infection of colostrums deprived piglets with porcine circovirus 2 (PCV2) and porcine reproductive and respiratory syndrome virus (PRRSV) potentiates PCV2 replication. Arch. Virol. **145**:2421-2429.
5. **Allan, G.M., F. McNeilly, S. Kennedy, B. Daft, E.G. Clarke, J.A. Ellis, D.M. Haines, B.M. Meehan, and B.M. Adair.** 1998. Isolation of porcine circovirus-like viruses from pigs with a wasting disease in the USA and Europe. J. Vet. Diagn. Invest. **10**:3-10.
6. **Allan, G.M., F. McNeilly, S. Kennedy B. Meeham, J. Ellis, S. Krakowka**. 2000. Immunostimulation, PCV-2 and PMWS. Vet. Rec. **147:**170-171
7. **Allan, G.M., F. McNeilly, I. McNair, M. O'Conner, B.M. Meehan, D. Gilpin, J.A. Ellis, H. Townsend. C. Lasagna, G. Boriosi, and S. Krakowka.** 2001.

Neonatal vaccination for Mycoplasma hyopneumoniae and postweaning multisystemic wasting syndrome: a field trial. Pig J. **48**:34-41.

8. **Allan, G.M., F. McNeilly, B.M. Meehan, J.A. Ellis, T.J. Conner, I. McNair, S. Krakowka, and S. Kennedy.** 2000. A sequential study of experimental infection of pigs with porcine circovirus and porcine parvovirus: immunostaining of cryostat sections and virus isolation. J. Vet. Med. B Infect. Dis. Vet. Public Health. **47**:81-94.
9. **Allan, G.M., F. McNeilly, B.M. Meehan, S. Kennedy, D.P. Mackie, J.A. Ellis, E.G. Clark, E. Espuna, N. Saubi, P. Riera, A. Botner, and C.E. Charreyre.** 1999. Isolation and characterisation of circoviruses from pigs with wasting syndromes in Spain, Denmark and Northern Ireland. Vet. Microbiol. **66**:115-23.
10. **Allan, G.M., S. Kennedy, F. McNeilly, J.C. Foster, J.A. Ellis, S.J. Krakowka, B.M. Meehan, and B.M. Adair.** 1999. Experimental reproduction of severe wasting disease by co-infection of pigs with porcine circovirus and porcine parvovirus. J. Comp. Pathol. **121**:1-11.
11. **Allan, G.M. and J.A. Ellis.** 2000. Porcine circoviruses: a review. J. Vet. Diagn. Invest. **12**:3-14.
12. **Bassami, M.R., D. Berryman, G.E. Wilcox, and S.R. Raidal.** 1998. Psittacine beak and feather disease virus nucleotide sequence analysis and its relationship to porcine circovirus, plant circoviruses, and chicken anaemia virus. Virology **249**:453-459.
13. **Balasch, M., J. Segales, C. Rosell, M. Domingo, A. Mankertz, A. Urniza, and J. Plana-Duran.** 1999. Experimental inoculation of conventional pigs with tissue

homogenates from pigs with post-weaning multisystemic wasting syndrome. J. Comp. Pathol. **121**:139-148.

14. **Biagini, P., P. Gallian, H. Attoui, M. Touinssi, J-F. Cantaloube, P. de Micco, and X. de Lamballerie.** 2001. Genetic analysis of full-length genomes and subgenomic sequences of TT virus-like mini virus human isolates. J. Gen. Virol. **82**: 379-383.

15. **Bøtner, A., S. Ladekjaer-Mikkelsen, J. Nielsen, S. Krakowka, J. Ellis, F. McNeilly, G. Allan, and Y. Storgaard.** 2002. Reproduction of PMWS in immunostimulated and non-immunostimulated conventional 3-week-old piglets experimentally infected with PCV2. Proceedings of the conference for ssDNA viruses of plant, birds, pigs, and primates-Saint-Malo, France, p. 132.

16. **Cheung, A.K.** 2003. Transcriptional analysis of porcine circovirus. J. Virol. **305**:168-180.

17. **Choi, C., C. Chae, and E.G. Clark.** 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.

18. **Dulac, G.C. and A. Afshar.** 1989. Porcine circovirus antigens in PK-15 cell line (ATCC CCL-33) and evidence of antibodies to circovirus in Canadian pigs. Can. J. Vet. Res. **53**:431-433.

19. **Edwards, S. and J.J. Sands.** 1994. Evidence of circovirus infection in British pigs. Vet. Rec. **134**:680-1.

20. **Ellis, J., L. Hassard, E. Clark, J. Harding, G. Allan, P. Willson, J. Strakappe, K. Martin, F. McNeilly, B. Meehan, D. Todd, and D. Haines.** 1998. Isolation of circovirus from lesions of pigs with postweaning multisystemic wasting syndrome. Can. Vet. J. **39**:44-51.

21. **Ellis, J., S. Krakowka, M. Lairmore, D. Haines, A. Bratanich, E. Clark, G. Allan, C. Konoby, L. Hassard, B. Meehan, K. Martin, J. Harding, S. Kennedy, and F. McNeilly.** 1999. Reproduction of lesions of postweaning multisystemic wasting syndrome in gnotobiotic piglets. J. Vet. Diagn. Invest. **11**:3-14.

22. **Ellis, J.A., A. Bratanich, E.G. Clark, G. Allan, B. Meehan, D.M. Haines, J. Harding, K.H. West, S. Krakowka, C. Konoby, L. Hassard, K. Martin, and F. McNeilly.** 2000. Coinfection by porcine circoviruses and porcine parvovirus in pigs with naturally acquired postweaning multisystemic wasting syndrome. J. Vet. Diagn. Invest. **12**:21-27.

23. **Fenaux M, P. G. Halbur, G. Haqshenas, R. Royer, P. Thomas, P. Nawagitgul, M. Gill, T. E. Toth, and X. J. Meng.** 2002. Cloned genomic DNA of type 2 porcine circovirus is infectious when injected directly into the liver and lymph nodes of pigs: characterization of clinical disease, virus distribution, and pathologic lesions. J. Virol. **76**: 541-551.

24. **Fenaux M, P.G. Halbur, M. Gill, T.E. Toth, and X.J. Meng.** 2000. Genetic characterization of type 2 porcine circovirus (PCV-2) from pigs with postweaning multisystemic wasting syndrome in different geographic regions of North America and development of a differential PCR-restriction fragment length polymorphism

assay to detect and differentiate between infections with PCV-1 and PCV-2. J. Clin. Microbiol. **38**: 2494-503.

25. **Halbur P.G., P.S. Paul, M.L. Frey, J. Landgraf, K. Eernisse, X.J. Meng, M.A. Lum, J.J. Andrews, and J.A. Rathje.** 1995. Comparison of the pathogenicity of two U.S. porcine reproductive and respiratory syndrome virus isolates with that of the Lelystad virus. Vet. Patlhol. **32**:648-660.

26. **Halbur P.G.**2001. PMWS Improved models and findings of clinical significance. Proc. of Swine Disease Conference for Swine Practitioners. **9**:162-166.

27. **Hamel, A.L., L.L. Lin, and G.P. Nayar.** 1998. Nucleotide sequence of porcine circovirus associated with postweaning multisystemic wasting syndrome in pigs. J. Virol. **72**:5262-5267.

28. **Harding, J.C. and E.G. Clark.** 1997. Recognizing and diagnosing postweaning multisystemic wasting syndrome (PMWS). Swine Health and Production. **5**:201-203

29. **Harms, P.A., S.D. Sorden, P.G. Halbur, S.R. Bolin, K.M. Lager, I. Morozov, P.S. Paul.** 2001. Experimental reproduction of severe disease in CD/CD pigs concurrently infected with type 2 porcine circovirus and porcine reproductive and respiratory syndrome virus. Vet. Pathol. **38**:528-539.

30. **Harms, P.A., S.D. Sorden, P.G. Halbur, P. Nawagitgal, K. Lagger, S.R. Bolin, P.S. Paul.** 2002. Role of maternal immunity to PCV2 and PRRSV co-infection in the pathogenesis of PMWS. Proc. Am. Assoc. Swine. Vet. **33**: 307-311.

31. **Hines, R.K. and P.D. Lukert.** 1995. Porcine circovirus: a serological survey of swine in the United States. Swine Health and Production. **3**:71-73.

32. **Joo H.S., C.R. Donaldson-Wood, R.H. Johnson.** 1976**.** A standardized haemagglutination inhibition test for porcine parvovirus antibody. Aust. Vet. J. **52**:422-4.

33. **Kennedy, S., G. Allan, F. McNeilly, B.M. Adair, A. Hughes, and P. Spillane.** 1998. Porcine circovirus infection in Northern Ireland. Vet. Rec. **142**:495-6.

34. **Kennedy, S., D. Moffett, F. McNeilly, B. Meehan, J. Ellis, S. Krakowka, and G.M. Allan.** 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.

35. **Kiupel, M., G.W. Stevenson, S.K. Mittal, E.G. Clark, and D.M. Haines.** 1998. Circovirus-like viral associated disease in weaned pigs in Indiana. Vet. Pathol. **35**:303-307.

36. **Krakowka, S., J.A. Ellis, F. McNeilly, S. Ringler, D.M. Rings, and G. Allen.** 2001. Activation of the immune system is the pivotal event in the production of wasting disease in pigs infected with porcine circovirus-2 (PCV-2). Vet. Pathol. **38**:31-42.

37. **Kyriakis, S.C., K. Saoulidis, S. Lekkas, Ch.C. Miliotis, P.A. Papoutsis, S. Kennedy.** 2002. The effects of immuno-modulation an the clinical and pathological expression of postweaning multisystemic wasting syndrome. J. Comp. Pathol. **126**: 38-46.

38. **Ladekjaer-Mikkelsen, A.S., J. Nielsen, T. Stadejek, T. Storgaard, S. Krakowka, J. Ellis, F. McNeilly, G. Allan, A. Botner.** 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.

39. **Larochelle, R., M. Morin, M. Antaya, and R. Magar.** 1999. Identification and incidence of porcine circovirus in routine field cases in Quebec as determined by PCR. Vet. Rec. **145**:140-142.

40. **Mankertz, J., H.J. Buhk, G. Blaess, and A. Mankertz.** 1998. Transcription analysis of porcine circovirus (PCV). Virus Genes **16**:267-276.

41. **Mankertz, A., M. Domingo, J.M. Folch, P. LeCann, A. Jestin, J. Segales, B. Chmielewicz, J. Plana-Duran, and D. Soike.** 2000. Characterisation of PCV-2 isolates from Spain, Germany and France. Virus Res. **66**:65-77.

42. **Mankertz A., K. Hattermann, B. Ehlers, and D. Soike.** 2000**.** Cloning and sequencing of columbid circovirus (CoCV), a new circovirus from pigeons. Arch. Virol. **145**:2469-2479.

43. **Meehan, B.M., J.L. Creelan, M.S. McNulty, and D. Todd.** 1997. Sequence of porcine circovirus DNA: affinities with plant circoviruses. J. Gen. Virol. **78**:221-227.

44. **Meehan, B.M., F. McNeilly, D. Todd, S. Kennedy, V.A. Jewhurst, J.A. Ellis, L.E. Hassard, E.G. Clark, D.M. Haines, and G.M. Allan.** 1998. Characterization of novel circovirus DNAs associated with wasting syndromes in pigs. J. Gen. Virol. **79**:2171-2179.

45. **Miyata, H., H. Tsunoda, A. Kazi, A. Yamada, M.A. Khan, J. Murakami, T. Kamahora, K. Shiraki, and S. Hino.** 1999. Identification of a novel GC-rich

113-nucleotide region to complete the circular, single-stranded DNA genome of TT virus, the first human circovirus. J. Virol. **73**:3582-3586.

46. **Moen, E.M., Huang, L., Grinde B.,** 2001. Molecular epidemiology of TTV-like mini virus in Norway. Arch. Virol. **147**: 181-185.

47. **Morozov, I., T. Sirinarumitr, S.D. Sorden, P.G. Halbur, M.K. Morgan, K.J. Yoon, and P.S. Paul.** 1998. Detection of a novel strain of porcine circovirus in pigs with postweaning multisystemic wasting syndrome. J. Clin. Microbiol. **36**:2535-2541.

48. **Muirhead, M.** 2002. Sources of information on PMWS/PDNS. Vet. Rec. **150:**456

49. **Nawagitgul, P., P.A. Harms, I. Morozov, B.J. Thacker, S.D. Sorden, C. Lekcharoensuk, and P.S. Paul.** 2002. Modified indirect porcine circovirus (PCV) type 2-based and recombinant capsid protein (ORF2)-based ELISA for the detection of antibodies to PCV. Clin Diagn Lab Immunol. **1**:33-40

50. **Nawagitgul, P., I. Morozov, S.R. Bolin, P.A. Harms, S.D. Sorden, and P.S. Paul.** 2000. Open reading frame 2 of porcine circovirus type 2 encodes a major capsid protein. J. Gen. Virol. **81**:2281-2287.

51. **Nayar, G.P., A.L. Hamel, L. Lin, C. Sachvie, E. Grudeski, and G. Spearman.** 1999. Evidence for circovirus in cattle with respiratory disease and from aborted bovine fetuses. Can. Vet. J. **40**:277-278.

52. **Nishizawa, T., H. Okamoto, K. Konishi, H. Yoshizawa, Y. Miyakawa, and M. Mayumi.** 1997. A novel DNA virus (TTV) associated with elevated transaminase levels in posttransfusion hepatitis of unknown etiology. Biochem. Biophys. Res. Commun. **241**:92-97.

53. **Onuki, A., K. Abe, K. Togashi, K. Kawashima, A. Taneichi, and H. Tsunemitsu.** 1999. Detection of porcine circovirus from lesions of a pig with wasting disease in Japan. J. Vet. Med. Sci. **61**:1119-1123.

54. **Opriessnig, T., M. Fenaux, S. Yu, R.B. Evans, D. Cavanaugh, J.M. Gallup, F.J. Pallares, E.L. Thacker, K.M. Lagger, X.J. Meng and P.G. Halbur.** 2003. Effect of porcine parvovirus vaccination on the development of PMWS in segregated early weaned pigs coinfected with type 2 porcine circovirus and porcine parvovirus. Vet. Microbiol. **98**: 209-220

55. **Opriessnig, T., S. Yu, J.M. Gallup, R.B. Evans, M. Fenaux, F.J. Pallares, E.L. Thacker, C.W. Brockus, M.R. Ackerman, P. Thomas, X.J. Meng and P.G. Halbur.** 2003. Effects of vaccination with selective bacterins on conventional pigs infected with wild type 2 porcine circovirus. Vet. Pathol. **40**: 521-9

56. **Pogranichnyy,R. M., K.-J. Yoon, P.A. Harms, S.L. Swenson, J.J. Zimmerman, and S.D. Sorden**. 2000. Characterization of immune response of young pigs to porcine circovirus type 2 infection. Viral Immunol.**13**:143-153.

57. **Quintana, J., M. Balasch, J. Segales, M. Calsamiglia, G.M. Rodriguez-Arrioja, J. Plana-Duran, M. Domingo.** 2002. Experimental inoculation of porcine circovirus type 1 (PCV1) and type 2 (PCV2) in rabbits and mice. Vet. Res. **33**:229-237.

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

59. **Rosell, C., J. Segales, J.A. Ramos-Vara, J.M. Folch, G.M. Rodriguez-Arrioja, C.O. Duran, M. Balasch, J. Plana-Duran, and M. Domingo.** 2000. Identification of porcine circovirus in tissues of pigs with porcine dermatitis and nephropathy syndrome. Vet. Rec. **146**:40-43.

60. **Sorden S.D., P.A. Harms, P. Nawagitgul, D. Cavanaugh, and P.S. Paul.** 1999. Development of a polyclonal-antibody-based immunohistochemical method for the detection of type 2 porcine circovirus in formalin-fixed, paraffin-embedded tissue. J. Vet. Diagn. Invest. **11**:528-530.

61. **Sparger, E.E., H. Louie, A.M. Ziomeck, and P.A. Luciw.** 1997. Infection of cats by injection with DNA of feline immunodeficiency virus molecular clone. Virology **238**:157-160

62. **Spillane, P., S. Kennedy, B. Meehan, and G. Allan.** 1998. Porcine circovirus infection in the Republic of Ireland. Vet. Rec. **143**:511-512**.**

63. **Takahashi, K., Y. Iwasa, M. Hijikata, and S. Mishiro.** 2000. Identification of a new human DNA virus (TTV-like mini virus, TLMV) intermediately related to TT virus and chicken anemia virus. Arch. Virol. **145**:979-93.

64. **Tischer, I., H. Gelderblom, W. Vettermann, and M.A. Koch.** 1982. A very small porcine virus with circular single-stranded DNA. Nature **295**:64-66.

65. **Tischer, I., W. Mields, D. Wolff, M. Vagt, and W. Griem.** 1986**.** Studies on epidemiology and pathogenicity of porcine circovirus. Arch. Virol. **91**:271-276.

66. **Tischer, I., L. Bode, D. Peters, S. Pociuli, and B. Germann.** 1995. Distribution of antibodies to porcine circovirus in swine populations of different breeding farms. Arch. Virol. **140**:737-743.

67. **Tischer, I., L. Bode, J. Apodaca, H. Timm, D. Peters, R. Rasch, S. Pociuli, and E. Gerike.** 1995. Presence of antibodies reacting with porcine circovirus in sera of humans, mice, and cattle. Arch. Virol. **140**:1427-1439.

68. **Tischer, I., R. Rasch, and G. Tochtermann.** 1974. Characterization of papovavirus and picornavirus-like particles in permanent pig kidney cell lines. Zentralbl. Bakteriol. Hyg. A **226**:153-167.

69. **Umemura T., A.E. Yeo, A. Sottini, D. Moratto, Y. Tanaka, R.Y. Wang, J.W. Shih, P. Donahue, D. Primi, H.J. Alter.** 2001. SEN virus infection and its relationship to transfusion-associated hepatitis. Hepathology. **33**: 1303-1311.

70. **Wellenberg G.J., S. Pesh, F.W. Berndsen, P.J. Steverink, W. Hunneman, T.J. Van der Vorst, N.H. Peperkamp, V.F. Ohlinger, R. Schippers, J.T. Van Oirschot, and M.F. de Jong.** 2000. Isolation and characterization of porcine circovirus type 2 from pigs showing signs of post-weaning multisystemic wasting syndrome in the Netherlands. Vet. Quart. **22**:167-72.

71. **Willems, L., D. Portetelle, P Kerkhofs, G. Chen, A. Burney, M Mammerickx, and R. Kettmann.** 1992. In vivo transfection of bovine leukemia provirus into sheep. Virology **189**:775-777.

72. **Wilson, L.E., T. Umemeru, J. Astemborski, S.C. Ray, H.J. Alter, S.A. Strathdee, D. Vlahov, D.L. Thomas**. 2001. Dynamics of SEN virus infection among injection drug users. J. infect. Dis. 184: 1315-1319.

Fig. 1

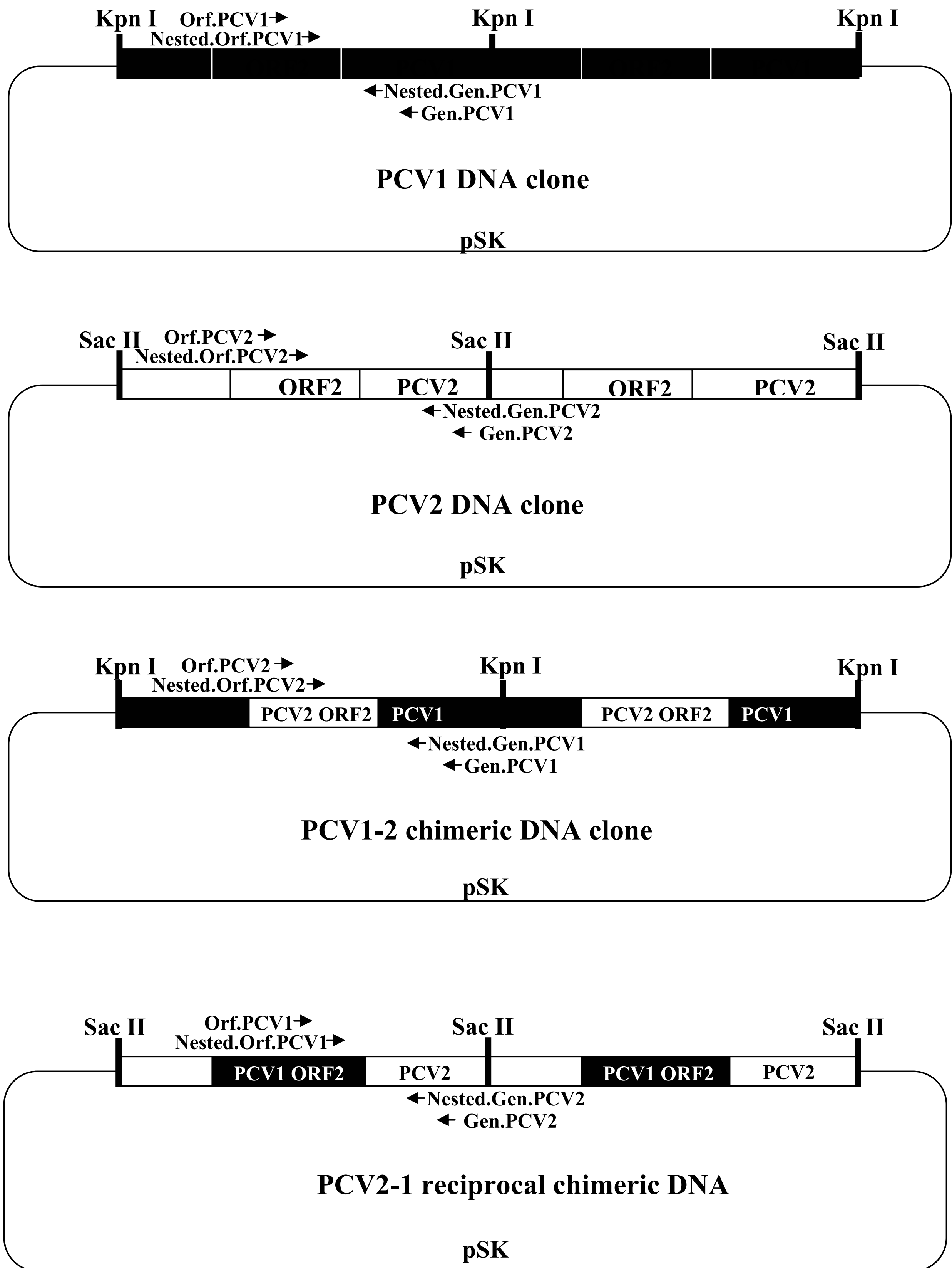

FIG. 1. Organization of the infectious DNA clones. The PCV2 DNA clone was constructed by ligating two full-length linear PCV2 genomes in tandem into the pBluescript SK vector (pSK) as described previously (23). PCV1 DNA clone was constructed by ligating two full-length linear PCV1 genomes in tandem into pSK vector. Chimeric PCV1-2 DNA clone was constructed by replacing the ORF2 capsid gene of PCV1 with that of the PCV2 in the non-pathogenic PCV1 genomic backbone in pSK vector. Reciprocal chimeric PCV2-1 DNA clone was constructed by replacing the ORF2 capsid gene of the pathogenic PCV2 with that of the non-pathogenic PCV1 in the PCV2 genomic backbone in pSK vector. Both chimeric clones were dimmers in pSK vector. The arrows represent the relative locations of the PCR primers used for the detection of PCV1, PCV2, PCV1-2, and PCV2-1 viremia in inoculated animals.

Fig. 2

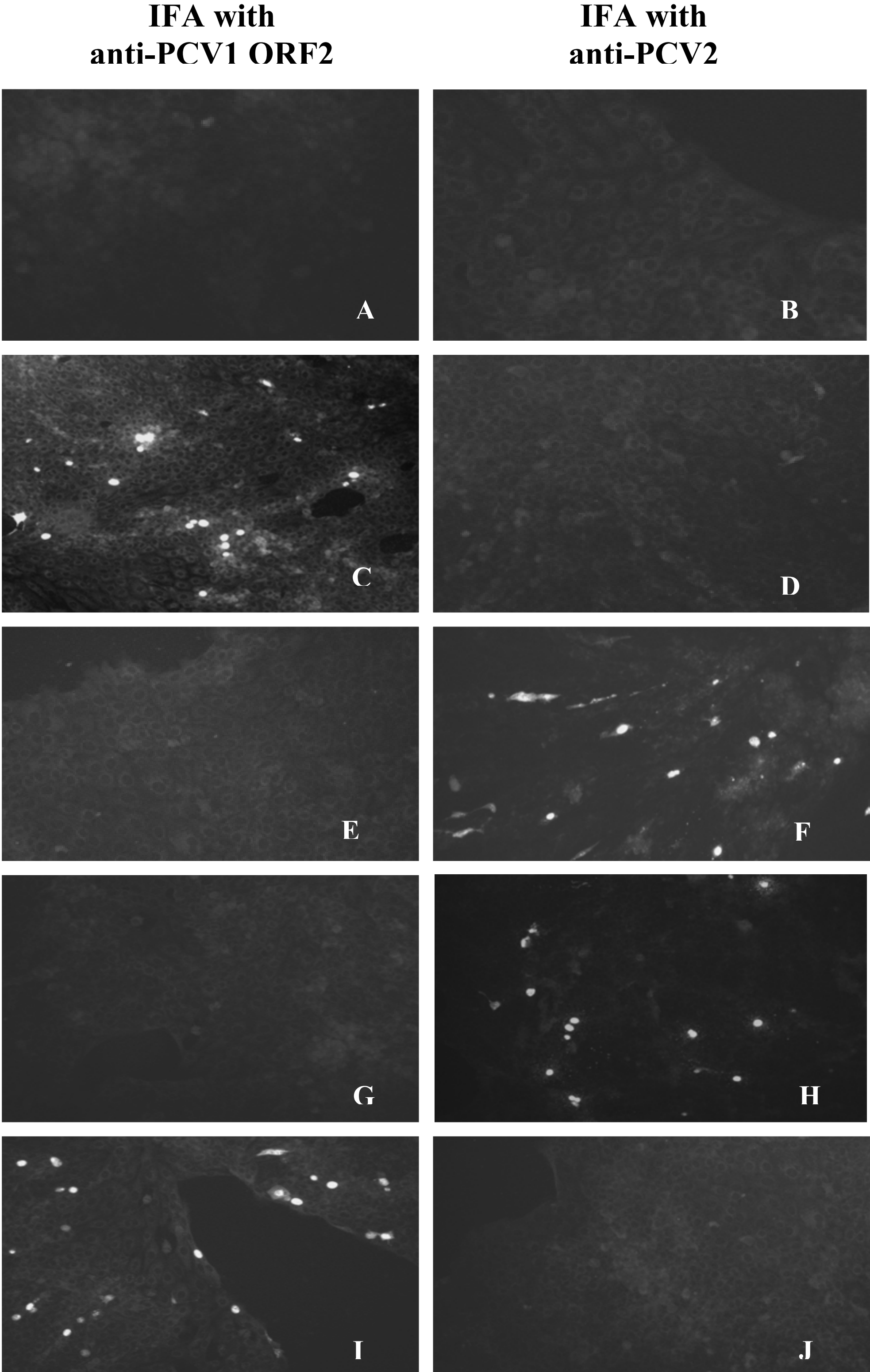

FIG. 2. The PCV1, PCV2, chimeric PCV1-2, and reciprocal chimeric PCV2-1 DNA clones are infectious and expressed respective viral antigens when transfected *in vitro* in PK-15 cells. The left panel (A, C, E, G, I) was stained with monoclonal antibody against the PCV1 ORF2. The right panel (B, D, F, H, J) was stained with antibody against PCV2. (A, B) Mock transfected PK-15 cells. (C, D) PK-15 cells transfected with the PCV1 DNA clone. (E, F) PK-15 cells transfected with the PCV2 DNA clone. (G, H) PK-15 cells transfected with the chimeric PCV1-2 DNA clone. (I, J) PK-15 cells transfected with the reciprocal chimeric PCV2-1 DNA clone.

Fig. 3

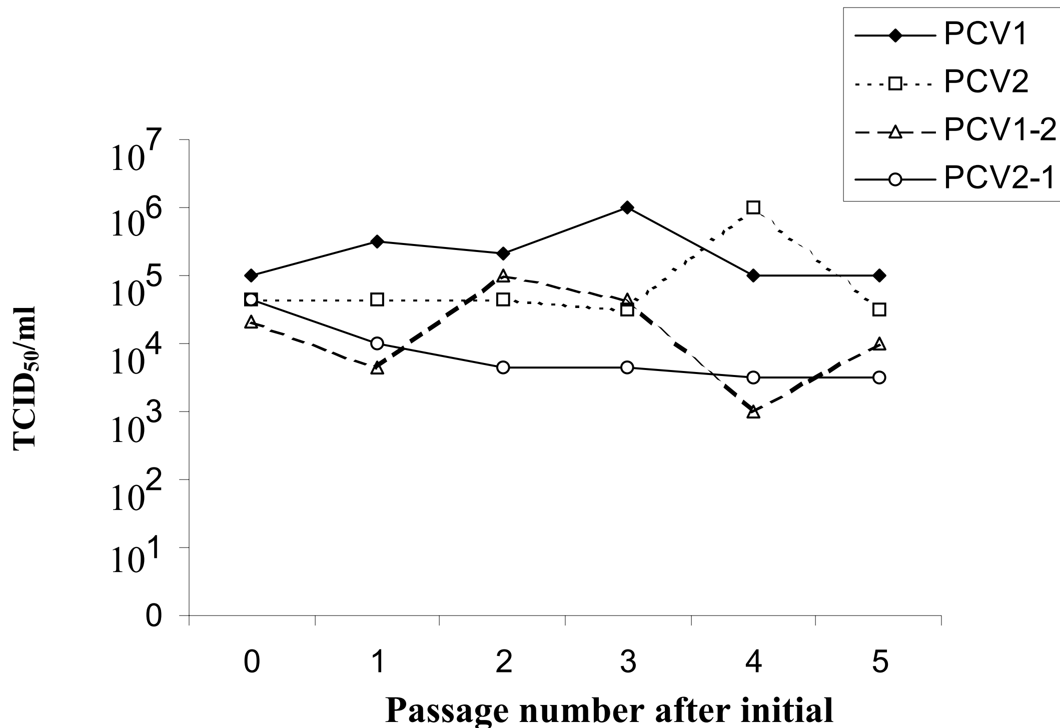

FIG. 3. *In vitro* transfection of PK-15 cells with PCV1, PCV2, PCV1-2 and PCV2-1 DNA clones followed by 5 serial passages of the 4 viruses in PK-15 cells. Synchronized PK-15 cells were transfected each with 44 μg of PCV1, PCV2, PCV1-2 or PCV2-1 DNA clones, respectively. The PCV1, PCV2, PCV1-2 and PCV2-1 had similar infectious viral titers after the initial transfection. The infectious titers were determined at each passage according to the Käber method.

Fig. 4

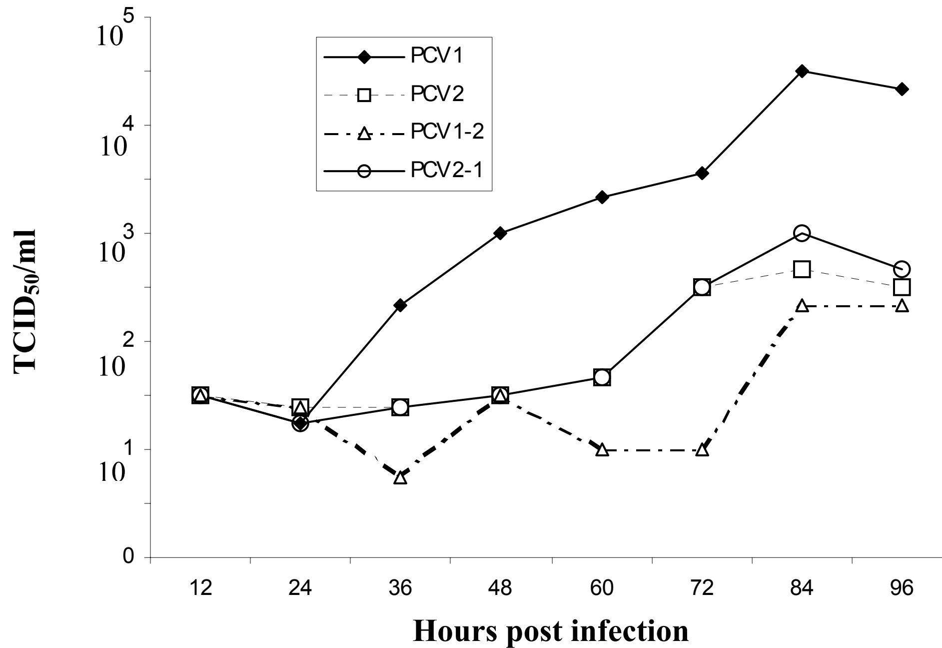

FIG. 4. One-step growth curve of PCV1, PCV2, PCV1-2 and PCV2-1 viruses. Synchronized PK-15 cell cultures were infected each with PCV1, PCV2, PCV1-2 or PCV2-1 virus all at a MOI of 0.1. All four viruses had a titer of about $10^{1.5}$ $TCID/_{50}$ at 12 hours post inoculation. PCV1 replicates more efficiently *in vitro* than PCV2, PCV1-2 and PCV2-1 viruses.

Table 1. Oligonucleotide primers used in this study

Primer	Primer sequence	Application
Primers for infectious DNA clone construction:		
KPNPCV1.U.	>[a]5’-TTTGGTACCCGAAGGCCGATT-‘3	PCV1 DNA clone construction
KPNPCV1.L.	<5’-ATTGGTACCTCCGTGGATTGTTCT-‘3	PCV1 DNA clone construction
Hpa I-2	<5’-GAAGTTAACCCTAAATGAATAAAAATAAAAACCATTACG-‘3	PCV1-2 DNA clone onstruction
Nar I-3	>5’-GGTGGCGCCTCCTTGGATACGTCATCCTATAAAAGTG-‘3	PCV1-2 DNA clone construction
Psi I-5	>5’-AGGTTATAAGTGGGGGGTCTTTAAGATTAA-‘3	PCV1-2 DNA clone construction
Acl I-6	<5’-GGAAACGTTACCGCAGAAGAAGACACC-‘3	PCV1-2 DNA clone construction
Bgl-II-ORF2	>5’-ACTATAGATCTTTATTCATTTAGAGGGTCTTTCAG-‘3	PCV2-1 DNA clone construction
SpH-I-ORF2	<5’-TACGGGCATGCATGACGTGGCCAAGGAGG-‘3	PCV2-1 DNA clone construction
Bgl-II-PCV2	<5’-AGACGAGATCTATGAATAATAAAAACCATTACGAAG-‘3	PCV2-1 DNA clone construction
SpH-I-PCV2	>5’-CGTAAGCATGCAGCTGAAAACGAAAGAAGTG-‘3	PCV2-1 DNA clone construction
Primers for detection of clone-specific viral sequences in pigs:		
MCV1	>5’-GCTGAACTTTTGAAAGTGAGCGGG-‘3	PCV1 and PCV2 detection
MCV2	<5’-TCACACAGTCTCAGTAGATCATCCCA-‘3	PCV1 and PCV2 detection
Orf.PCV1	<5’-CCAACTTTGTAACCCCCTCCA-‘3	PCV1 and PCV2-1 detection
Gen.PCV1	>5’-GTGGACCCACCCTGTGCC-‘3	PCV1 and PCV1-2 detection
Nested.Orf.PCV1	<5’-CCAGCTGTGGCTCCATTTAA-‘3	PCV1 and PCV2-1 detection
Nested.Gen.PCV1	>5’-TTCCCATATAAAATAAATTACTGAGTCTT-‘3	PCV1 and PCV1-2 detection
Orf.PCV2	<5’-CAGTCAGAACGCCCTCCTG-‘3	PCV2 and PCV1-2 detection
Gen.PCV2	>5’-CCTAGAAACAAGTGGTGGGATG-‘3	PCV2 and PCV2-1 detection
Nested.Orf.PCV2	<5’-TTGTAACAAAGGCCACAGC-‘3	PCV2 and PCV1-2 detection
Nested.Gen.PCV2	>5’-GTGTGATCGATATCCATTGACTG-‘3	PCV2 and PCV2-1 detection

[a]Primer direction.

Table 2. Detection of viremia by nested PCR in sera of inoculated and control pigs

Group	Inoculum[a]	DPI								Total
		-2	7	14	21	28	35	42	49	
1	PBS	0/8[b]	0/8	0/8	0/8	0/4	0/4	0/4	0/4	0/8
2	PCV1 DNA	0/8	1/8	1/8	2/8	0/4	2/4	0/4	0/4	5/8
3	PCV2 DNA	0/8	3/8	6/8	7/8	1/4	2/4	2/4	0/4	8/8
4	PCV1-2 DNA	0/8	0/7	1/7	2/7	2/4	2/4	2/4	0/4	4/7
5	PCV2-1 DNA	0/8	0/8	0/8	0/8	0/4	0/4	0/4	0/4	0/8

[a]Phosphate buffered saline (PBS) used as negative control; Cloned genomic PCV or chimeric PCV DNA in pSK plasmid
[b]Eight pigs in each group; number positive/number tested, one pig died in group 4 shortly after inoculation.

Table 3. Seroconversion to antibodies against PCV2 in pigs inoculated with PCV2 or chimeric PCV1-2 infectious DNA clones and seroconversion to antibodies against PCV1 in pigs inoculated with PCV1 or reciprocal chimeric PCV2-1 infectious DNA clones

Group	Inoculum[a]	Antibody tested for[b]	DPI[c] -2	7	14	21	28	35	42	49
1	PBS	PCV1	NA	2/8	2/8	1/8	0/4	0/4	0/4	0/4
		PCV2	5/8	5/8	2/8	0/8	0/4	0/4	0/4	0/4
2	PCV1 DNA	PCV1 ORF2	NA	3/8	2/8	8/8	4/4	4/4	4/4	4/4
3	PCV2 DNA	PCV2	3/8	2/8	0/8	0/8	0/4	3/4	4/4	4/4
4	PCV1-2 DNA	PCV2	2/8	1/7	1/7	0/7	1/4	1/4	3/4	4/4
5	PCV2-1 DNA	PCV1 ORF2	NA	3/8	3/8	7/8	3/4	3/4	4/4	4/4

[a]Phosphate buffered saline (PBS) used as negative control. The inocula were infectious DNA clones in pSK plasmid

[b]PCV1 antibody to ORF2 was measured with an indirect immunofluorescence assay specific for PCV1 antigen. PCV2 antibody was measured with an enzyme-linked immunosorbent assay.

[c]Days post inoculation (DPI), number positive/number tested, one pig died in group 4 shortly after inoculation

Table 4. Gross lymph node lesions in control and inoculated pigs

Group	Inoculum[a]	DPI[b]	
		21	49
1	PBS	0/4(0.0)[Ic]	0/4(0.0)[I]
2	PCV1 DNA	0/4(0.0)[I]	4/4(1.5)[II]
3	PCV2 DNA	4/4(2.5)[II]	4/4(2.25)[II]
4	PCV1-2 DNA	2/3(0.66)[I]	3/4(1.25)[II]
5	PCV2-1 DNA	1/4(0.25)[I]	0/4(0.0)[I]

[a]Phosphate buffered saline (PBS) used as negative control. The inocula were infectious DNA clones in pSK plasmid.
[b]Four pigs from each group were necropsied at 21 days post inoculation (DPI) and the remaining pigs were necropsied at 49 DPI;
Number with enlarged lymph nodes/number necropsied at each day post inoculation (mean scores for estimated enlargement).
[c]Different superscripts (I,II) within each column indicate significantly different mean value score between groups.

Table 5. Distribution of histopathological lesions in different tissues/organs from control and inoculated pigs

Group	Inoculum[a]	DPI[b]	Lung[c]	Liver[d]	Lymph nodes[e]	Spleen	Thymus	Ileum	Brain	Heart	Kidney	Tonsil
1	PBS	21	0/4(0.0)^{I}	0/4(0.0)^{I}	0/4(0.0)^{I}	0/4	0/4	0/4	0/4	0/4	0/4	0/4
		49	0/4(0.0)^{I}	0/4(0.0)^{I}	0/4(0.0)^{I}	0/4	0/4	0/4	0/4	0/4	0/4	0/4
2	PCV1 DNA	21	0/4(0.0)^{I}	0/4(0.0)^{I}	0/4(0.0)^{I}	0/4	0/4	0/4	0/4	0/4	0/4	0/4
		49	0/4(0.0)^{I}	0/4(0.0)^{I}	0/4(0.0)^{I}	0/4	0/4	0/4	0/4	0/4	0/4	0/4
3	PCV2 DNA	21	0/4(0.0)^{I}	4/4(1.5)^{II}	4/4(1.75)^{II}	3/4	0/4	0/4	1/4	1/4	2/4	3/4
		49	1/4(0.25)^{I}	3/4(0.75)^{II}	4/4(1.0)^{II}	0/4	0/4	0/4	0/4	1/4	2/4	2/4
4	PCV1-2 DNA	21	0/3(0.0)^{I}	1/3(0.33)^{I}	1/3(0.33)^{I}	0/3	0/3	0/3	0/3	0/3	0/3	0/3
		49	0/4(0.0)^{I}	1/4(0.25)^{I,II}	1/4(0.25)^{I}	0/4	0/4	0/4	0/4	0/4	2/4	0/4
5	PCV2-1 DNA	21	0/4(0.0)^{I}	0/4(0.0)^{I}	0/4 (0.0)^{I}	0/4	0/4	0/4	0/4	0/4	0/4	0/4
		49	0/4(0.0)^{I}	0/4(0.0)^{I}	0/4 (0.0)^{I}	0/4	0/4	0/4	0/4	0/4	1/4	0/4

[a]Phosphate buffered saline (PBS) used as negative control. The inocula were infectious DNA clones in pSK plasmid.
[b]Four pigs from each group were necropsied at 21 DPI and the remaining pigs were necropsied at 49 DPI.
[c]Number positive/number tested (mean histological lung score: 0, normal; 1, mild interstitial pneumonia; 2, moderate; 3, severe).
[d]Number positive/number tested (mean histiological liver sore: 0, normal; 1, mild hepatitis; 2, moderate; 3, severe.)
[e]Number positive/number tested (mean histiological lymphoid (lymph nodes) depletion score: 0, normal; 1, mild; 2, moderate; 3, severe.)
[c]Different superscripts (I,II) within each column indicate significantly different mean value score between groups.

Table 6. Immunohistiochemical detection of PCV2 antigen in lymph nodes of control, PCV2 and PCV1-2 inoculated pigs

Group	Inoculum[a]	DPI[b]	
		21	49
1	PBS	0/4[c](0 $\pm$ 0.00)[I d]	0/4 (0 $\pm$ 0.00)[I]
3	PCV2 DNA	4/4 (2 $\pm$ 0.67)[II]	3/4 (0.75 $\pm$ 0.25)[II]
4	PCV1-2 DNA	0/3 (0 $\pm$ 0.00)[I]	1/4 (0.25 $\pm$ 0.25)[I,II]

[a]Phosphate buffered saline (PBS) used as negative control. The inocula were infectious DNA clones in pSK plasmid.
[b]Four pigs from each group were necropsied at 21 days post inoculation (DPI) and the remaining pigs were necropsied at 49 DPI;
mean estimated amount of PCV2 antigen in lymphiod tissue (ranging from 0 = no antigen detected to 3 = high levels of antigen)
[c]Number of pigs positive for PCV2 antigen in lymphoid tissues / number of pigs tested.
[d]Different superscripts (I,II) within each column indicate significantly different mean value score between groups.

Chapter 5

A Chimeric Porcine Circovirus (PCV) with the Immunogenic Capsid Gene of the Pathogenic PCV2 Cloned Into the Genomic Backbone of the Non-Pathogenic PCV1 Induces Protective Immunity Against PCV2 Infection in Pigs

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ABSTRACT

Porcine circovirus type 2 (PCV2) is associated with postweaning multisystemic wasting syndrome (PMWS) in pigs, whereas PCV1 is non-pathogenic. We previously demonstrated that a chimeric PCV1-2 virus (with the immunogenic capsid gene of PCV2 cloned into the backbone of PCV1) induces an antibody response to the PCV2 capsid protein and is attenuated in pigs. Here, we report that the attenuated chimeric PCV1-2 induces protective immunity in pigs against wild-type PCV2 challenge. Forty-eight specific-pathogen-free (SPF) piglets were randomly and equally assigned to four groups of twelve pigs each. Pigs in group 1 were vaccinated by intramuscular injection with 200 µg of the chimeric PCV1-2 infectious DNA clone. Pigs in group 2 were vaccinated by intra-lymphoid injection with 200 µg of the chimeric PCV1-2 infectious DNA clone. Pigs in group 3 were vaccinated by intramuscular injection with $10^{3.5}$ $TCID_{50}$ of the chimeric PCV1-2 live virus. Pigs in group 4 were not vaccinated and served as controls. By 42 days post-vaccination (DPV), the majority of pigs had seroconverted to PCV2

capsid antibody. At 42 DPV, all pigs were challenged intranasally and intramuscularly with 2 x $10^{4.5}$ $TCID_{50}$ of a wild-type pathogenic PCV2 virus. By 21 days post-challenge (DPC), nine out of the twelve group 4 pigs were viremic for PCV2. Vaccinated animals in groups 1-3 had no detectable PCV2 viremia after challenge. At 21 DPC the lymph nodes in the non-vaccinated pigs were larger ($p < 0.05$) than those of vaccinated pigs. The PCV2 genomic copy loads in lymph nodes were reduced ($p < 0.0001$) in vaccinated pigs. Moderate amounts of PCV2 antigen were detected in most lymphoid tissues of non-vaccinated pigs but only in 1 of 36 vaccinated pigs. Mild to severe lymphoid depletion (LD) and histiocytic replacement (HR) were detected in lymphoid tissues in the majority of non-vaccinated group 4 pigs but only in a few vaccinated groups 1-3 pigs. The data from this study indicate that the attenuated chimeric PCV1-2 live virus as well as the chimeric PCV1-2 infectious DNA clone, when given intramuscularly in pigs, induces protective immunity against PCV2 infection and could potentially serve as an effective vaccine.

INTRODUCTION

Postweaning multisystemic wasting syndrome (PMWS) was first described in weaning piglets of a high health herd in Canada in 1991 (14). Since then PMWS has been reported in many swine producing regions of North America, Europe and Asia (2, 5, 6, 21, 28). The primary causative agent of PMWS is thought to be type 2 porcine circovirus (PCV2) (2, 7, 9, 10, 15, 20, 29).

The type 1 porcine circovirus (PCV1) was initially discovered as a persistent contaminant of the porcine kidney cell culture (PK-15) (36). PCV1 is a small non-enveloped icosahedral virus, with a single-stranded circular DNA genome of about 1.76 kb. PCV1 has not been found to cause disease and is generally considered to be non-pathogenic (1, 35). Pathogenic PCV2 and non-pathogenic PCV1 share only about 76% nucleotide sequence identity but have similar genomic organization (10). Two open reading frames (ORFs) have been characterized: ORF1 encodes rep proteins required for viral replication (4) and ORF2 encodes the immunogenic capsid protein (25). Both PCV1 and PCV2 are members of the *Circoviridae* family, along with psittacine beak and feather disease virus (BFDV) (3), and the tentative members columbid circovirus (CoCV), goose circovirus and canary circovirus (23, 31, 37). The human circoviruses TT virus, TTV-like minivirus and the SEN virus have genomic organization similar to PCV (27, 34, 38).

Accumulated evidence indicated that PCV2 is the primary, but not the sole, causative agent of PMWS (2, 7, 9, 10, 15, 20, 29, 30). Clinical PMWS has been reproduced in conventional pigs co-infected with PCV2 and either porcine parvovirus (PPV) or porcine reproductive and respiratory syndrome virus (PRRSV) (15, 29). Ladekjaer-Mikkelsen et al. (20) recently reproduced PMWS in 3-week old specific-

pathogen-free (SPF) piglets inoculated with PCV2 alone. PMWS was also reproduced in PCV2 inoculated piglets immunostimulated with keyhole limpet hemocyanin in incomplete Freund's adjuvant (18). Opriessnig et al. (30) showed that pigs vaccinated with *M. hyopneumoniae* and *A. pleuropneumoniae* prior to PCV2 inoculation had increased length of PCV2 viremia and more severe lymphoid lesions compared to unvaccinated pigs but vaccination coupled with PCV2 infection was unable to induce clinical PMWS. It is generally believed that immunostimulation either by vaccination or secondary viral infection plays a role in the occurrence of PMWS. However, the exact role of immunostimulation in the progression to clinical PMWS is not known.

There is a need for a vaccine to prevent PCV2 infections and its role in the progression to clinical PMWS. We previously showed that chimeric PCV1-2 infectious DNA clone (with the immunogenic capsid gene of PCV2 cloned into the backbone of the non-pathogenic PCV1) is infectious when injected directly into the lymph nodes of SPF piglets and induces a strong antibody response to PCV2 capsid antigen while remaining attenuated in pigs (11). Therefore, the chimeric PCV1-2 appears to be a good candidate vaccine. In this study, we evaluated the efficacy of this candidate vaccine by immunizing SPF pigs intramuscularly and intra-lymphoidly with chimeric PCV1-2 virus and infectious chimeric PCV1-2 DNA clone followed by challenge with wild-type pathogenic PCV2. We showed that the PCV1-2 chimeric virus and infectious DNA clone both induce protective immunity against wild-type PCV2 challenge in SPF pigs.

MATERIALS AND METHODS

PCV2 and chimeric PCV1-2 infectious DNA clones. The constructions of PCV2 and chimeric PCV1-2 infectious DNA clones were reported previously (9, 11). The original wild-type PCV2 was from a pig with naturally occurring PMWS on an Iowa farm (Isolate #40895) (10). The PCV2 infectious DNA clone was constructed by cloning two tandem copies of the complete PCV2 genomes into pBluescript vector. The PCV2 infectious DNA clone and the PCV2 virus generated by transfection of PK-15 cells with the PCV2 infectious DNA clone have been shown to induce the hallmark pathological lesions of PMWS (9). The chimeric PCV1-2 infectious DNA clone was constructed by replacing the ORF2 capsid gene of non-pathogenic PCV1 with that of PCV2 in the genomic backbone of PCV1 (11). The PCV2 and PCV1-2 infectious clone plasmids used in this study were prepared essentially as previously described (9, 11). The concentration of the plasmid DNA used in vaccination of pigs was determined by spectrophotometry.

Generation and infectivity titration of PCV1-2 and PCV2 virus stocks. PCV2 and chimeric PCV1-2 live viruses were generated by transfection of PK-15 cells with the respective infectious DNA clone as previously described (9, 11). To determine the infectivity titers of the PCV2 and chimeric PCV1-2 virus stocks, PK-15 cells were cultivated on 8-well LabTek chamber slides (Nalge Nunc International). When the PK-15 cells reached 70-80% confluency, the cells were infected with a 10-fold serial dilution of either PCV2 or PCV1-2 virus stock. After 3 days incubation, the infected cells were stained by an immunofluorescence assay (IFA) to determine the infectivity titers as previously described (9, 11). Briefly, the infected cells were fixed to the LabTek

chamber slides using an 80% acetone and 20% methanol fixing solution. Both PCV2 and PCV1-2 infected cells were then incubated with PCV2 polyclonal rabbit antibody. After washing three times with phosphate buffered saline buffer (PBS), the cells were incubated with a secondary FITC-labeled goat anti-rabbit IgG antibody (KPL Inc., Gaithersburg, MD). Slides were mounted by flouromount-G and cover-slipped. Viral infectivity titers were calculated using the Kärber method.

Vaccination of SPF pigs with chimeric PCV1-2 infectious DNA clone as well as PCV1-2 chimeric live virus. Forty-eight 9-week-old SPF piglets were randomly assigned to 4 groups of 12 pigs each, and each group was housed seperately. Pigs in group 1 were vaccinated with 200 µg of chimeric PCV1-2 infectious DNA clone by intramuscular injection. Pigs in group 2 were vaccinated with 200 µg of chimeric PCV1-2 infectious DNA clone by intra-superficial inguinal lymph node injections. Pigs in group 3 were vaccinated with 1 x $10^{3.5}$ $TCID_{50}$ of the chimeric PCV1-2 live virus by intramuscular injection. Pigs in group 4 were not vaccinated and served as controls. All animals were monitored daily for clinical signs, and serum samples were collected at –1, 7, 14, 21, 28, 35 and 42 days post vaccination (DPV), and weekly post challenge (DPC) until necropsy at 21 DPC.

Challenge of vaccinated pigs with wild-type pathogenic PCV2. At 42 DPV, all pigs were challenged with 2 x $10^{4.5}$ $TCID_{50}$ of the wild-type pathogenic PCV2. To maximize the challenge conditions each animal received one third of the PCV2 challenge inoculum

intramuscularly and two thirds intranasally. All animals were necropsied at 21 DPC (63 DPV).

Clinical evaluation. Pigs were weighed at 0 DPV and at the time of necropsy. Rectal temperatures and clinical respiratory scores, ranging from 0 to 6 (0 = normal; 6 = severe) (13), were recorded every other day from –1 to 63 DPV (21 DPC). Clinical observations, including evidence of central nervous system disease, icterus, muscoskeletal disease, and changes in body condition, were recorded daily.

Gross pathology and histopathology. The necropsy team was blinded to vaccination status of the pigs at necropsy. Complete necropsies were performed on all pigs at 21 DPC. An estimated percentage of the lung with grossly visible pneumonia was recorded for each pig based on a previously described scoring system (13). The degree of enlargement of lymph nodes (ranging from 0 = normal to 3 = three times the size) was estimated. Sections for histopathologic examination were taken from lungs (five sections), heart, lymph nodes (tracheobronchial, iliac, mesenteric, subiliac, superficial inguinal), tonsil, thymus, liver, spleen, small intestine, colon, pancreas, and kidney. The tissues were examined in a blinded fashion and given a score for severity of lung, lymph node, and liver lesions (13). Lung scores ranged from 0 (normal) to 6 (severe lymphohistiocytic interstitial pneumonia). Liver scores ranged from 0 (normal) to 3 (severe lymphohistiocytic hepatitis). Lymph nodes were scored for the estimated amount of lymphoid depletion (LD) of follicles ranging from 0 (normal or no lymphoid

depletion) to 3 (severe lymphoid depletion) and for the degree of histiocytic replacement (HR) of follicles (0 = none, 3 = large amount) (13).

Immunohistochemistry (IHC). IHC detection of PCV2-specific antigen was performed on lymph node, spleen, tonsil and thymus tissues collected during necropsies at 21 DPC. A rabbit polyclonal antiserum against PCV2 was used for the IHC, according to the procedures described previously (33). The amount of PCV2 antigen distributed in the lymphoid tissues was scored in a blinded fashion by assigning a score of 0, if no signal, to 3 for a strong positive signal.

Serology. Serum samples were collected from all pigs at –1, 7, 14, 21, 28, 35 and 42 DPV, and at 7, 14 and 21 DPC. Serum antibodies to PRRSV were assayed using Herd Check PRRSV ELISA (IDEXX Laboratories, Westbrook, MA). Serum antibodies to PPV were detected by a hemaglutination inhibition (HI) assay (16). Serum antibodies to PCV2 were detected by a modified indirect ELISA based on the recombinant ORF2 capsid protein of PCV2 (24). Serum samples with a sample positive (S/P) ratio above 0.20 were considered seropositive (24).

Quantitative real-time PCR assay. To determine virus genomic copy loads of chimeric PCV1-2 and PCV2 in sera and tissues of vaccinated and challenged swine, serum samples were tested at –1, 7, 14, 21, 28, 35 and 42 DPV, as well as at 7, 14 and 21 DPC by a quantitative real-time PCR. Briefly, a pair of primers MCV1 (5'-GCTGAACTTTTGAAAGTGAGCGGG-3') and MCV2 (5'-

TCACACAGTCTCAGTAGATCATCCCA-3') were synthesized (10) and used for the quantitative real-time PCR. The MCV1 and MCV2 primer pair was designed to amplify known PCV1 and PCV2 sequences including the chimeric PCV1-2 (11). Primers MCV1 and MCV2 amplify a 220 bp fragment when using chimeric PCV1-2 or PCV2 as a template. Viral DNA was extracted from a 100 μl serum sample or 50 μg of homogenized thoracic lymph node (TBLN) tissues using DNAzol reagent according to the manufacturer's protocol (Molecular Research Center, Cincinnati, OH). The DNA extracted from serum samples was resuspended in 100 μl DNase, RNase, and proteinase-free water. The DNA extracted from thoracic lymph node tissues was resuspended in 300 μl DNase, RNase, and proteinase-free water. The PCR reaction was performed in the presence of intercalating SYBR green dye (Molecular Probes Inc., Eugene, OR). The PCR parameters consisted of 38 cycles of denaturation at 94°C for 15 sec, annealing at 48°C for 15 sec, and extension at 72 °C for 30 sec. To quantify the viral genomic copy numbers, a standard dilution series with a known amount of plasmid containing a single copy of the PCV2 genome (9) was run simultaneously with samples in each reaction. After the reaction was completed, a melt curve cycle was included to confirm the size of the PCR product. The geometric mean of recovered genomic copies per reaction on TBLN homogenates was calculated for each group after setting results for negative samples to 1 copy per sample.

PCR. DNA extracts from thoracic lymph nodes of selected animals in each group were amplified using PCR primer sets specific for PCV2 or PCV1-2. To amplify chimeric PCV1-2 specific sequences, the PCR reaction employed primer pair Gen.PCV1 (5'-

GTGGACCCACCCTGTGCC-‘3) and Orf.PCV2 (5’-CAGTCAGAACGCCCTCCTG-‘3) to amplify a fragment of 580 bp (11). To amplify PCV2 specific sequences, the PCR reaction employed primer pair Gen.PCV2 (5’-CCTAGAAACAAGTGGTGGGATG-‘3) and Orf.PCV2 (5’- CAGTCAGAACGCCCTCCTG-‘3) to amplify a fragment of 900 bp (11). The PCR products were sequenced to confirm the identity of the virus recovered from pigs.

Statistical analyses. All statistical analyses described below were performed using the SAS® system (Version 8.02, SAS institute Inc., Cary NC). Serum samples S/P ratios determined by ELISA were compared between vaccinated and non-vaccinated groups by analysis of variance, using the MIXED procedure. The model included effects of vaccine, days post vaccination, and their interaction. Chimeric PCV1-2 vaccination effects for each DPV were evaluated by the slice option. S/P ratios were dichotomized to presence/absence of seroconversion at S/P = 0.20, and analyzed by logistic regression using the LOGISTIC procedure. Mean viral genomic copy loads in lymph nodes were compared by the Kruskal-Wallis test using the NPAR1WAY procedure and/or by analysis of variance of ranked data, followed by a Bonferroni test of multiple mean ranks, using GLM procedure. Gross pathologic and histopathologic scores were compared by the Kruskal-Wallis test using the NPAR1WAY procedure and/or by analysis of variance using the GLM procedure followed by a Bonferroni test of multiple means. Proportion of pigs with gross and histopathologic lesions in various tissues was compared between groups by Fisher’s Exact Test using the FREQ procedure.

RESULTS

Chimeric PCV1-2 live virus and chimeric PCV1-2 infectious DNA clone both replicate in pigs when vaccinated intramuscularly or intra-lymphoid and induce specific antibody response against PCV2 capsid antigen. Prior to inoculation at –1 DPV, serum samples from all animals tested negative for PCV1 or PCV2 nucleic acids by real-time PCR.

Group 1 pigs vaccinated intramuscularly with the chimeric PCV1-2 DNA clone did not develop PCV1-2 viremia for the duration of the study as no PCV1-2 DNA was detected in sera (Table 1). Three pigs in group1 had detectable PCV2 maternal antibody titers at –1 DPV, which waned by 14 DPV. Seroconversion to PCV2 specific antibodies occurred in seven out of twelve pigs by 42 DPV, the day of challenge with wild-type pathogenic PCV2 (Table 2).

We have previously shown that pigs can be easily infected by intra-lymphoid injection of PCV2 or PCV1-2 infectious DNA clone (9, 11). Therefore, as positive controls, group 2 pigs were vaccinated with the chimeric PCV1-2 infectious DNA clone by intra-lymphoid injection. Like pigs in group 1, none of the vaccinated pigs in group 2 developed PCV1-2 viremia (Table 1). Three of the twelve pigs had detectable PCV2 maternal antibody titers at –1 DPV, which waned in all by 14 DPV. Seroconversion to PCV2 capsid antibodies was first detected at 21 DPV, and by 42 DPV seven of the twelve pigs had seroconverted (Table 2).

Animals in group 3 were vaccinated with the chimeric PCV1-2 live virus by intramuscular route of injection. PCV1-2 viremia was not detected in any of the immunized pigs for the duration of the study. Four pigs in group 3 had detectable

maternal PCV2 antibodies at –1 DPV, which waned in all by 7 DPV. Seroconversion to PCV2 capsid antibodies was first detected at 28 DPV in three of the twelve animals, and by 42 DPV all pigs in group 3 had seroconverted (Table 2).

In the non-vaccinated group 4 pigs, PCV1-2 viremia was not detected, and none of the animals seroconverted to PCV2 antibodies prior to PCV2 challenge (Tables 1 and 2). Three pigs in group 4 had detectable PCV2 maternal antibodies at –1 DPV. By 7 DPV, the maternal antibody had waned in all but one animal.

PCV2 antibody S/P ratios differed between treatment groups ($p < 0.0001$) and over time ($p < 0.0001$) (data not shown). Following vaccination, up to 42 DPV (the day of PCV2 challenge) pigs in group 1, 2 and 3 were 1.89 (95% CI:[0.694;5.129]), 3.46 (95% CI:[1.307;9.134]) and 5.38 (95% CI:[2.043;14.148]) times more likely to have an S/P ratio greater than 0.20 compared to non-vaccinated pigs in group 4 (overall vaccine effect $p = 0.004$).

The chimeric PCV1-2 candidate vaccine prevents PCV2 viremia and reduces virus loads in lymph nodes after challenge with wild-type pathogenic PCV2. After challenging the vaccinated pigs with wild-type pathogenic PCV2, PCV2 viremia was not detected by real-time PCR in any of the vaccinated pigs in groups 1, 2 and 3. In group 4 non-vaccinated pigs, after PCV2 challenge, PCV2 viremia was first detected at 7 DPC in one of the twelve pigs, and by 14 DPC nine of the twelve pigs had detectable PCV2 viremia (Table 1). Seroconversion to PCV2 antibodies was first detected at 14 DPC in six of the twelve group 4 pigs, and by 21 DPC all pigs in group 4 had seroconverted to PCV2

(Table 2). The PCV2 viral genome loads in serum samples of group 4 pigs peaked at 14 DPC, ranging from 2,800 to 240,800 PCV2 genomic copies per ml of serum.

At necropsy (21 DPC), PCV2 genomic DNA was detected in the tracheobronchial lymph nodes (TBLN) in 3/12 group 1 pigs, 2/12 group 2 pigs, 5/12 group 3 pigs and 9/12 group 4 pigs ($p = 0.057$). The range of PCV2 viral genomic copy loads per 10 μg of homogenized TBLN in positive samples was 1023 to 61,119 in group 1, 10,532 to 82,152 in group 2, 1652 to 5,419,774 in group 3 and 363 to 621,285,534 in group 4. The median genomic copy loads differed between groups 1, 2, 3 and 4 ($p = 0.012$). Median PCV2 copy loads in TBLN did not differ between groups 1, 2 and 3 or groups 3 and 4 ($p > 0.05$), however were different between groups 1 and 4, and 2 and 4 ($p < 0.05$). PCR amplification using PCV2 and PCV1-2 specific primers followed by DNA sequencing confirmed that the genomic sequence detected by real-time PCR in the TBLN of animals in group 1, 2, 3 and 4 originated from the challenging PCV2 pathogenic virus and not from the chimeric PCV1-2 vaccine virus.

The chimeric PCV1-2 candidate vaccine reduces macroscopic and microscopic lesions, as well as viral antigen loads in tissues of vaccinated pigs after challenge with wild-type pathogenic PCV2.

Clinical evaluation. Clinical signs characteristic of PMWS were not observed in any animals of groups 1, 2, 3 and 4 for the duration of the study.

Gross lesions. All pigs were necropsied at 21 DPC. The enlargement of lymph nodes of the vaccinated pigs in groups 1, 2 and 3 generally ranged from mild to moderate with 1 or 2 pigs with severely enlarged (3x normal size) lymph nodes in each group

(Table 3). The lymph nodes of all non-vaccinated group 4 pigs were moderately to severely enlarged (Table 3). The mean gross lesion scores of the lymph nodes differed between groups 1, 2, 3 and 4 ($p = 0.0007$). Vaccinated groups 1, 2 and 3 were not different ($p > 0.05$) from each other, but were less than the mean score of non-vaccinated group 4 pigs ($p < 0.05$; Table 3).

Microscopic lesions. Microscopic lung lesions characterized as mild peribronchiolar lymphoplasmacytic and histiocytic bronchointerstitial pneumonia and liver lesions characterized by mild lymphoplasmacytic hepatitis were detected in pigs in all groups (Table 4). Mild lymphoid depletion (LD) of lymph node follicles was detected in 4/12 group 1 pigs, 1/12 group 2 pigs, 1/12 group 3 pigs, and mild to moderate LD in 11/12 pigs in group 4 ($p < 0.001$; Table 4). Mild histiocytic replacement (HR) of lymph node follicles was observed in 3/12 pigs in group 1, 1/12 pigs in both groups 2 and 3, and mild to moderate HR in 10/12 pigs in group 4 ($p = 0.001$; Table 4). Mild LD and HR of the tonsil follicles were found in 1/12 pigs in group 1, 0/24 pigs in groups 2 and 3, in 7/12 pigs in group 4 ($p = 0.0003$; Table 4). Mild LD and HR of the spleen follicles were observed in 2/12 pigs in group 1, none in groups 2 and 3, but in 10/12 pigs with mild to moderate LD and 9/12 pigs with mild to moderate HR in group 4 ($p < 0.0001$; Table 4). Presence of lesions in other tissues and organs are summarized in Table 4.

Detection of PCV2 antigen. At necropsy (21 DPC), PCV2 antigen was not detected in the lymph node tissues of pigs in vaccinated groups 1, 2 and 3, except for one pig in group 3. In the non-vaccinated group 4 pigs, low to high amounts of PCV2 antigen were detected in the lymph nodes ($p < 0.0001$; Table 5). No PCV2 antigen was detected in the tonsil of group 1, 2 and 3 pigs. However, low-to-high amounts of PCV2 antigen

were detected in the tonsil of 8/12 non-vaccinated group 4 pigs (p < 0.0001;Table 5). PCV2 antigen was not detected in spleen tissues of vaccinated groups 1, 2 or 3 pigs. Low-to moderate amounts of PCV2 antigen were detected in the spleen tissue of 5/12 pigs in non-vaccinated group 4 ($p = 0.002$;Table 5). No PCV2 antigen was detected in thymus tissues of any pigs (Table 5).

DISCUSSION

Postweaning multisystemic wasting syndrome has become a serious global pig disease, and hence there is an urgent need for the development of a vaccine against PCV2-associated diseases including PMWS. We previously reported that a chimeric PCV1-2 virus is attenuated when inoculated into SPF pigs but was capable of inducing a humoral immune response against PCV2 capsid protein, suggesting that the chimeric PCV1-2 may serve as a candidate vaccine against PCV2 infection (11). In this study, we demonstrated that pigs vaccinated with the chimeric PCV1-2 candidate vaccine developed protective immunity against wild-type pathogenic PCV2 challenge. We also demonstrated that pigs can be effectively vaccinated by intramuscular route of injections with both infectious chimeric PCV1-2 DNA clone and chimeric PCV1-2 live virus.

The majority of the vaccinated pigs in all 3 groups seroconverted to PCV2 antibodies within 4 to 6 weeks post-vaccination. The remaining seronegative pigs at the time of challenge had elevated PCV2 antibody titers with rising S/P ratios. Statistical analysis showed that there is a significant overall vaccine effect on S/P ratios ($p = 0.004$). No chimeric PCV1-2 viremia was detected throughout the study in the vaccinated pigs, which is in agreement with our earlier study (11). Most importantly, no PCV2 viremia

was detected in vaccinated pigs after challenge with wild-type pathogenic PCV2. In contrast, PCV2 viremia was detected in 9/12 non-vaccinated pigs after challenge. After PCV2 challenge, PCV2 antigen was detected in low-to-high amounts in lymph node, tonsil and spleen tissues of non-vaccinated pigs, but not in the vaccinated pigs with the exception of one pig in group 3. Vaccinated pigs also had reduced PCV2 genomic copy viral loads in the lymph nodes. These data indicate that the chimeric PCV1-2 candidate vaccine can prevent PCV2 viremia and significantly reduce the amount of PCV2 virus in the lymphoid tissues, which are important factors in pathogenesis of PCV2 associated diseases.

The mean scores of microscopic lesions in lymph node, spleen and tonsil tissues of the three vaccinated groups were less severe ($p < 0.05$) than those of the non-vaccinated group, indicating protection against PCV2 challenge by the candidate vaccine. The vaccinated pigs had significantly less enlargement of lymph nodes when compared to the non-vaccinated pigs. The enlargement of the lymph nodes observed in vaccinated pigs may be attributed to normal vaccine activation of the immune system in response to the PCV2 challenge since the enlarged lymph nodes in vaccinated pigs had no detectable microscopic lesions or viral DNA. In non-vaccinated pigs, lymphoid depletion and histiocytic replacement of follicles associated with PCV2 antigen were observed in lymph node, spleen and tonsil consistent with the hallmark PCV2-associated pathological lesions observed in natural and experimental cases of PMWS. In contrast, only one of the vaccinated pigs had evidence of PCV2-associated lymphoid lesions. These results strongly indicate that chimeric PCV1-2 candidate vaccine is effective in protecting pigs

from PCV2-associated lymphoid lesions and thus preventing the detrimental effects on the immune system.

The occurrence of lymphoid depletion during initial PCV2 infection may be linked to the eventual occurrence of leukopenia prior to the onset of clinical PMWS (26, 32). Therefore, the chimeric PCV1-2 candidate vaccine may have the ability to stop the eventual progression to clinical PMWS, by preventing the initial lymphoid depletion of lymphoid tissues.

No significant differences were found among the three different routes of vaccination with the PCV1-2 candidate vaccine. Intramuscular vaccination with PCV1-2 DNA clone, intra-lymphoid vaccination with PCV1-2 DNA clone and intramuscular vaccination with PCV1-2 live virus were all effective in inducing protective immunity against PCV2 infection. However, only the intramuscular vaccination route is likely the route acceptable to swine producers. The intra-lymphoid route of vaccination with chimeric PCV1-2 infectious DNA clone was included as a positive control since we had previously shown that this route has the ability to induce an infection (9, 11).

Low levels of maternal antibody found in a few animals in groups 1, 2 and 3 had no apparent effect on inducing protective immunity by the chimeric PCV1-2 candidate vaccine. Since there were only a few animals with low levels of maternal antibodies in this study, a definitive answer cannot be drawn as to whether or not the low level of maternal antibodies has any effect on vaccination with a live vaccine. Since many newborns, following colostrum uptake, in commercial swine farms have PCV2 maternal antibodies, future studies with larger numbers of animals with different levels of maternal antibody are warranted to confirm our preliminary results.

Although not all the vaccinated animals seroconverted to PCV2 by the time of challenge, they were all protected against the pathogenic PCV2 challenge. This suggests that high S/P ratios of PCV2 antibody responses are not absolutely required for protection. It is possible that the chimeric PCV1-2 candidate vaccine induces a cell-mediated immune response, which may be equally or more important for induction of protection against PCV2. Further research is warranted to determine the exact role and the extent of cell-mediated immunity induced by the candidate vaccines in protection against PCV2 infections.

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REFERENCES

1. **Allan, G.M., F. McNeilly, J.P. Cassidy, G.A. Reilly, B. Adair, W.A. Ellis, and M.S. McNulty.** 1995. Pathogenesis of porcine circovirus; experimental infections of colostrum deprived piglets and examination of pig foetal material. Vet. Microbiol. **44**:49-64.

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

3. **Bassami, M.R., D. Berryman, G.E. Wilcox, and S.R. Raidal.** 1998. Psittacine beak and feather disease virus nucleotide sequence analysis and its relationship to porcine circovirus, plant circoviruses, and chicken anaemia virus. Virology **249**:453-459.

4. **Cheung, A.K**. 2003. Transcriptional analysis of porcine circovirus. Virology **305**: 168-180

5. **Choi, C., C. Chae, and E.G. Clark.** 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.

6. **Edwards, S. and J.J. Sands.** 1994. Evidence of circovirus infection in British pigs. Vet. Rec. **134**:680-681.

7. **Ellis, J.A., A. Bratanich, E.G. Clark, G. Allan, B. Meehan, D.M. Haines, J. Harding, K.H. West, S. Krakowka, C. Konoby, L. Hassard, K. Martin, and F.

McNeilly. 2000. Coinfection by porcine circoviruses and porcine parvovirus in pigs with naturally acquired postweaning multisystemic wasting syndrome. J. Vet. Diagn. Invest. **12**:21-27.

8. **Darwich L., P. Sandrine, A. Rovira, J. Segales, M.Domingo, I.P. Oswald, and E. Mateu.** 2003. Cytokine mRNA expression profiles in lymphoid tissues of pigs naturally affected by postweaning multisystemic wasting syndrome. J. Gen. Virol. **84**:2117-2125.
9. **Fenaux M, P.G. Halbur, G. Haqshenas, R, Royer, P. Thomas, P. Nawagitgul, M. Gill, T.E. Toth, and X.J. Meng.** 2002. Cloned genomic DNA of type 2 porcine circovirus is infectious when injected directly into the liver and lymph nodes of pigs: characterization of clinical disease, virus distribution, and pathological lesions. J. Virol. **76**:541-551.
10. **Fenaux M, P.G. Halbur, M. Gill, T.E. Toth, and X.J. Meng.** 2000**.** Genetic characterization of type 2 porcine circovirus (PCV-2) from pigs with postweaning multisystemic wasting syndrome in different geographic regions of North America and development of a differential PCR-restriction fragment length polymorphism assay to detect and differentiate between infections with PCV-1 and PCV-2. J. Clin. Microbiol. **38**: 2494-2503.
11. **Fenaux M., T. Opriessnig, P.G. Halbur, and X.J. Meng**. 2003. Immunogenicity and pathogenicity of the chimeric infectious DNA clones between pathogenic type 2 porcine circovirus (PCV2) and non-pathogenic PCV1 in weaning pigs. J. Virol. **77**:11232-11243.

12. **Gilpin D.F., K. McCullough, B.M. Meeham, F. McNeilly, I. McNair, L.S. Stevenson, J.C. Foster, J.A. Ellis, S. Krakowka, B.M. Adair, and G.M. Allan**. 2003. In vitro studies on the infection and replication of porcine circovirus type 2 in cells of porcine immune system. Vet. Immunol. Immunopathol. **94**:149-161.
13. **Halbur P.G., P.S. Paul, M.L. Frey, J. Landgraf, K. Eernisse, X.J. Meng, M.A. Lum, J.J. Andrews, and J.A. Rathje.** 1995. Comparison of the pathogenicity of two U.S. porcine reproductive and respiratory syndrome virus isolates with that of the Lelystad virus. Vet. Pathol. **32**:648-660
14. **Harding, J.C. and E.G. Clark.** 1997. Recognizing and diagnosing post-weaning multisystemic wasting syndrome (PMWS). Swine Health Prod. **5**:201-203
15. **Harms P.A., S.D. Sorden, P.G. Halbur, S.R. Bolin, K.M. Lager, L. Morozov, and P.S. Paul.** 2001. Experimental reproduction of severe disease in CD/CD pigs concurrently infected with type 2 porcine circovirus and porcine reproductive and respiratory syndrome virus. Vet. Pathol. **38**:528-539.
16. **Joo H.S., C.R. Donaldson-Wood, and R.H. Johnson.** 1976. A standardized haemagglutination inhibition test for porcine parvovirus antibody. Aust. Vet. J. **52**:422-424.
17. **Krakowka, S., J.A. Ellis, F. McNeilly, D. Gilpin, B. Meeham, K. McCullough, and G. Allan.** 2002. Immunologic features of porcine circovirus type 2 infection. Viral Immunol. **15**:567-582
18. **Krakowka, S., J.A. Ellis, F. McNeilly, S. Ringler, D.M. Rings, and G. Allen.** 2001. Activation of the immune system is the pivotal event in the production of

wasting disease in pigs infected with porcine circovirus-2 (PCV-2). Vet. Pathol. **38**:31-42.

19. **Kyriakis, S.C., K. Saoulidis, S. Lekkas, Ch. C. Miliotis, P.A. Papoutsis, and S. Kennedy.** 2002. The effects of immuno-modulation on the clinical and pathological expression of postweaning multisystemic wasting syndrome. J. Comp. Pathol. **126**:38-46.
20. **Ladekjaer-Mikkelsen, A.S., J. Nielsen, T. Stadejek, T. Storgaard, S. Krakowka, J. Ellis, F. McNeilly, G. Allan, and A. Botner.** 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.
21. **Larochelle, R., M. Morin, M. Antaya, and R. Magar.** 1999. Identification and incidence of porcine circovirus in routine field cases in Quebec as determined by PCR. Vet. Rec. **145**:140-142.
22. **Liu, Q., L. Wang, P. Willison, and L.A. Babiuk.** 2000. Quantitative, competitive PCR analysis of porcine circovirus DNA in serum from pigs with postweaning multisystemic wasting syndrome. J. Clin. Microbiol. **38**:3474-3477.
23. **Mankertz A., K. Hattermann, B. Ehlers, and D. Soike.** 2000**.** Cloning and sequencing of columbid circovirus (CoCV), a new circovirus from pigeons. Arch. Virol. **145**:2469-2479
24. **Nawagitgul, P., P.A. Harms, I. Morozov, B.J. Thacker, S.D. Sorden, C. Lekcharoensuk, and P.S. Paul.** 2002. Modified indirect porcine circovirus (PCV)

type 2-based and recombinant capsid protein (ORF2)-based ELISA for the detection of antibodies to PCV. Clin. Diagn. Lab. Immunol. **9:**33-40.

25. **Nawagitgul, P., I. Morozov, S.R. Bolin, P.A. Harms, S.D. Sorden, and P.S. Paul.** 2000. Open reading frame 2 of porcine circovirus type 2 encodes a major capsid protein. J. Gen. Virol. **81**:2281-2287.
26. **Nielson, J., I.E. Vincent, A. Bøtner, A-S. Ladekjaer-Mikkelsen, G. Allan, A. Summerfield, and K.C. McCullough**. 2002. Association of lymphopenia with porcine circovirus type 2 induced postweaning multisystemic wasting syndrome (PMWS). Vet. Immunol. Immunopathol. **92**:97-111.
27. **Nishizawa, T., H. Okamoto, K. Konishi, H. Yoshizawa, Y. Miyakawa, and M. Mayumi.** 1997. A novel DNA virus (TTV) associated with elevated transaminase levels in posttransfusion hepatitis of unknown etiology. Biochem. Biophys. Res. Commun. **241**:92-97.
28. **Onuki, A., K. Abe, K. Togashi, K. Kawashima, A. Taneichi, and H. Tsunemitsu.** 1999. Detection of porcine circovirus from lesions of a pig with wasting disease in Japan. J. Vet. Med. Sci. **61**:1119-1123
29. **Opriessnig, T., M. Fenaux, S. Yu, R.B. Evans, D. Cavanaugh, J.M. Gallup, F.J. Pallares, E.L. Thacker, K.M. Lager, X.J. Meng, and P.G. Halbur.** 2003. Effect of porcine parvovirus vaccination on the development of PMWS in segregated early weaned pigs coinfected with type 2 porcine circovirus and porcine parvovirus. Vet. Microbiol. **98**: 209-220
30. **Opriessnig, T., S. Yu, J.M. Gallup, R.B. Evans, M. Fenaux, F.J. Pallares, E.L. Thacker, C.W. Brockus, M.R. Ackermann, P. Thomas, X.J. Meng, and P.G.**

Halbur. 2003. Effects of vaccination with selective bacterins on conventional pigs infected with wild type 2 porcine circovirus. Vet. Pathol. **40**:521-9.

31. **Phenix, K.V., J.H. Weston, I. Ypelaar, A. Lavazza, J.A. Smyth, D. Todd, G.E. Wilcox, and S.R. Raidal.** 2001. Nucleotide sequence analysis of a novel circovirus of canaries and its relationship to other members of the genus circovirus of the family Circoviridae. J. Gen. Virol. **82**:2805-2809.
32. **Segales J., C. Alfonso, C. Rosell, J. Pastor, F. Chianini, E. Campos, L. Lopez-Fuertes, J. Quinana, G. Rodriguez-Arrioja, M. Calsamiglia, j. Pujols, J. Dominguez, and M. Domingo.** 2001. Changes in peripheral blood leukocyte populations in pigs with natural postweaning multisystemic wasting syndrome (PMWS). Vet. Immunol. Immunopathol. **81**:37-44.
33. **Sorden S.D., P.A. Harms, P. Nawagitgul, D. Cavanaugh, and P.S. Paul.** 1999. Development of a polyclonal-antibody-based immunohistochemical method for the detection of type 2 porcine circovirus in formalin-fixed, paraffin-embedded tissue. J. Vet. Diagn. Invest. **11**:528-530.
34. **Takahashi, K., Y. Iwasa, M. Hijikata, and S. Mishiro.** 2000. Identification of a new human DNA virus (TTV-like mini virus, TLMV) intermediately related to TT virus and chicken anemia virus. Arch. Virol. **145**:979-93.
35. **Tischer, I., W. Mields, D. Wolff, M. Vagt, and W. Griem.** 1986**.** Studies on epidemiology and pathogenicity of porcine circovirus. Arch. Virol. **91**:271-276.
36. **Tischer, I., R. Rasch, and G. Tochtermann.** 1974. Characterization of papovavirus and picornavirus-like particles in permanent pig kidney cell lines. Zentralbl. Bakteriol. Hyg. A **226**:153-167.

37. **Todd, D., J.H. Weston, D. Soike, and J.A. Smyth.** 2001. Genome sequence determinations and analyses of novel circoviruses from goose and pigeon. Virology. **286**:354-362

38. **Wilson, L.E., T Umemeru, J. Astembroski, S.C. Ray, H.J. Alter, S.A. Strathdee, D. Vlahov, and D.L. Thomas**. 2001. Dynamics of SEN virus infection among injection drug users. J. Infect. Dis. **184**:1315-1319.

Fig. 1

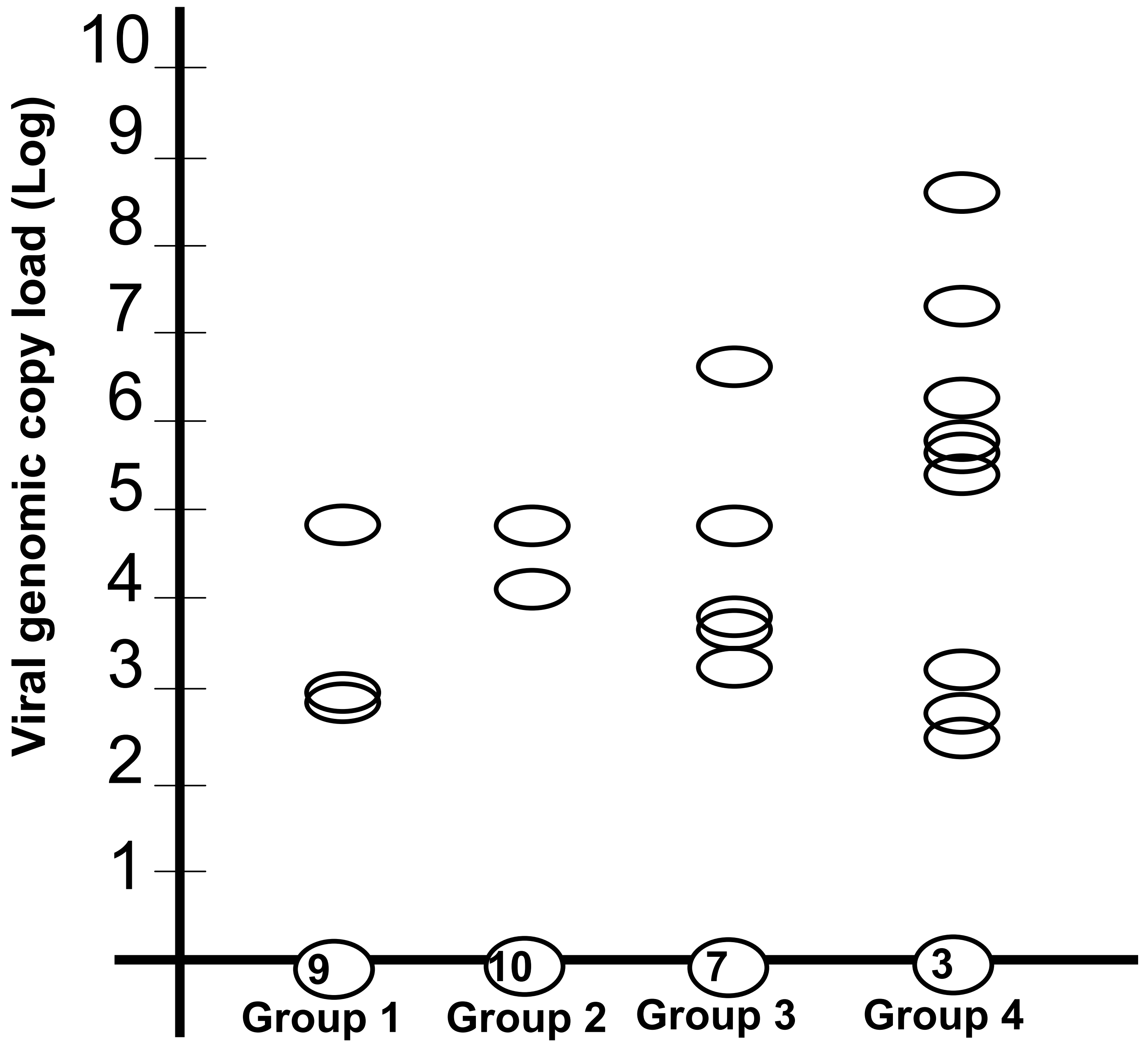

FIG. 1. Quantitative real-time PCR results of wild-type PCV2 viral genomic copy loads in tracheobronchial lymph node (TBLN) tissues collected during necropsys at 21 days post challenge (DPC) from vaccinated pigs in groups 1, 2 and 3 and from non-vaccinated pigs in group 4. PCV2 DNA was extracted from 50 μg of homogenized TBLN lymph node tissues using DNAzol reagent, and subjected to real-time PCR amplification. Pigs positive for PCV2 genomic DNA in each group are indicated with a "○". The numbers (9, 10, 7, 3) in the X-axis indicate the number of animals in each group that were negative for PCV2 genomic DNA in the TBLN. The PCV2 genomic copy loads were determined by real-time PCR and represented as a log of PCV2 genomic copy loads per 10 μg of TBLN tissue value (Y-axis).

Table 1. Detection of chimeric PCV1-2 and PCV2 viremia by real-time PCR in sera of vaccinated and non-vaccinated pigs

Group	Vaccine	Route of vaccination	Days post vaccination [a]							Total	PCV2 challenge [b]: Days post challenge [a]			Total
			-2	7	14	21	28	35	42	(PCV1-2)	7	14	21	(PCV2)
1	PCV1-2 DNA[c]	Intramuscular	0/12	0/12	0/12	0/12	0/12	0/12	0/12	0/12	0/12	0/12	0/12	0/12
2	PCV1-2 DNA	Intralymphoid	0/12	0/12	0/12	0/12	0/12	0/12	0/12	0/12	0/12	0/12	0/12	0/12
3	PCV1-2 Virus[d]	Intramuscular	0/12	0/12	0/12	0/12	0/12	0/12	0/12	0/12	0/12	0/12	0/12	0/12
4	None		0/12	0/12	0/12	0/12	0/12	0/12	0/12	0/12	1/12	9/12	4/12	9/12

[a]Detection by real-time PCR of chimeric PCV1-2 and wild-type PCV2 nucleic acid

[b]At 42 days post vaccination (DPV), the animals in all four groups were challenged with the wild-type PCV2 virus.

[c]Cloned PCV1-2 genomic DNA in pSK plasmid.

[d]A PCV1-2 live vaccine virus stock generated by transfection of PK-15 cells with PCV1-2 infectious DNA clone.

Table 2. Seroconversion to PCV2 specific antibodies in pigs vaccinated with PCV1-2 live virus or with PCV1-2 infectious DNA clone before and after PCV2 challenge.

			Days post vaccination						PCV2 challenge[a] ↓	Days post challenge		
Group	Vaccine	Route of vaccination	-1	7	14	21	28	35	42	7	14	21
1	PCV1-2 DNA[d]	Intramuscular	0/9[b]	0/9	1/9	1/9	1/9	1/9	6/9	8/9	9/9	9/9
			3/3[c]	1/3	0/3	0/3	0/3	2/3	1/3	2/3	2/3	2/3
2	PCV1-2 DNA	Intralymphoid	0/9	0/9	0/9	1/9	2/9	5/9	5/9	8/9	9/9	8/9
			3/3[c]	1/3	0/3	1/3	1/3	2/3	2/3	3/3	3/3	3/3
3	PCV1-2 Virus[e]	Intramuscular	0/8	0/8	0/8	0/8	2/8	6/8	8/8	7/8	8/8	8/8
			4/4[c]	0/4	0/4	0/4	2/4	3/4	4/4	4/4	4/4	4/4
4	None		0/9	0/9	0/9	0/9	0/9	0/9	0/9	0/9	5/9	9/9
			3/3[c]	1/3	1/3	1/3	1/3	1/3	1/3	1/3	1/3	3/3

[a]At 42 days post vaccination (DPV), the animals in all four groups were challenged with the wild-type PCV2 virus.
[b]PCV2 antibody was measured with an enzyme-linked immunosorbent assay with the recombinant PCV2 capsid antigen: number positive/number tested.
[c]Animals with maternal antibodies: number positive / number of animals with maternal antibodies at -1 DPV.
[d]Cloned chimeric PCV1-2 genomic DNA in pSK plasmid.
[e]A PCV1-2 candidate vaccine live virus stock generated by transfection of PK-15 cells with the chimeric PCV1-2 infectious DNA clone.

Table 3. Gross lymph node lesions in vaccinated and non-vaccinated pigs

Group	Vaccine[a]	Route of vaccination	Gross lesions Lymph nodes
1	PCV1-2 DNA	Intramuscular	12/12[b](2.0)^{I}
2	PCV1-2 DNA	Intra-lymphoid	12/12 (1.8)^{I}
3	PCV1-2 Virus	Intramuscular	12/12 (1.6)^{I}
4	None		12/12 (2.7)^{II}

[a]Animals in group 1-3 were vaccinated with chimeric PCV1-2 infectious DNA clone or chimeric PCV1-2 live virus. At 42 days post vaccination (DPV), all animals in groups 1-4 were challenged with wild-type pathogenic PCV2.
[b]All 12 pigs in each group were necropsied at 21 days post challenge (DPC); Number of animals with enlarged lymphnodes/number of animals in each group (mean score of estimated enlargement). Different superscripts (I,II) indicate different mean value score between groups ($p < 0.05$).

Table 4. Distribution of histological lesions in different tissues and organs from vaccinated and non-vaccinated pigs challenged with wild-type PCV2

			No. of pigs positive/no. tested											
		Route of	Lung	Liver	Lymph node		Tonsil		Spleen		Kidney	Heart	Thymus	Intestine
Group	Vaccine[a]	vaccination			LD	HR	LD	HR	LD	HR				
1	PCV1-2 DNA	Intramuscular	4/12(0.33)^{I,II}[b]	2/12(0.16)^{I}	4/12(0.33)^{I}	3/12(0.25)^{I}	1/12(0.08)^{I}	1/12(0.08)^{I}	2/12(0.17)^{I}	1/12(0.08)^{I}	1/12	0/12	0/12	0/12
2	PCV1-2 DNA	Intralymphoid	1/12(0.08)^{I}	3/12(0.25)^{I}	1/12(0.08)^{I}	1/12(0.08)^{I}	0/12^{I}	0/12^{I}	0/12^{I}	0/12^{I}	0/12	0/12	0/12	0/12
3	PCV1-2 Virus	Intramuscular	2/12(0.17)^{I}	2/12(0.16)^{I}	1/12(0.08)^{I}	1/12(0.08)^{I}	0/12^{I}	0/12^{I}	0/12^{I}	0/12^{I}	2/12	0/12	0/12	0/12
4	None		8/12(0.67)^{II}	6/12(0.5)^{I}	11/12(0.92)^{II}	10/12(0.83)^{II}	7/12(0.58)^{II}	7/12(0.58)^{II}	10/12(0.83)^{II}	9/12(0.75)^{II}	4/12	0/12	0/12	0/12

[a]The candidate vaccines were chimeric PCV1-2 live virus or chimeric PCV1-2 infectious DNA clone.
[b]Values in parentheses are mean histiological scores (0, normal to 6, sever multifocal interstitial pneumonia) for interstitial pneumonia for lung, (0, normal; 1, mild; 2, moderate; 3, severe) hepatitis for liver, and lymphoid depletion (LD) and histological replacement (HR) of follicles in lymphoid tissues. Different superscripts (I and II) within each column indicate mean value scores differences between groups ($p < 0.05$).

Table 5. Immunohistochemical detection of PCV2 antigen in lymph nodes, tonsils, spleen and thymus of vaccinated and non-vaccinated pigs at 21 days post-challenge with wild-type PCV2

Group	Vaccine[a]	Route of vaccination	No. of pigs positive/no. tested			
			Lymph node	Tonsils	Spleen	Thymus
1	PCV1-2 DNA	Intramuscular	0/12[b](0.0)^{I}	0/12(0.0)^{I}	0/12(0.0)^{I}	0/12(0.0)
2	PCV1-2 DNA	Intralymphoid	0/12 (0.0)^{I}	0/12(0.0)^{I}	0/12(0.0)^{I}	0/12(0.0)
3	PCV1-2 virus	Intramuscular	1/12 (0.08)^{I}	0/12(0.0)^{I}	0/12(0.0)^{I}	0/12(0.0)
4	None		9/12 (0.75)^{II}	8/12(0.67)^{II}	5/12(0.42)^{II}	0/12(0.0)

[a] The candidate vaccine was either chimeric PCV1-2 infectious DNA clone or chimeric PCV1-2 live virus.
[b] All pigs were necropsied at 21days post-challenge (DPC). Values in parentheses are the mean estimated amounts of PCV2 antigen in lymphoid tissue (ranging from 0, no antigen detected, to 3, high amounts of antigen. Different superscripts (I,II) indicate mean value score differences between groups ($p < 0.05$).

Chapter 6

Two Amino Acid Mutations in the Capsid Protein of Type 2 Porcine Circovirus (PCV2) Enhanced PCV2 Replication *In Vitro* and Attenuated the Virus *In Vivo*

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ABSTRACT

Porcine circovirus type 2 (PCV2) is the primary causative agent of postweaning multisystemic wasting syndrome (PMWS), whereas the PK-15 cell culture-derived porcine circovirus type 1 (PCV1) is non-pathogenic to pigs. To identify potential genetic determinants for virulence and replication, we serially passaged a PCV2 isolate for 120 times in PK-15 cells. The viruses at passage 1 (VP1) and 120 (VP120) were biologically, genetically and experimentally characterized. A one-step growth curve was used to compare the growth characteristics of PCV1, PCV2 VP1, and PCV2 VP120. The results showed that the PCV2 VP120 virus replicated to a similar titer as PCV1 but more efficiently than PCV2 VP1 in PK-15 cells with at least 1 log difference. The complete genomic sequences of viruses at passages 0, 30, 60, 90, and 120 were determined. A total of 2 amino acid mutations were identified after 120 passages. The first mutation occurred at passage 30, in which a proline at position 110 of the capsid protein was substituted for an alanine (P110A), and this mutation remained in the subsequent passages. The second mutation, a substitution of an arginine for a serine at position 191 of the capsid protein

(R191S), appeared at passage 120 but not in earlier passages. To experimentally characterize the pathogenicity of the VP120 virus, 31 specific-pathogen-free (SPF) pigs were randomly divided into three groups. Ten pigs in group 1 received phosphate buffered saline as negative controls. Eleven group 2 pigs were inoculated intramuscularly and intranasally with $10^{4.9}$$TCID_{50}$ of PCV2 VP120. Ten pigs in group 3 were inoculated with $10^{4.9}$$TCID_{50}$ of PCV2 VP1. PCV2 viremia was detected in 9/10 pigs in the PCV2 VP1 group, but only in 4/11 pigs in PCV120 group. The viremia in VP1 group (mean 3 weeks) lasted longer than that of VP120 group (mean 1.6 weeks). In addition, the PCV2 genomic copy loads in serum, as determined by quantitative real-time PCR, in the PCV2 VP1 group were higher than those in PCV2 VP120 group ($p < 0.0001$). Gross and histopathologic lesions found in pigs inoculated with PCV2 VP1 were more severe than those inoculated with PCV2 VP120 at both 21 and 42 DPI necropsies ($p = 0.0032$ and $p = 0.0274$, respectively). Taken together, the results from this study suggest that the P110A and R191S mutations in the capsid of PCV2 enhanced the growth ability of PCV2 *in vitro* and attenuated the virus *in vivo*.

INTRODUCTION

Postweaning multisystemic wasting syndrome (PMWS) was initially recognized in weaning piglets of a high health Canadian herd in 1991 (16). Since then, PMWS has become a disease in all swine producing regions of the world (3, 8, 9, 12, 22, 25). The primary causative agent of PMWS has been determined to be type 2 porcine circovirus (PCV2) (2, 3, 4, 8, 9, 10, 11, 12, 17, 18, 19,). Type 1 porcine circovirus (PCV1) was discovered as a noncytopathic contaminant of the PK-15 porcine kidney cell line (28, 30). PCV1 does not cause any disease in pigs and is considered to be non-pathogenic (1, 29).

Both PCV1 and PCV2 are small, non-enveloped viruses with a single stranded circular DNA genome of about 1.76 kb. The PCV genome contains at least two functional open reading frames (ORFs): ORF1 (930 bp) encodes the Rep proteins involved in viral replication (7) and ORF2 (690 bp) encodes the immunogenic capsid protein (7, 21, 24). PCVs are members of the *Circoviridae* family along with chicken anemia virus (CAV), Psittacine beak and feather disease virus, and tentative members columbid circovirus, goose circovirus and canary circovirus (5, 23, 26, 31).

The complete genomic sequences of PMWS-associated PCV2 and non-pathogenic PCV1 have been determined (12, 20). Sequence analyses revealed that PCV1 and PCV2 share about 76% nucleotide sequence identity and have a very similar genomic organization. We have previously demonstrated that cell culture-derived PCV1 replicated more efficiently in PK-15 cells than PCV2 (14), and that PCV2 caused pathological lesions characteristic of PMWS in specific-pathogen-free (SPF) pigs whereas PCV1 did not (11, 14). The genetic determinants for PCV2 pathogenicity in pigs and for the enhanced growth ability of PCV1 in PK-15 cells are not known.

The objectives of this study are to identify the genetic determinants for PCV2 pathogenicity *in vivo* and for replication *in vitro*. A pathogenic PCV2 isolate was serially passaged in PK-15 cells for 120 times, and the viruses harvested from passages 1 and 120 were biologically, genetically and experimentally characterized. Two amino acid mutations were identified in the capsid gene after 120 passages, and these two mutations may be responsible for the increased growth rate *in vitro* and the attenuation of virulence *in vivo*.

MATERIALS AND METHODS

Virus and cell. The PCV1 virus used in this study was originally isolated from a PK-15 cell line (ATCC CCL-33) (11). The PCV2 virus used in this study was originally isolated from a spleen tissue sample of a pig with naturally occurring PMWS (isolate 40895)(11, 12). The PK-15 cell line used in this study was free of PCV1 contamination (11).

Serial passages of PCV2 *in vitro*. A homogenous PCV2 virus stock, designated passage 1 (VP1), was generated by transfection of PK-15 cells with the PCV2 infectious DNA clone as previously described (11). The VP1 PCV2 virus stock was then serially passaged for 120 times in PK-15 cells. Briefly, the infected cells, when reaching confluency, were subcultured at a 1 to 3 ratio in minimum essential medium (MEM) with Earle's salts and L-glutamine supplemented with 2% fetal calf serum (FCS) and 1 X antibiotic (Invitrogen, Inc., CA). For every 10 to 15 passages, the infected cells were harvested by the repeated freeze-thaw protocol, and used to inoculate a new PK-15 culture. The newly infected culture was then passed 10 to 15 times by subculturing

before repeating the freeze-thaw procedure. This procedure was repeated until it reached passage 120 (VP120). The virus harvested at each passage was stored at –80 for further analyses.

Biological characterization of PCV1, PCV2 VP1, and PCV2 VP120 viruses in PK-15 cells. A one-step growth curve was performed to determine the comparative growth ability of PCV1, PCV2 VP1, and PCV2 VP120 *in vitro*. Briefly, PK-15 cells were grown on six 12-well plates. The plates were infected, in duplicate, with PCV1, PCV2 VP1 or PCV2 VP120 at a multiplicity of infection (M.O.I.) of 0.1. After 1 hour absorption, the inoculum was removed and the cell monolayer was washed five times with phosphate buffered saline (PBS). Maintenance MEM media (2% bovine calf serum and 1X antibiotics) was subsequently added to each well, and the infected cell cultures were continuously incubated at 37°C with 5% CO2. Every 12 hours, the media and cells from duplicate wells of each inoculated group were harvested and stored at –80 °C until virus titration. The infectious titers of PCV1 and PCV2 viruses collected at different time points were determined by immunofluorescent assays (IFA) specific for PCV1 or PCV2 as previously described (11, 14).

Genetic characterization of PCV2 viruses at different passages. PCV2 viruses harvested from passages 1, 30, 60, 90, and 120 were genetically characterized by determining the complete genomic sequences of the viruses from each passage. Briefly, viral DNA was extracted from 100 µl of the cell culture materials collected at passages 1, 30, 60, 90, and 120 by using DNAzol reagent according to the manufacturer's protocol

(Molecular Research Center, Cincinnati, Ohio). The extracted DNA was resuspended in DNase-, RNase- and proteinase-free water. To amplify the entire genome, three pairs of PCV2 specific primers were used to amplify three overlapping fragments: primer pair PCV2.2B (''5-TCCGAAGACGAGCGCA-'3) and PCV2.2A (''5-GAAGTAATCCTCCGATAGAGAGC-'3), primer pair PCV2.3B (''5-GTTACAAAGTTATCATCTAGAATAACAGC-'3) and PCV2.3A (''5-ATTAGCGAACCCCTGGAG-'3), and primer pair PCV2.4B (''5-AGAGACTAAAGGTGGAACTGTACC-'3) and PCV2.4A (''5-AGGGGGGACCAACAAAAT-'3). The PCR reaction consisted of 38 cycles of denaturation at 94°C for 1 min, annealing at 46°C for 30 sec, and extension at 72°C for 2 min, followed by a final extension at 72°C for 7 min. The PCR products of expected size were excised from 0.8% agarose gels followed by purification with a Geneclean Kit (Bio 101, Inc., La Jolla, CA). The PCR products were directly sequenced for both strands using the PCR primers. The nucleotide and amino acid sequences were compiled and analyzed with the MacVector program (Oxford Molecular Ltd., Beaverton, OR) using Clustal alignment. The complete sequence of PCV2 VP120 was compared to PCV2 VP1 and 31 other PCV2 isolates as well as 4 PCV1 isolates available in the GenBank database.

Experimental characterization of the serially-passaged PCV2 VP120, and VP1. To determine the pathogenic potential of the VP120 PCV2, thirty-one SPF pigs of 3 to 4 weeks of age were randomly assigned to 3 groups, and housed separately. Prior to the inoculation, serum samples from all piglets were tested by PCR for the presence of

PCV1 or PCV2 DNA. To maximize the efficiency of inoculation (13), each pig was inoculated with 1 ml of the inoculum intramuscularly and 3 ml intra-nasally. The ten pigs in group 1 were each inoculated with PBS buffer as negative controls. Eleven pigs in group 2 each received $10^{4.9}$ $TCID_{50}$ of PCV2 VP120, and ten pigs in group 3 each received $10^{4.9}$ $TCID_{50}$ of PCV2 VP1. All pigs were monitored for clinical signs of disease. Serum samples were collected from each pig at –1, 7, 14, 21, 28, 35 and 42 days post inoculation (DPI). At 21 DPI, 5 randomly selected pigs from each group were necropsied. The remaining pigs in each group were necropsied at 42 DPI.

Clinical evaluation. Pigs were weighed at –1, 7, 14, 21, 28, 35 and 42 DPI. Rectal temperatures and clinical scores, ranging from 0 to 6 (0 = normal; 6 = severe) (11), were recorded every other day from 0 to 42 DPI. Clinical observations, including evidence of central nervous system disease, liver disease (icterus), musculoskeletal disease, and changes in body condition, were recorded at two day intervals. All clinical evaluations were performed by a team of two people.

Gross pathology and histiopathology. Complete necropsies were performed on all pigs. The necropsy team was blinded to the infection status of the pigs. The percentage of lung with grossly visible pneumonia was estimated for each pig based on a previously described scoring system (11). Lesions such as the enlargement of the lymph nodes (ranging from 0 for normal to 3 for three times normal size) were scored separately. Sections for histopathologic examination were taken from the nasal turbinate, lungs (five sections; 11), heart, brain, lymph nodes (tracheobronchial, iliac, mesenteric,

subiliac, and superficial inguinal), tonsil, liver, thymus, spleen, pancreas, and kidney. The tissues were examined in a blinded fashion and given a subjective score for severity of lung, lymph node, and liver lesions (11). Lung scores ranged from 0 (normal) to 3 (severe lymphohistiocytic interstitial pneumonia). Liver scores ranged from 0 (normal) to 3 (severe lymphohistiocytic interstitial hepatitis). Lymph node scores were an estimated amount of lymphoid depletion of follicles ranging from 0 (normal or no lymphoid depletion) to 3 (severe lymphoid depletion and histiocytic replacement of follicles) (11).

Serology. Blood samples were collected from all pigs at –1, 7, 14, 21, 28, 35, and 42 DPI. Serum antibodies to PCV2 were detected by a modified indirect ELISA based on the recombinant ORF2 capsid protein of PCV2 (24). Serum samples with a sample/positive (S/P) ratio above 0.2 were considered seropositive for PCV2.

Quantitative real-time PCR. Quantitative real-time PCR was performed to determine PCV2 virus loads in serum samples collected at –1, 7, 14, 21, 28, 35, and 42 DPI and in lymphoid tissue samples collected at 21 DPI and 42 DPI. Primer pair MCV1 (5'-GCTGAACTTTTGAAAGTGAGCGGG-3') and MCV2 (5'-TCACACAGTCTCAGTAGATCATCCCA-3') (12) was used for the quantitative real-time PCR. The PCR reaction was performed in the presence of intercalating SYBR green dye (Molecular Probes, Inc. Eugene, OR) as previously described (13). A standard dilution series with a known amount of pBluescript plasmid containing a single copy of the PCV2 genome (11) was run simultaneously in each real-time PCR reaction to quantify the virus genomic copy numbers (13).

Immunohistochemistry (IHC). IHC detection of PCV2 specific antigen was performed on lymph node, spleen, tonsil and thymus tissues collected during necropsy at 21 and 42 DPI as previously described (11,13, 14). The amount of PCV2 antigen distributed in the lymphoid tissues was scored in a blinded fashion by assigning a score of 0, if no signal, to 3 for a strong positive signal (27).

Statistical analysis. All statistical analyses were performed using the SAS®-system (Version 8.02, SAS institute Inc. Cary NC 27513). Growth characteristics of viruses were compared by regressive analyses using the GLM procedure. Serum samples S/P ratios were compared by analysis of variance, with the MIXED procedure. The model included effects of inoculum, DPI, and their interaction. S/P ratios were dichotomized to presence/absence of seroconversion at S/P = 0.20 and analyzed by logistic regression using the method of generalized equations in the SENROD procedure. Mean viral genomic copy numbers in serum and lymph nodes of piglets in group 2 and 3 were compared by the Kruskal-Wallis test using the NPAR1WAY procedure and/or by analysis of variance of ranked data, followed by a Bonferroni test of multiple mean ranks, using the GLM procedure. Serology and viremia data were analyzed for all pigs up to 21 DPI, and separately for those pigs necropsied at 42 DPI. Clinical sign scores were dichotomized to presence/absence of clinical signs for each examination date, and per pig over the entire period of study and compared between groups by Fisher's Exact test using the FREQ procedure, and by logistic regression using LOGISTIC procedure. Gross pathologic and histopathologic scores were compared by the Kruskal-Wallis test using

the NPAR1WAY procedure and/or by analysis of variance using the GLM procedure, followed by a Bonferroni test of multiple means. Proportion of pigs with gross and histopathologic lesions in various tissues were compared between groups by Fisher's Exact Test using the FREQ procedure.

RESULTS

PCV2 VP120 replicated more efficiently in PK-15 cells than PCV2 VP1. To determine the growth characteristics of PCV1, PCV2 VP1 and VP120, one-step growth curves were performed in duplicate simultaneously for PCV1, PCV2 VP1 and PCV2 VP120. The infectious titers of viruses collected at 12 hour intervals were determined by IFA (Fig. 1). The initial titers after infection at 12 h postinoculation were about $10^{1.5}$ $TCID_{50}$/ml for all three viruses. The infectious titers of PCV1 and PCV2 VP120 compared to PCV2 VP1 increased differently ($p = 0.0053$) from 12 to 96 h. By 96 h postinfection, PCV1 and PCV2 VP120 had titers of $10^{3.66}$ and $10^{3.75}$ $TCID_{50}$/ml, whereas the PCV2 VP1 was $10^{2.83}$ $TCID_{50}$/ml (Fig. 1).

Identification of two amino acid mutations within the PCV2 capsid protein during serial passages. The complete genomes of PCV2 passage numbers 1, 30, 60, 90, and 120 were amplified and sequenced. Sequence analyses revealed that there were a total of 2 amino acid mutations in the entire genome after 120 passages. The first mutation was detected in passage 30 (VP30) in which an alanine was substituted for an proline at position 110 of the capsid (P110A) (Fig. 2). This mutation was also present during the remaining passages. A second mutation from arginine to serine at position 191

of the capsid (R191S) was identified at passage 120 but not in lower passages (Fig. 2). By comparing all known PCV1 and PCV2 sequences in the Genbank including 31 PCV2 and 4 PCV1 isolates, we found that the P110A mutation is unique (Fig. 2) as all known PCV1 and PCV2 isolates have a proline at residue 110 of the capsid protein. The R191S mutation, however, is variable: PCV2 isolates of North American origin have an arginine, PCV2 isolates of Canadian and French origins have glycine, and PCV2 isolates of Spanish, Taiwanese and German origins have an alanine. All non-pathogenic PCV1 isolates have a threonine residue (Fig. 2).

PCV2 VP120 virus reduced viremia length and virus loads in sera of infected pigs compared to PCV2 VP1 virus. Serum samples were collected from all control and inoculated pigs at –1, 7, 14, 21, 28, 35, and 42 DPI and assayed for PCV2 viremia by quantitative real-time PCR and for anti-PCV2 antibody by ELISA. Prior to inoculation at –1 DPI, serum samples from all pigs were tested negative for PCV2 DNA.

The group 1 negative control pigs were negative for PCV2 viremia throughout the study (Table 1). All pigs in group 1 had detectable PCV2 maternal antibodies at –1 DPI, which all waned by 21 DPI. Seroconversion to PCV2 was not detected in any of the ten negative control pigs (Table 2). In the PCV2 VP120 inoculated group 2 pigs, viremia was first detected in one of eleven pigs at 7 DPI (Table 1, Fig. 3). A total of 4 pigs in group 2 were viremic during the study. The average length of continuous viremia was 1.6 weeks. By 35 DPI, all group 2 pigs seroconverted to PCV2 (Table 2). In the PCV2 VP1 inoculated group 3 pigs, viremia was first detected in seven of ten pigs at 7 DPI (Table 1, Fig. 3). Nine out of the ten pigs in group 3 became viremic for PCV2 during the study and the

average length of continuous viremia was 3 weeks. All animals in group 3 seroconverted to PCV2 by 35 DPI (Table 2). The range of PCV2 genomic copy numbers per ml of serum in positive samples was 8,840 to 274,800 in PCV2 VP120 inoculated group 2 pigs, and 26,520 to 120,000,000 in PCV2 VP1 inoculated group 3 pigs (Fig. 3). PCV2 genomic copy loads per ml of serum were greater in group 3 than that in group 2 pigs up to 21 DPI ($p = 0.0003$) and 42 DPI ($p = 0.039$). However, PCV2 DNA was recovered from lymph nodes of only 3/11 group 2 and 2/10 group 3 pigs, and the median PCV2 genomic copy loads per mg of tracheobronchial lymph node (TBLN) did not differ between group 2 and 3 ($p = 0.72$). The virus recovered from the sera and TBLN of 4 selected pigs in group 2 and 3 were sequenced, and sequence analyses revealed that the recovered viruses originated from the inocula. The S/P ratios of PCV2 antibodies differed between groups 1, 2 and 3 ($p < 0.0001$) and over time ($p < 0.0001$).

PCV2 VP120 virus is attenuated in pigs. Mild clinical signs (sneezing and rough coat) were noted in some animals from all three groups (data not shown). Two of 10 non-inoculated, and all of 21 inoculated pigs developed clinical signs ($p = 0.051$). Up to 21 DPI, group 2 and 3 pigs were 58 (95% C.I.: [13.1; 255.0]) and 41 [9.3;178.0] times more likely to show mild clinical signs at any examination date than negative control pigs, with no difference between pigs of group 2 and 3 (OR_{3vs2}: 1.4[0.8;2.6]). For the 16 pigs that were necropsied at 42 DPI, when evaluated over the entire study period, group 2 and 3 pigs again were more likely to show mild clinical signs than negative control pigs (OR_{2vs1}: 20.4[4.6;90.1]; OR_{3vs1}: 71.6 [16.0;320.8]) with group 3 pigs being 3.5 [1.9;6.6] times more likely to show mild clinical signs than group 2 pigs. There were no

differences in weight gain ($p = 0.081$) or mean rectal temperatures ($p > 0.05$) among any of the groups (data not shown). At necropsies, lymph nodes of 2/5 pigs in group 1 were mildly enlarged, however this was not associated with PCV2 infection as evidenced by the lack of PCV2 DNA or seroconversion. At 42 DPI necropsy, all group 1 pigs had normal lymph nodes (Table 3). Group 2 pigs inoculated with PCV2 VP120 had mild to moderately enlarged lymph nodes at both 21 and 42 DPI (Table 3). The lymph nodes in group 3 pigs were moderately to severely enlarged at both 21 and 42 DPI (Table 3). Pigs inoculated with PCV2 VP1 had visible gross pneumonia at 21 DPI. Visible gross pneumonia was not found in group 1 or 2 pigs at either 21 or 42 DPI.

Microscopic lung lesions characterized by mild peribronchiolar lymphoplasmacytic and histiocytic bronchointerstitial pneumonia and liver lesions characterized by mild lymphoplasmacytic hepatitis were detected in pigs of all groups (Table 4). Mild lymphoid depletion (LD) of lymph node follicles was detected in 0/5 group 1 pigs at 21 and 42 DPI, in 3/5 (21 DPI) and in 2/6 (42 DPI) group 2 pigs, in 4/5 (21 DPI) and 5/5 (42 DPI) group 3 pigs (Table 4). Mild histiocytic replacement (HR) of lymph node follicles was not observed in group 1 pigs. In group 2, mild HR was observed in the lymph nodes of 0/5 pigs at 21 DPI and 2/6 pigs at 42 DPI. In group 3, mild to moderate HR of the lymph nodes was observed in 3/5 pigs at both 21 and 42 DPI. The tonsil and spleen tissue follicles of the group 1 pigs were free of LD and HR at 21 or 42 DPI. Mild LD of the tonsil follicles was found in 2/5 pigs in group 2 at 21 DPI. Mild to moderate LD of the tonsil follicles was found in 2/5 pigs in group 3 at 21 DPI and mild HR of the tonsil tissue in 1/5 pigs in group 3 at both 21 and 42 DPI. Mild LD of the spleen follicles was observed in 2/5 and 1/6 pigs in group 2 at 21 and 42 DPI,

respectively. Mild to moderate LD of the spleen follicles was noted in 4/5 in group 3 pigs at both 21 and 42 DPI. In group 2 pigs, mild HR of the spleen tissue follicles was found in 1/6 pigs at 42 DPI. In group 3, 4/5 pigs at 21 DPI and 3/5 pigs at 42 DPI had mild to moderate HR of the spleen tissue follicles (Table 4). Presence of lesions in other tissues and organs are summarized in Table 4.

At necropsies (21 and 42 DPI), PCV2 antigen was not detected by IHC in the lymphoid tissues of the negative control group 1 pigs. In the PCV2 VP120 inoculated group 2, low amounts of PCV2 antigen was detected in spleen tissues of 1/5 pigs at 21 DPI, in lymph node tissues of 2/6 pigs at 42 DPI, and in tonsil tissues of 3/6 pigs at 42 DPI (Table 5). In the PCV2 VP0 inoculated group 3, low-to-high amounts of PCV2 antigen were detected in lymph node tissues of 5/5 pigs, in tonsil tissues of 4/5 pigs, and in spleen tissues of 4/5 pigs at 21 DPI. At 42 DPI, low-to-moderate amounts of PCV2 antigen were detected in PCV2 VP0 inoculated group 3 (Table 5).

All gross pathologic and histopathologic scores at 21 DPI and 42 DPI were compared by analysis of variance using the GLM procedure followed by a Bonferroni test of multiple means. At 21 DPI, groups 1 and 2 mean scores are similar ($p = 1.00$) but differ from the mean scores of group 3 ($p = 0.0032$). By 42 DPI, the mean scores of group 1 differ from group 2 (p = 0.0083) and group 3 (p = 0.0001), and the group 2 mean scores are milder than those of group 3 (p = 0.0274) (Tables 3-5).

DISCUSSION

PCV2 infection and PMWS have become an economically important global disease. The molecular mechanisms of PCV2 replication and pathogenesis are poorly

understood. We report here the identification of two amino acid mutations within the PCV2 capsid protein that are important for in PCV2 pathogenicity *in vivo* and improved growth ability in PK-15 cells. The passage 120 of a PCV2 isolate contained two amino acid mutations in the capsid protein, P110A and R191S, compared to the passage 1 of the virus. As a result of these two mutations, PCV2 VP120 replicated more efficiently ($p = 0.0053$) in PK-15 cells with at least 1 log difference in infectious titer compared to the passage 1 virus. PCV2 VP120 replicated to a similar level with the PK-15 cell culture-adapted PCV1, and thus these two mutations either alone or collectively are responsible for the enhanced growth of PCV2 VP120 *in vitro*. Allan et al (1) attempted to infect human Vero cells with PCV1. Intranuclear immune staining, characteristic of PCV1 replication, was not detected until the 6th cell culture passage in Vero cells. By passage 15, PCV1 replicated in Vero cells similarly to PK-15 cells, suggesting that efficient PCV1 replication in Vero cells depended upon the number of virus passages.

When SPF piglets were inoculated with the PCV2 VP120, fewer pigs developed viremia with shorter duration and lower PCV2 genomic copy loads compared to PCV2 VP1 inoculated pigs. We previously showed that the non-pathogenic PK-15 cell-adapted PCV1 had a short average viremia length of 0.625 weeks in infected pigs (14). Analyses of the gross, microscopic and IHC mean scores revealed that the PCV2 VP120 virus inoculated pigs had milder pathological lesions and clinical signs than the PCV2 VP1 virus inoculated pigs. Taken together, the results from this study showed that PCV2 VP 120 had adapted to grow better in PK-15 cells and is attenuated in pathogenicity *in vivo*.

After 120 passages in PK-15 cells, only two amino acid mutations were detected in the entire PCV2 genome, suggesting that the PCV2 genome is relatively stable. This

may explain why the sequences of all known PCV2 field isolates identified to date are very conserved (12, 20). The P110A mutation occurred early (passage 30) during the serial passage, and involved in two hydrophobic amino acids, proline and alanine. The change from proline to alanine may alter the tertiary structure of the capsid protein as proline is often involved in the bending regions of protein structures. The uniqueness of the P110A mutation in passage 120 compared to the sequences of known PCV1 and PCV2 field isolates strongly suggests a biological role of this mutation. The R191S mutation occurred very late during the serial passage (between passages 90 and 120), and is also unique to the VP120 as the known PCV2 and PCV1 isolates do not have a serine residue at this position. However, glycine, alanine and threonine substitutions at this position have been identified in field isolates of PCV1 and PCV2. Lekcharoensuk et al (21) identified at least 3 conformational neutralizing epitopes within the PCV2 capsid protein, and the R191S mutation falls within one of these immunoreactive epitopes, suggesting that the R191S mutation may also play an important role in PCV2 replication and pathogenesis.

Amino acid substitutions induced by cell culture passage or chemical mutagenesis techniques have been routinely used for the attenuation of many viruses, and have led to the productions of many vaccines (6). A single amino acid substitution could lead to the attenuation of a virus. For example, substitution of a proline for a leucine at residue 101 of the nonstructural 4B protein of the mosquito-borne Dengue 4 (DEN4) virus resulted in decreased viral replication in mosquito but a proportionally increased replication in human Vero cells (15). Hence, the balancing control of efficient replication of DEN4 virus in either mosquito or human Vero cells was maintained by a single amino acid

change. In the *Circoviridae* family, a single amino acid mutation in the VP1 capsid protein of CAV was found to be responsible for the pathogenicity of the virus in chickens (32). Taken together, the results from this study suggested that either P110A or R191S or collectively are responsible for the attenuation of PCV2 VP120 in pigs and for the improved growth ability of PCV2 in PK-15 cells.

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REFERENCES

1. **Allan, G.M., D.P. Mackie, J. McNair, B.M. Adair, and M.S. McNulty.** 1994. Production, preliminary characterization and applications of monoclonal antibodies to porcine circovirus. Vet. Immunol. Immunopathol. **43**:357-371.
2. **Allan, G.M., F. McNeilly, J.P. Cassidy, G.A. Reilly, B. Adair, W.A. Ellis, and M.S. McNulty.** 1995. Pathogenesis of porcine circovirus; experimental infections of colostrum deprived piglets and examination of pig foetal material. Vet. Microbiol. **44**:49-64.
3. **Allan, G.M., F. McNeilly, S. Kennedy, B. Daft, E.G. Clarke, J.A. Ellis, and D.M. Haines.** 1998. Isolation of porcine circovirus-like viruses from pigs with a wasting disease in the USA and Europe. J. Vet. Diagn. Invest. **10**:3-10.
4. **Allan, G.M. and J.A. Ellis.** 2000. Porcine circoviruses: a review. J. Vet. Diagn. Invest. **12**:3-14.
5. **Bassami, M.R., D. Berryman, G.E. Wilcox, and S.R. Raidal.** 1998. Psittacine beak and feather disease virus nucleotide sequence analysis and its relationship to porcine circovirus, plant circoviruses, and chicken anaemia virus. Virology **249**:453-459.
6. **Brooks, G.F., J.S. Butel, S.A. Morse.** 1998. Pathogenesis & control of viral diseases. In: Jawetz, Melnick, % Adelberg's Medical Microbiology. Appelton & Lange **30**: 363-365.
7. **Cheung, A.K**. 2003. Transcriptional analysis of porcine circovirus. Virology **305**: 168-180.

8. **Choi, C., C. Chae, and E.G. Clark.** 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.

9. **Edwards, S. and J.J. Sands.** 1994. Evidence of circovirus infection in British pigs. Vet. Rec. **134**:680-681.

10. **Ellis, J., L. Hassard, E. Clark, J. Harding, G. Allan, P. Willson, J. Strakappe, K. Martin, F. McNeilly, B. Meehan, D. Todd, and D. Haines.** 1998. Isolation of circovirus from lesions of pigs with postweaning multisystemic wasting syndrome. Can. Vet. J. **39**:44-51.

11. **Fenaux M, P.G. Halbur, G. Haqshenas, R, Royer, P. Thomas, P. Nawagitgul, M. Gill, T.E. Toth, and X.J. Meng.** 2002. Cloned genomic DNA of type 2 porcine circovirus is infectious when injected directly into the liver and lymph nodes of pigs: characterization of clinical disease, virus distribution, and pathological lesions. J. Virol. **76**:541-551.

12. **Fenaux M, P.G. Halbur, M. Gill, T.E. Toth, and X.J. Meng.** 2000**.** Genetic characterization of type 2 porcine circovirus (PCV-2) from pigs with postweaning multisystemic wasting syndrome in different geographic regions of North America and development of a differential PCR-restriction fragment length polymorphism assay to detect and differentiate between infections with PCV-1 and PCV-2. J. Clin. Microbiol. **38**: 2494-2503.

13. **Fenaux M., T. Opriessnig, P.G. Halbur, F. Elvinger, and X.J. Meng**. 2004. A chimeric porcine circovirus (PCV) with the immunogenic capsid gene of the

pathogenic PCV2 cloned into the genomic backbone of the non-pathogenic PCV1 induces protective immunity against PCV2 infection in pigs. J. Virol. In press.

14. **Fenaux M., T. Opriessnig, P.G. Halbur, and X.J. Meng**. 2003. Immunogenicity and pathogenicity of the chimeric infectious DNA clones between pathogenic type 2 porcine circovirus (PCV2) and non-pathogenic PCV1 in weaning pigs. J. Virol. **77**:11232-11243.
15. **Hanley K.A., L.R. Manlucu, L.E. Gilmore, J.E. Blaney, C.T. Hanson, B.R. Murphy, S.S. Whitehead.** 2003. A trade-off in replication in mosquito versus mammalian systems conferred by a point mutation in the NS4B protein of dengue virus type 4. Virology. **312**:222-232
16. **Harding, J.C. and E.G. Clark.** 1997. Recognizing and diagnosing post-weaning multisystemic wasting syndrome (PMWS). Swine Health Prod. **5**:201-203
17. **Harms P.A., S.D. Sorden, P.G. Halbur, S.R. Bolin, K.M. Lager, L. Morozov, and P.S. Paul.** 2001. Experimental reproduction of severe disease in CD/CD pigs concurrently infected with type 2 porcine circovirus and porcine reproductive and respiratory syndrome virus. Vet. Pathol. **38**:528-539.
18. **Krakowka, S., J.A. Ellis, B. Meehan, S. Keenedy, F. McNeilly, and G. Allan.** 2000. Viral wasting syndrome of swine: experimental reproduction of postweaning multisystemic wasting syndrome in gnotobiotic swine by coinfection with porcine circovirus 2 and porcine porvovirus. Vet Pathol. **37**:254-263.
19. **Ladekjaer-Mikkelsen, A.S., J. Nielsen, T. Stadejek, T. Storgaard, S. Krakowka, J. Ellis, F. McNeilly, G. Allan, and A. Botner.** 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.

20. **Larochelle, R., R. Magar, S. D'Allaire.** 2002. Genetic characterization and phylogenetic analysis of porcine circovirus type 2 (PCV2) strains from cases presenting various clinical conditions. Virus Res. **90**:101-112
21. **Lekcharoensuk, P., I. Morozov, P.S. Paul, N. Thangthumniyom, W. Wajjawalku, and X.J. Meng.** 2004. Epitope mapping of the major capsid protein of type 2 porcine circovirus (PCV2) using chimeric PCV1 and PCV2 viruses. J. Virol. Submitted.
22. **Mankertz, A., M. Domingo, J.M. Folch, P. LeCann, A. Jestin, J. Segales, B. Chmielewicz, J. Plana-Duran, and D. Soike.** 2000. Characterisation of PCV-2 isolates from Spain, Germany and France. Virus Res. **66**:65-77.
23. **Mankertz A., K. Hattermann, B. Ehlers, and D. Soike.** 2000**.** Cloning and sequencing of columbid circovirus (CoCV), a new circovirus from pigeons. Arch. Virol. **145**:2469-2479
24. **Nawagitgul, P., P.A. Harms, I. Morozov, B.J. Thacker, S.D. Sorden, C. Lekcharoensuk, and P.S. Paul.** 2002. Modified indirect porcine circovirus (PCV) type 2-based and recombinant capsid protein (ORF2)-based ELISA for the detection of antibodies to PCV. Clin. Diagn. Lab. Immunol. **9:**33-40.
25. **Onuki, A., K. Abe, K. Togashi, K. Kawashima, A. Taneichi, and H. Tsunemitsu.** 1999. Detection of porcine circovirus from lesions of a pig with wasting disease in Japan. J. Vet. Med. Sci. **61**:1119-1123

26. **Phenix, K.V., J.H. Weston, I. Ypelaar, A. Lavazza, J.A. Smyth, D. Todd, G.E. Wilcox, and S.R. Raidal.** 2001. Nucleotide sequence analysis of a novel circovirus of canaries and its relationship to other members of the genus circovirus of the family Circoviridae. J. Gen. Virol. **82**:2805-2809.

27. **Sorden S.D., P.A. Harms, P. Nawagitgul, D. Cavanaugh, and P.S. Paul.** 1999. Development of a polyclonal-antibody-based immunohistochemical method for the detection of type 2 porcine circovirus in formalin-fixed, paraffin-embedded tissue. J. Vet. Diagn. Invest. **11**:528-530.

28. **Tischer, I., H. Gelderblom, W. Vettermann, and M.A. Koch.** 1982. A very small porcine virus with circular single-stranded DNA. Nature **295**:64-66.

29. **Tischer, I., W. Mields, D. Wolff, M. Vagt, and W. Griem.** 1986**.** Studies on epidemiology and pathogenicity of porcine circovirus. Arch. Virol. **91**:271-276.

30. **Tischer, I., R. Rasch, and G. Tochtermann.** 1974. Characterization of papovavirus and picornavirus-like particles in permanent pig kidney cell lines. Zentralbl. Bakteriol. Hyg. A **226**:153-167.

31. **Todd, D., J.H. Weston, D. Soike, and J.A. Smyth.** 2001. Genome sequence determinations and analyses of novel circoviruses from goose and pigeon. Virology. **286**:354-362

32. **Yamaguchi, S., T. Imada, N. Kaji, M. Mase, K. Tsukamoto, N. Tanimura and N. Yuasa.** 2001. Identification of a genetic determinant of pathogenicity in chicken anaemia virus. J. Gen. Virol. **82**: 1233-1238.

Fig. 1

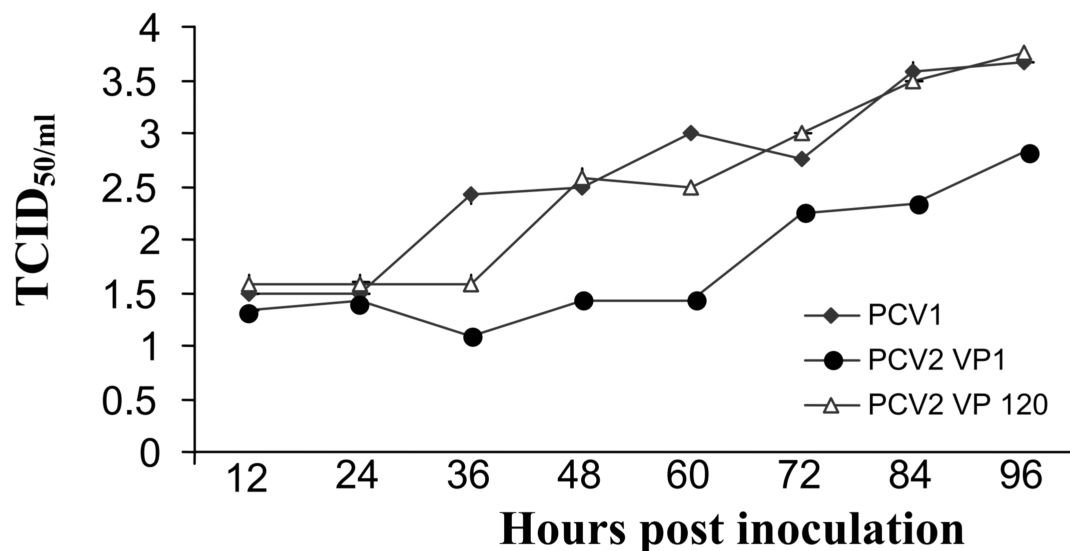

FIG. 1. One–step growth curves of PCV1, PCV2 VP1 and PCV2 VP120. Duplicate synchronized PK-15 cell cultures were each infected with PCV1, PCV2 VP1, or PCV2 VP120, all at an M.O.I. of 0.1. All three viruses had a titer of about $10^{1.5}$ $TCID_{50}$/ml at 12 h postinoculation. PCV1 and PCV2 VP120 replicated more efficiently *in vitro* than did PCV2 VP1 ($p = 0.0053$).

Fig. 2

	110	191	
	P	R	VP 0
	A	R	VP 30
	A	R	VP 60
	A	R	VP 90
	A	S	VP 120
	P	R	PCV2: US & Canada
	P	G/A	PCV2: Canada, France, Spain, Germany & Taiwan
	P	T	PCV1

FIG. 2. Schematic diagram of amino acid mutations in the capsid protein during serial passages of PCV2 in PK-15 cells. Serial passage numbers are indicated as VP1, VP30, VP60, VP90 and VP120. Known field isolates of PCV2 and PCV1 from different geographic origins are also compared for these two mutations.

Fig. 3

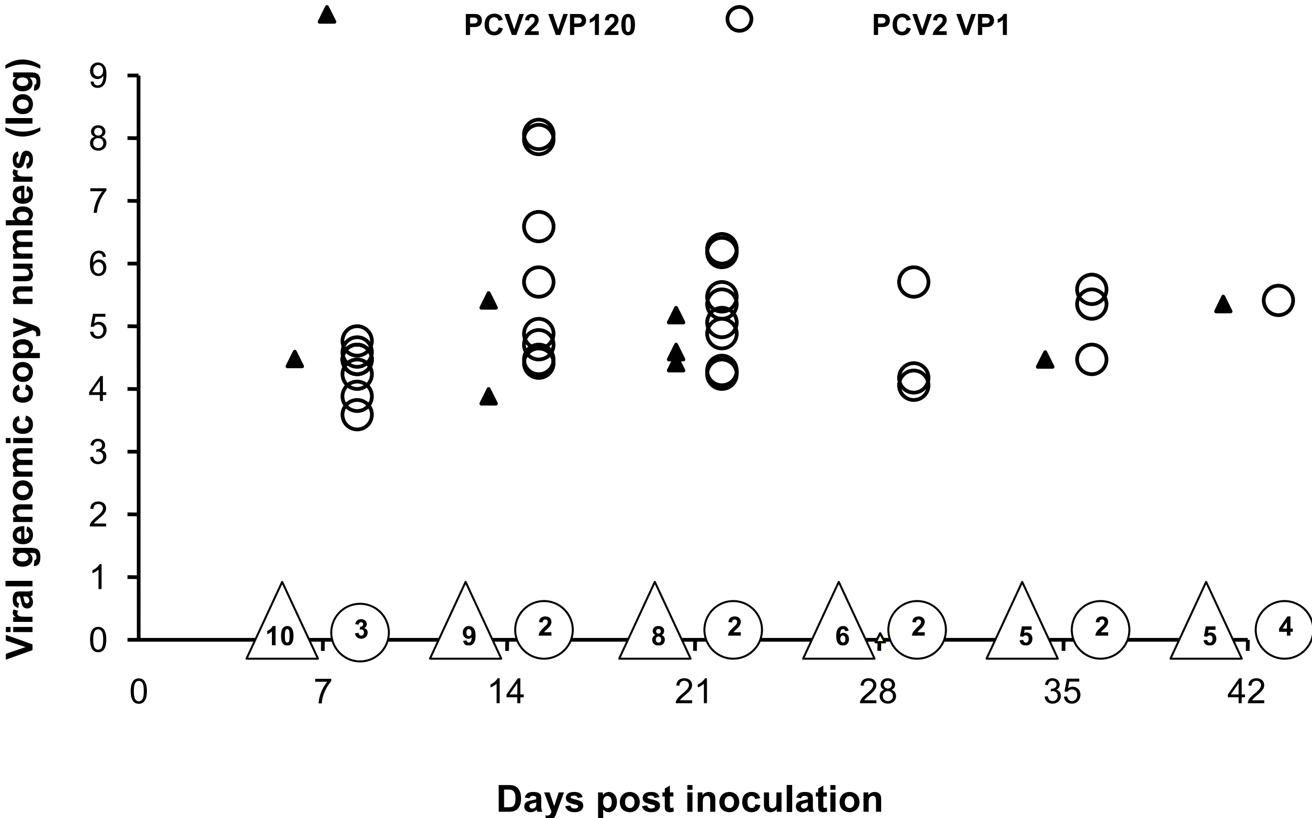

FIG. 3. Quantitative real-time PCR results of PCV2 VP1 and PCV2 VP120 viral genomic copy loads in 1 ml of serum sample collected at –1, 7, 14, 21, 28, 35 and 42 days post inoculation (DPI) from groups 1, 2 and 3 pigs. Group 2 pigs inoculated with PCV2 VP120 that were positive for PCV2 DNA are indicated with a ▲. The numbers (10, 9, 8, 6, 5, 5) inside the symbol △ on the X-axis indicate the number of pigs in group 2 that were negative for PCV2 viremia on the respective DPI. Group 3 pigs inoculated with PCV2 VP1 that were positive for PCV2 DNA are indicated with symbol O. The numbers (3, 2, 2, 2, 2, 4) inside the symbol O on the X-axis indicate the number of pigs in group 3 that were negative for PCV2 viremia on the respective DPI. The PCV2 genomic copy loads were represented as a log of copy numbers per 1 ml of serum (Y-axis).

Table 1. Detection of viremia by real-time PCR in sera of inoculated and control pigs

Group	Inocula	**No. of pigs positive/no. tested**[a]							
		Days post inoculation							
		-1	7	14	21	28	35	42	total
1	Control	0/10	0/10	0/10	0/10	0/5	0/5	0/5	0/10
2	PCV2 VP120	0/11	1/11	2/11	3/11	0/6	1/6	1/6	4/11
3	PCV2 VP 1	0/10	7/10	8/10	8/10	3/5	3/5	1/5	9/10

[a]Five pigs per group were necropsied at 21 day post inoculation (DPI) and the remaining pigs were necropsied at 42 DPI.

Table 2. Seroconversion to PCV2 antibodies in pigs inoculated with PCV2 passages 1 (VP1) and 120 (VP120)

Goup	Inoculum	**No. of pigs positive/no. tested[a]** Days post inoculation						
		-1	7	14	21	28	35	42
1	Control	10/10[b]	1/10	1/10	0/10	0/5	0/5	0/5
2	PCV2 VP120	3/11 [b]	0/11	0/11	0/11	0/6	6/6	6/6
3	PCV2 VP 1	1/10 [b]	0/10	1/10	2/10	4/5	5/5	5/5

[a]Five pigs per group were necropsied at 21 day post inoculation (DPI) and the remaining pigs were necropsied at 42 DPI.
[b]Maternal antibodies were detectable at –1 DPI but waned in all groups between 7 and 21 DPI.

Table 3. Gross lymph node and lung lesions in control and inoculated pigs

Group	Inoculum	No. of pigs with enlarged lymph nodes[a]		No. of pigs with gross pneumonia lesions	
		21 DPI	42 DPI	21 DPI	42 DPI
1	Control	2/5(0.4[b])^{I}[c]	0/5(0.0)^{I}	0/5(0.0)^{I}	0/5(0.0)
2	PCV2 VP120	2/5(0.4)^{I}	4/6(1.3)^{II}	0/6(0.0)^{I}	0/5(0.0)
3	PCV2 VP1	5/5(2.2)^{II}	5/5(2.6)^{II}	2/5(2.6)^{II}	0/5(0.0)

[a]Five pigs from each group was necropsied at 21 days post inoculation (DPI) and the remaining pigs were necropsied at 42 DPI.
[b] Values in parentheses are the mean scores of estimated lymph node enlargement (0 = normal to 3 severely enlarged and dicolored) and mean percentage of lungs affected by gross visible pneumonia (0 – 100%)
[c] Different superscripts (I,II) indicate different mean value score between groups ($p < 0.05$).

Table 4. Distribution of histopathological lesions in different tissues and organs from control and inoculated pigs

Group	Inocula	DPI[a]	No. of pigs positive/no. of pigs tested									
			Lung	Lymph nodes		Tonsil		Spleen		liver	Kidney	Heart
				LD¶[c]	HR	LD†	HR	LD¶	HR†			
1	Control	21	0/5(0.0)[b]	0/5(0.0)	0/5(0.0)	0/5(0.0)	0/5(0.0)	0/5(0.0)	0/5(0.0)	0/5(0.0)	1/5	1/5
		42	3/5(0.6)	0/5(0.0)	0/5(0.0)	0/5(0.0)	0/5(0.0)	0/5(0.0)	0/5(0.0)	1/5(0.2)	1/5	0/5
2	PCV2 VP120	21	2/5(0.4)	3/5(0.6)	0/5(0.0)	2/5(0.4)	0/5(0.0)	2/5(0.4)	0/5(0.0)	3/5(0.6)	2/5	0/5
		42	5/6(1.0)	2/6(0.3)	2/6(0.3)	0/6(0.0)	0/6(0.0)	1/6(0.2)	1/6(0.2)	0/6(0.0)	2/6	0.5
3	PCV2 VP0	21	2/5(0.4)	4/5(1.2)	3/5(1.0)	5/5(1.2)	1/5(0.2)	4/5(1.0)	4/5(1.0)	3/5(1.2)	3/5	3/5
		42	5/5(1.4)	5/5(1.4)	3/5(0.8)	0/5(0.0)	1/5(0.2)	4/5(1.0)	3/5(0.6)	3/6(0.6)	1/5	2/5

[a]DPI, Days postinoculation
[b] Values in parentheses are mean histological scores for interstitial pneumonia and for interstitial hepatitis and lymphoid depletion (LD) and histioctyic replacement (HR) for lymph nodes, tonsils and spleen.
[c] Indicates difference ($p < 0.05$) using Fisher's Exact test between groups 1, 2, and 3 in severity of respective histopatholigical lesion with symbol † at 21 DPI and symbol ¶ at 42 DPI necropsies.

Table 5. Immunohistiochemical detection of PCV2 antigen in lymph nodes, tonsils and spleen of inoculated and control pigs

Group	Inocula	DPI [a]	No. of pigs positive/no. tested [b]		
			Lymph node†[b]	Tonsil†	Spleen
1	Control	21	0/5 (0.0)[c]	0/5 (0.0)	0/5 (0.0)
		42	0/5 (0.0)	0/5 (0.0)	0/5 (0.0)
2	PCV2 VP120	21	0/5 (0.0)	0/5 (0.0)	1/5 (0.2)
		42	2/6 (0.3)	3/6 (0.5)	0/6 (0.0)
3	PCV2 VP1	21	5/5 (1.6)	4/5 (1.0)	4/5 (1.2)
		42	3/5 (0.8)	2/5 (0.4)	2/5 (0.4)

[a]DPI, days postinoculation
[b]Indicates difference ($p < 0.05$) using Fisher's Exact test between groups 1, 2, and 3 of PCV2 antigen presence in respective tissues with symbol † at 21 DPI.
[c]Value in parentheses are the mean scores of the amounts of PCV2 antigen in lymphoid tissues (ranging from 0, no antigen detected, to 3, high levels of antigen).

General Conclusions

PMWS is an emerging disease of swine. Since the recognition of the disease in 1991 (7), PMWS has emerged to be an economically important global swine disease (1, 4, 5, 8, 13, 14, 17). The clinical and pathological presentations and etiology of PMWS are poorly understood, although PCV2 is generally believed to be the primary causative agent of PMWS (1, 6, 15, 17).

I genetically characterized six North American isolates of PCV2 (one Canadian isolate and five U.S. isolates) from pigs with PMWS from different geographic regions. Sequence analysis of the complete genome indicated that the six North American isolates of PCV2 are closely related to other known PCV2 isolates worldwide. The highest variability in the PCV genome was in the capsid gene (ORF2). Phylogenetic analysis revealed that all PCV2 isolates sequenced form a major genotype, whereas all PCV1 isolates are closely related and form another genotype. On the basis of phylogenetic analysis, it is evident that both PCV1 and PCV2 evolved from the same ancestor, but they likely have undergone divergent evolution. Within the major genotype of PCV2, several minor branches were also identified. The French PCV2 isolates diverge the most from all other PCV2 isolates. These data suggest that, although the genome of PCV2 is relatively stable in general, minor genetic differences do exist among PCV2 isolates from different geographic regions. This observed difference has important implications for the diagnosis of PCV2 infection by nucleic acid-based assays such as PCR.

On the basis of genetic analyses of all PCV isolates, I developed a universal PCR restriction fragment length polymorphism (RFLP) assay for the diagnosis of PCV2

infection and for the differentiation between infections by PCV1 and PCV2 in pigs. This assay uses a pair of PCR primers selected from two conserved regions of the PCV1 and PCV2 genomes and a unique *Nco*I restriction enzyme site that exists only in PCV2 isolates. Our results indicate that this PCR-RFLP assay is accurate and rapid in diagnosing PCV2 infection in pigs with PMWS from different geographic regions, and in differentiating PCV1 and PCV2 infections as well as detecting dual infections by PCV1 and PCV2.

Due to the difficulty in producing a biologically pure form of PCV2 because of the presence of other common swine agents in the tissue homogenates of diseased pigs, an infectious cDNA clone of PCV2 was constructed. I showed that the PCV2 DNA clone is infectious when injected directly into the livers and superficial iliac lymph nodes of specific-pathogen-free (SPF) pigs. Pigs directly injected with the infectious DNA clone developed an infection and disease resembling those induced by infection via the intranasal route of inoculation with PCV2 infectious live virus stock. By using this infectious PCV2 DNA clone, the clinical disease, pathological lesions, and virus distribution exclusively attributable to PCV2 infection were more definitively characterized. PCV2 is clearly responsible for the PMWS-like histopathological lesions, but PCV2 may require a secondary agent or immunostimulant to induce the full spectrum of the clinical PMWS disease (2, 9, 10, 11, 12, 18).

The construction of an infectious PCV2 infectious DNA clone and the demonstration of infection by direct injection of the infectious PCV2 DNA clone into the liver and lymph nodes of pigs should be very advantageous for future PCV2 pathogenesis studies. The replication and pathogenesis of PCV2 can be studied *in vivo* without having

to first produce infectious virus stocks in cell culture. This is advantageous, as serial cell culture passages may select for variants (3). Direct injection of animals with the PCV2 infectious DNA clone for pathogenesis studies eliminates the problems associated with the presence of other indigenous swine agents in tissue homogenate inocula.

PCV2-associated PMWS has increasingly become a more serious economic problem for the swine industry resulting in an urgent need for the development of a vaccine against PCV2. We constructed three additional infectious PCV DNA clones: PCV1 DNA clone, chimeric PCV1-2 DNA clone, and a reciprocal chimeric PCV2-1 DNA clone. The availability of the PCV2, PCV1 chimeric PCV1-2 and reciprocal chimeric PCV2-1 infectious DNA clones enabled the study of the structural and functional relationship of PCV genes. We found that the PCV1 replicated more efficiently than PCV2, chimeric PCV1-2, and reciprocal chimeric PCV2-1 in PK-15 cells. This is likely due to the fact that the PCV1 isolate originated from PK-15 cell line and thus may be adapted to *in vitro* replication.

The chimeric PCV1-2 infectious DNA clone, constructed by cloning the immunogenic capsid gene (ORF2) of the pathogenic PCV2 into the non-pathogenic PCV1 genome, when transfected into cell culture or live pigs resulted in virus replication and expression of the immunogenic capsid antigen of PCV2. Pigs inoculated with the chimeric PCV1-2 retained the non-pathogenic nature of PCV1 with only a mild infection resembling that of PCV1-inoculated animals while seroconverting to antibodies specific to the ORF2 capsid protein of PCV2. Therefore, the chimeric PCV1-2 infectious DNA clone could serve as a vaccine candidate against PCV2 infection and PMWS.

We subsequently demonstrated that pigs vaccinated with the chimeric PCV1-2 candidate vaccine developed protective immunity against a wild-type pathogenic PCV2 challenge. The data indicated that the chimeric PCV1-2 candidate vaccine can prevent PCV2 viremia and significantly reduce the amount of PCV2 virus in the lymphoid tissue, which are important factors in pathogenesis of PCV2 associated diseases (1). The lack of hallmark microscopic PMWS lesions in vaccinated and challenged pigs such as lymphocytic depletion (LD) and histiocytic replacement (HR) of the lymphoid tissues strongly indicates that the chimeric PCV1-2 candidate vaccine is effective in protecting pigs from PCV2-associated lymphoid lesions and thus preventing the detrimental effects to the immune system. The occurrence of LD and HR during initial PCV2 infection may be linked to the eventual occurrence of leukopenia prior to the onset of clinical PMWS (16, 19). Therefore, the chimeric PCV1-2 candidate vaccine may have the ability to stop the eventual progression to clinical PMWS by preventing the initial damage to the lymphoid tissues.

Intramuscular vaccination with PCV1-2 DNA clone, intra-lymphoid vaccination with PCV1-2 DNA clone and intramuscular vaccination with PCV1-2 live virus were all effective in inducing protective immunity against PCV2 infection. However, the intramuscular vaccination route is likely the only route acceptable by swine producers when the vaccine is licensed.

Although not all the vaccinated pigs seroconverted to PCV2 by the time of challenge, they were all protected against the pathogenic PCV2 challenge, suggesting that high level of PCV2 antibody titers responses are not absolutely required for protection. It is also possible that the chimeric PCV1-2 candidate vaccine induces a cell-mediated

immune response, which may be equally or more important for the induction of protection against PCV2.

The molecular mechanisms of PCV2 replication and pathogenesis are poorly understood. In an attempt to identify PCV2 genetic determinants for virulence and replication, we serially passaged a pathogenic PCV2 isolate for 120 times in PK-15 cells. After 120 passages, two amino acid mutations were detected within the PCV2 capsid protein, P110A and R191S, that may be involved in PCV2 virulence and adaptation to PK-15 cell growth. PCV2 passage 120 (VP120) replicated to a similar level with the PK-15 cell culture-adapted PCV1 but more efficiently than the passage 1 PCV2 (VP1), and thus these two mutations either alone or collectively are responsible for the enhanced growth of PCV2 VP120 *in vitro*. When SPF piglets were inoculated with the PCV2 VP120, fewer pigs developed viremia and those that did had shorter viremia and lower PCV2 genomic copy loads compared to wild-type PCV2 inoculated pigs. Analyses of the gross, microscopic and IHC mean scores revealed that the PCV2 VP120 inoculated pigs had milder pathological lesions and milder clinical signs than the PCV2 VP1 inoculated pigs. Taken together, the results from this study showed that PCV2 VP120 adapted to replicate better in PK-15 cells and became attenuated *in vivo*. The two mutations, P110A and R191S, are unique compared to known PCV1 and PCV2 sequences, suggesting a role in PCV2 virulence and replication. These mutations could be used to further improve the safety of the chimeric PCV1-2 vaccine. Since only two mutations were detected in the PCV2 genome after 120 passages, this suggests that the PCV2 genome is relatively stable.

In summary, we have genetically characterized PCV1 and PCV2 from different geographic origins and found a high DNA sequence homology and close phylogenetic relationship among PCV1 and PCV2 isolates, respectively. We developed a PCR-RFLP diagnostic assay capable of detecting and differentiating between infections causes by PCV1 or PCV2. We have constructed infectious DNA clones of PCV1, PCV2 and two chimeric infectious DNA clones. We have definitively characterized the pathological lesions associated with PCV2 and PCV1 infections and confirmed the primary etiological role of PCV2 infection in PMWS. We developed a genetically engineered chimeric PCV1-2 vaccine and showed that the chimeric vaccine retains the nonpathogenic nature of PCV1 but induces a strong protective immune response against PCV2. In addition, we identified the putative genetic determinants for PCV2 replication and/or virulence by serially passaging PCV2 120 times in PK-15 cells. The VP120 of PCV2 increased the growth rate of PCV2 *in vitro* and attenuated the virus *in vivo*.

References

1. **Allan, G.M. and J.A. Ellis.** 2000. Porcine circoviruses: a review. J. Vet. Diagn. Invest. **12**:3-14.
2. **Allan, G.M., S. Kennedy, F. McNeilly, J.C. Foster, J.A. Ellis, S.J. Krakowka, B.M. Meehan, and B.M. Adair.** 1999. Experimental reproduction of severe wasting disease by co-infection of pigs with porcine circovirus and porcine parvovirus. J. Comp. Pathol. **121**:1-11.
3. **Allan, G.M., D.P. Mackie, J. McNair, B.M. Adair, and M.S. McNulty.** 1994. Production, preliminary characterization and applications of monoclonal antibodies to porcine circovirus. Vet. Immunol. Immunopathol. **43**:357-371.
4. **Allan, G.M., F. McNeilly, S. Kennedy, B. Daft, E.G. Clarke, J.A. Ellis, D.M. Haines, B.M. Meehan, and B.M. Adair.** 1998. Isolation of porcine circovirus-like viruses from pigs with a wasting disease in the USA and Europe. J. Vet. Diagn. Invest. **10**:3-10.
5. **Ellis, J., L. Hassard, E. Clark, J. Harding, G. Allan, P. Willson, J. Strakappe, K. Martin, F. McNeilly, B. Meehan, D. Todd, and D. Haines.** 1998. Isolation of circovirus from lesions of pigs with postweaning multisystemic wasting syndrome. Can. Vet. J. **39**:44-51.
6. **Hamel, A.L., L.L. Lin, and G.P. Nayar.** 1998. Nucleotide sequence of porcine circovirus associated with postweaning multisystemic wasting syndrome in pigs. J. Virol. **72**:5262-5267.

7. **Harding, J.C. and E.G. Clark.** 1997. Recognizing and diagnosing postweaning multisystemic wasting syndrome (PMWS). Swine Health and Production. **5**:201-203.

8. **Kennedy, S., G. Allan, F. McNeilly, B.M. Adair, A. Hughes, and P. Spillane.** 1998. Porcine circovirus infection in Northern Ireland. Vet. Rec. **142**:495-6.

9. **Kennedy, S., D. Moffett, F. McNeilly, B. Meehan, J. Ellis, S. Krakowka, and G.M. Allan.** 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.

10. **Krakowka, S., J.A. Ellis, F. McNeilly, S. Ringler, D.M. Rings, and G. Allen.** 2001. Activation of the immune system is the pivotal event in the production of wasting disease in pigs infected with porcine circovirus-2 (PCV-2). Vet. Pathol. **38**:31-42.

11. **Kyriakis, S.C., K. Saoulidis, S. Lekkas, Ch. C. Miliotis, P.A. Papoutsis, and S. Kennedy.** 2002. The effects of immuno-modulation on the clinical and pathological expression of postweaning multisystemic wasting syndrome. J. Comp. Pathol. **126**:38-46.

12. **Ladekjaer-Mikkelsen, A.S., J. Nielsen, T. Stadejek, T. Storgaard, S. Krakowka, J. Ellis, F. McNeilly, G. Allan, and A. Botner.** 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.

13. **Larochelle, R., M. Morin, M. Antaya, and R. Magar.** 1999. Identification and incidence of porcine circovirus in routine field cases in Quebec as determined by PCR. Vet. Rec. **145**:140-142.
14. **Mankertz, A., M. Domingo, J.M. Folch, P. LeCann, A. Jestin, J. Segales, B. Chmielewicz, J. Plana-Duran, and D. Soike.** 2000. Characterisation of PCV-2 isolates from Spain, Germany and France. Virus Res. **66**:65-77.
15. **Meehan, B.M., F. McNeilly, D. Todd, S. Kennedy, V.A. Jewhurst, J.A. Ellis, L.E. Hassard, E.G. Clark, D.M. Haines, and G.M. Allan.** 1998. Characterization of novel circovirus DNAs associated with wasting syndromes in pigs. J. Gen. Virol. **79**:2171-2179.
16. **Nielson, J., I.E. Vincent, A. Bøtner, A-S. Ladekjaer-Mikkelsen, G. Allan, A. Summerfield, and K.C. McCullough**. 2002. Association of lymphopenia with porcine circovirus type 2 induced postweaning multisystemic wasting syndrome (PMWS). Vet. Immunol. Immunopathol. **92**:97-111.
17. **Onuki, A., K. Abe, K. Togashi, K. Kawashima, A. Taneichi, and H. Tsunemitsu.** 1999. Detection of porcine circovirus from lesions of a pig with wasting disease in Japan. J. Vet. Med. Sci. **61**:1119-1123.
18. **Opriessnig, T., M. Fenaux, S. Yu, R.B. Evans, D. Cavanaugh, J.M. Gallup, F.J. Pallares, E.L. Thacker, K.M. Lager, X.J. Meng, and P.G. Halbur.** 2003. Effect of porcine parvovirus vaccination on the development of PMWS

in segregated early weaned pigs coinfected with type 2 porcine circovirus and porcine parvovirus. Vet. Microbiol. **98**: 209-220

19. **Segales J., C. Alfonso, C. Rosell, J. Pastor, F. Chianini, E. Campos, L. Lopez-Fuertes, J. Quinana, G. Rodriguez-Arrioja, M. Calsamiglia, j. Pujols, J. Dominguez, and M. Domingo.** 2001. Changes in peripheral blood leukocyte populations in pigs with natural postweaning multisystemic wasting syndrome (PMWS). Vet. Immunol. Immunopathol. **81**:37-44.

Addendum

Detection and *In Vitro* and *In Vivo* Characterizations of Porcine Circoviruses from Porcine-Derived Commercial Pepsin Product

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ABSTRACT

Porcine circovirus type 1 (PCV1) and type 2 (PCV2) are widespread in swine herds. PCV1 is nonpathogenic, whereas PCV2 is associated with postweaning multisystemic wasting syndrome in pigs. We report here the detection, biological, experimental, and genetic characterizations of PCV1 and PCV2 from a porcine-derived commercial pepsin. The complete genomic sequences of the pepsin-derived PCV1 and PCV2 were determined and found to share 98%-99% nucleotide sequence identity with respective North American PCV1 and PCV2 isolates. Detection of PCV DNA in pepsin does not necessary mean that the contaminating viruses are still infectious. Thus, PK-15 cells were inoculated with the PCV-contaminated pepsin to biologically characterize the contaminating PCVs. However, no evidence of PCV replication in cells inoculated with the contaminated pepsin was detected. Since naked PCV genomic DNA has been shown to be infectious when directly injected into pigs, we therefore conducted an *in vivo* study to further determine the infectivity of the contaminating PCVs in pepsin. Sixteen, 5-week-old, specific-pathogen-free pigs were randomly divided into three groups. The five

pigs in group 1 were each inoculated with 3 ml of PBS buffer as negative controls. The six pigs in group 2 were each inoculated with 400 mg of the contaminated pepsin dissolved in 4 ml of PBS. The five pigs in group 3 were each inoculated with $10^{4.9}TCID_{50}$ of PCV2 as positive controls. PCV2 viremia, seroconversion, gross and microscopic lesions were detected in group 3 positive control pigs but not in group 1 and 2 pigs, confirming that the contaminating PCVs in pepsin is non-infectious. Nevertheless, the detection of PCV DNA in commercial pepsin raises a potential concern for human infection through xenotransplantation. It is important to test other porcine-derived medical products such as heparin, factor VIII, and insulin for PCV contamination.

INTRODUCTION

Porcine circovirus type 1 (PCV1) and type 2 (PCV2) are widespread in commercial swine populations worldwide (2, 4, 5, 7, 20). PCV1 was discovered as a contaminant of PK-15 cell line (29). Experimental inoculation of pigs with PCV1 did not produce clinical disease, and thus PCV1 is generally considered to be nonpathogenic (1, 28). PCV2 is the primary causative agent of postweaning multisystemic wasting syndrome (PMWS) in weaning piglets (4, 6, 7, 11, 12, 15). The PMWS-associated PCV2 shares about 76% nucleotide sequence identity with the non-pathogenic PCV1 (7). Both PCV1 and PCV2 are non-enveloped icosahedral viruses with a single stranded circular DNA genome of about 1.76 kb. Like porcine parvoviruses, PCV virions are resistant to inactivation and are difficult to remove from the environment (22).

Xenotransplantation with porcine organs, cells and other porcine-derived products has received considerable attention in the last few years, and could potentially alleviate the problem associated with the shortage of human organ donors (17). However, inadvertent transmission of potentially zoonotic porcine viruses to humans is of major concern. Antibodies to PCV1 have been detected in many animal species including humans (1, 28), and PCV2 has been shown to infect BALB/C mice (14). The ability of potential cross-species infections by PCVs raise potential concerns for human infections via xenotransplantation of porcine cells as well as porcine-derived medical and research products such as factor VIII, heparin, insulin and pepsin. The objective of this study was to determine if porcine-derived commercial pepsin product was contaminated with PCVs, and if so, whether or not the contaminating viruses are still infectious *in vitro* and *in vivo*.

MATERIALS AND METHODS

Cell, virus, and pepsins. The PK-15 cell line used in this study was free of PCV1 contamination (6). The PCV2 virus (Isolate #40895) used in the study was originally isolated from a spleen tissue sample of a pig with naturally occurring PMWS (6). The PCV1 virus used in the study was isolated from the PK-15 cell culture (ATCC CCL-33) (9). The PCV1 and PCV2 virus stocks were generated by transfection of PK-15 cells with the PCV1 infectious DNA clone or with the PCV2 infectious DNA clone as previously described (6). The lyophilized porcine-derived pepsins were obtained from a commercial company.

DNA extraction and PCR. Ten mg of lyophilized porcine-derived pepsin was dissolved in 100 μl of phosphate buffered saline (PBS). DNA was extracted from two lots of pepsin products with the QIAamp DNA mini kit (Qiagen, Inc., Valencia, CA) according to the protocols supplied by the manufacturer. The resulting DNA was resuspended in DNase-, RNase-, and proteinase-free water. The MCV1 (5'-GCTGAACTTTTGAAAGTGAGCGGG-3') and MCV2 (5'-TCACACAGTCTCAGTAGATCATCCCA-3') primer pair (7) was used to detect PCV1 or PCV2 DNA in the pepsin. The PCR consisted of 38 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 30 seconds, and extension at 72°C for 1 min, followed by final extension at 72°C for 7 min. To amplify the complete genomes of PCV1 and PCV2 from the contaminated pepsin, eleven nested sets of primer pairs were designed (Table 1) based on the published sequence of PCV1 and PCV2 (7) and used for PCR amplification. The PCR reactions consisted of 38 cycles of denaturation at 94°C for 1 min, annealing at

46°C for 30 seconds, and extension at 72°C for 2 min, followed by a final extension at 72°C for 7 min.

Nucleotide sequencing, sequence and phylogenetic analyses. The PCR products of expected size were purified from agarose gel using the glassmilk procedure with a Geneclean Kit (Bio 101, Inc., La Jolla, CA), and directly sequenced using the nested PCR primers (Table 1). The sequences were compiled and analyzed with the MacVector computer program (Oxford Molecular Ltd., Beaverton, OR). The percentages of sequence identity between the pepsin-derived PCV1 and PCV2 and those published PCVs were determined with the Clustal alignment program in the MacVector package. Phylogenetic analysis was conducted with the aid of the PAUP program (David L. Swofford, Smithsonian Institution, Washington, D.C., and distributed by Sinauer Associates, Inc., Sunderland, MA.). The heuristic search and midpoint rooting options were used to produce a consensus tree.

Biological characterization of the contaminating PCVs in PK-15 cells. Detection of PCV DNA in commercial pepsin does not necessarily mean that the contaminating viruses are still infectious. To biologically characterize the infectivity of the contaminating PCVs, PK-15 cells grown in T-25 flasks were inoculated with the lot of pepsin contaminated with PCV1 and PCV2 as well as with a lot of pepsin that is negative for PCV1 or PCV2. The pepsin was dissolved in PBS buffer prior to inoculation. Mock-infected PK-15 cells served as the negative control. As positive controls, PK-15 cells were inoculated with PCV1 or PCV2, respectively. After 1 hour absorption, the

inoculated cells were washed with PBS buffer and maintained in minimum essential medium (MEM) with Earle's salts and L-glutamine supplemented with 2% fetal calf serum (FCS) and 1 X antibiotic (Invitrogen, Inc., CA). After five blind passages, the inoculated cells were plated on eight-well LabTek chamber slides (Nalge Nunc International, Roskilde, Denmark), and subsequently fixed with a solution containing 80% acetone and 20% methanol at 4°C for 20 min. Evidence of PCV1 and PCV2 replication in inoculated cells was detected by immunofluorescence assay (IFA) with a PCV1 monoclonal antibody (1) as previously described (8) or with PCV2-specific polyclonal rabbit antibody as previously described (6).

Experimental characterization of the contaminating PCVs in specific-pathogen-free (SPF) pigs. Sixteen SPF pigs, 5 weeks of age, were randomly assigned to into three groups. The piglets used in this study were derived from pregnant sows that were negative for antibodies to PCV, PRRSV, PPV, or swine hepatitis E virus (6, 13). Preinoculation serum samples from all piglets were tested by PCR (7) for the presence of PCV1 and PCV2 DNA to confirm that the pigs used in this study were not naturally infected by PCV1 or PCV2. To maximize the inoculation efficiency, each pig was given one-fourth of the inoculum intramuscularly and three-fourths intranasally. The 5 pigs in group 1 were each inoculated with 4 ml of PBS buffer and served as negative controls. The 6 pigs in group 2 were each inoculated with 400 mg of the PCV-contaminated pepsin dissolved in 4 ml of PBS. The 5 pigs in group 3 each received 4 x $10^{4.3}$$TCID_{50}$ of the wild-type PCV2 as positive controls. All pigs were monitored for clinical signs of

disease. Serum samples were collected from each pig at –1, 7, 14, 21, 28, 35 and 42 days postinoculation (DPI).

Clinical evaluations. Pigs were weighed on a weekly basis. Rectal temperatures and clinical respiratory scores, ranging from 0 to 6 (0 = normal; 6 = severe) (10), were recorded every other day from 5 to 39 DPI. Clinical observations, including evidence of central nervous system disease, liver disease (icterus), musculoskeletal disease, and changes in body condition, were recorded daily. All clinical evaluations were performed by a team of two people.

Gross pathology and histopathology. All animals received a complete necropsy at 42 DPI. The necropsy team was blinded to the infection status of the pigs at necropsy. An estimated percentage of the lung with grossly visible pneumonia was recorded for each pig based on a previously described scoring system (10). Other lesions such as enlargement of lymph nodes (ranging from 0 for normal to 3 for three times of the normal size) were scored separately. Sections for histopathologic examination were taken from lungs (five sections) (10), heart, lymph nodes (tracheobronchial), tonsil, thymus, liver, spleen, small intestine and kidney. The tissues were examined in a blinded fashion and given a score for severity of lung, lymph node, tonsil, spleen, and liver lesions (10). Lung scores ranged from 0 (normal) to 3 (severe lymphohistiocytic interstitial pneumonia). Liver scores ranged from 0 (normal) to 3 (severe lymphohistiocytic hepatitis). Lymphoid tissue scores were for an estimated amount of lymphoid depletion (LD) of and histiocytic replacement (HR) of follicles ranging from 0 (normal) to 3 (severe LD and HR of follicles).

Serology and PCR. Sera collected from all pigs at –1, 7, 14, 21, 28, 35 and 42 DPI were tested for PCV2 antibodies by a modified indirect ELISA based on the recombinant ORF2 capsid protein of PCV2 (18). Serum samples with a sample/positive (S/P) ratio above 0.2 were considered positive for PCV2 antibodies. Serum samples were also tested by PCR for the presence of PCV1 and PCV2 DNA using the MCV1 and MCV2 primer pair as previously described (9). The MCV1 and MCV2 primer pair can detect and differentiate PCV1 and PCV2. Briefly, viral DNA was extracted from a 100 μl of serum sample or 50 μg of homogenized thoracic lymph node tissue using DNAzol reagent according to the manufacturer's protocol (Molecular Research Center, Cincinnati, OH). The extracted DNA was resuspended in 100 μl DNase, RNase, and proteinase-free water. The PCR parameters for primers MCV1 and MCV2 were discussed previously (7).

Immunohistochemistry (IHC). IHC was used to detect PCV2 specific antigen in lymph node, spleen, tonsil and thymus tissues collected during necropsy at 42 DPI. A PCV2-specific rabbit polyclonal antiserum was used for the IHC, according to the procedures described previously (24). The amount of PCV2 antigen distributed in the lymphoid tissues was scored in a blinded fashion by assigning a score of 0, if no signal, to 3 for a strong positive signal.

RESULTS

Genetic characterizations of pepsin-derived PCV1 and PCV2. Of the two batches of pepsins tested in the study, one is positive for both PCV1 and PCV2 DNA by PCR, and the other one is negative. The complete genomes of the pepsin-derived PCV1 and PCV2 were amplified and sequenced, and found to be 1,765 bp and 1,768 bp in length,

respectively. To determine the extent of sequence identity between the pepsin-derived PCV1 and PCV2 and other known PCVs, the complete genomic sequences of 4 PCV1 and 31 PCV2 isolates available in GenBank database were compared with pepsin-derived PCV1 and PCV2. Pepsin-derived PCV2 displayed 95-99% sequence identity with published known PCV2 sequences. Similarly, the pepsin-derived PCV1 shared 98-99% sequence identity with those published PCV1 sequences.

To gain a better understanding of the genetic relationship between the pepsin-derived PCVs and other known PCVs, a phylogenetic tree was constructed on the basis of the complete genomic sequences of 37 different isolates of PCV1 and PCV2 from different geographic regions (Fig. 1). Phylogenetic analysis showed that the pepsin-derived PCV2 is closely related to North American PCV2 isolates, and formed a minor branch (Fig.1). Similarly, the pepsin-derived PCV1 clustered with other PCV1 isolates but formed a distinct branch.

The contaminating PCVs in commercial pepsin are non-infectious *in vitro*. The *in vitro* infectivity of the contaminating PCV1 and PCV2 in pepsin was biologically characterized by inoculating PK-15 cells with the contaminated pepsin dissolved in PBS. IFA on inoculated cells with PCV1 monoclonal antibody revealed that the contaminating PCV1 in pepsin was not infectious *in vitro*, as there was no PCV1 antigens detected in inoculated PK-15 cells after the 5 blind passages. Similarly, the contaminating PCV2 also lacked infectivity *in vitro* as determined by IFA with a PCV2-specific rabbit polyclonal antibody. In the positive control experiment, PK-15 cells inoculated with PCV1 and PCV2 virus stocks were both infected as demonstrated by IFA for the presence of PCV1

or PCV2 antigen in the nucleus. The mock-infected PK-15 cells were negative for PCV1 or PCV2 antigen.

The contaminating PCV1 and PCV2 in pepsin lack infectivity when inoculated into SPF pigs. Serum samples collected from all pigs at –1, 7, 14, 21, 28, 35 and 42 DPI were tested for both PCV1 and PCV2 DNA by PCR and for anti-PCV2 antibody by an ELISA. Since an ELISA for PCV1 antibody is not available, seroconversion to PCV1 was not tested. Prior to inoculation at –1 DPI, serum samples from all pigs in groups 1, 2 and 3 were tested negative for PCV1 or PCV2 DNA.

Group 1 negative control pigs remained negative for PCV1 and PCV2 viremia throughout the study (Table 2). No seroconversion to PCV2 antibodies was detected in any of the five control pigs throughout the study (Table 3). In the contaminated pepsin inoculated group 2 pigs, PCV1 or PCV2 viremia was not detected (Table 2). No seroconversion to PCV2 antibodies was detected in any of the group 2 pigs throughout the study (Table 3). In the positive control PCV2 inoculated group 3 pigs, PCV2 viremia was first detected on 7 DPI, and 4 out of 5 pigs became viremic for PCV2 (Table 2). Seroconversion to PCV2 antibodies was first detected at 21 DPI in one pig, and by 42 DPI, all pigs inoculated with the PCV2 virus had seroconverted to PCV2 antibodies (Table 3).

Lack of pathological lesions or viral antigens in pigs inoculated with the contaminating PCV2 from commercial pepsin. We examined the pathological lesions induced by PCV2 infection only, since PCV1 is nonpathogenic (1) and the lesions for

PCV2 are well characterized (7, 11, 12). From 19 to 42 DPI, the mean clinical scores recorded for positive control PCV2 inoculated group 3 pigs became more severe ($p<0.05$) than those recorded for animals in groups 1 and 2 (data not shown). There was no difference in weight gain or mean rectal temperatures among any of the groups (data not shown).

Gross pathological lesions were evaluated during necropsy at 42 DPI. The negative control group 1 pigs has no evidence of gross lymph node or lung gross lesions. Two out of six pepsin-inoculated group 2 pigs had moderate enlargement of lymph nodes. The positive control PCV2 inoculated group 3 pigs all had mild to severe enlargement of lymph nodes. The mean gross lesion scores in the lymph nodes of group 1 and 2 pigs were not significantly different from each other, but were significantly less severe than that of PCV2 inoculated group 3 pigs (data not shown). Lungs were free of gross visible pneumonia in all three groups (data not shown).

Mild lymphoplasmacytic and histiocytic bronchointersitial pneumonia was observed in 3/5 pigs in group 1 and in 5/6 pigs in the pepsin inoculated group 2 (Table 4). All PCV2 inoculated group 3 pigs had mild to moderate lymphoplasmacytic and histiocytic bronchointersitial pneumonia. One of the five group 1 pigs had mild lymphoplasmacytic hepatitis and lymphoplasmacytic interstitial nephritis. In the pepsin inoculated group 2 pigs, one animal had very mild lymphoid depletion and histiocytic replacement of the lymph node follicles. All PCV2 inoculated group 3 pigs had mild to moderate lymphoid depletion and histiocytic replacement of the follicles in lymph nodes and spleen tissues (Table 4).

IHC staining for PCV2–specific antigen was performed on lymph nodes of all pigs necropsied at 42 DPI. Lymphoid tissues from the negative control group 1 pigs as well as the pepsin-inoculated pigs were negative for PCV2 antigen. In the positive control PCV2 inoculated group 3 pigs, mild to moderate levels of PCV2 antigen was detected in lymph node tissues of 3/5 pigs, and in tonsil and spleen tissues of 2/5 pigs (data not shown).

DISCUSSION

Xenozoonosis due to the inadvertent transmission of porcine viruses from pig xenografts to human transplant recipients and the potential subsequent transmission of the virus to others are possible (17). Tischer *et al* (28) detected PCV1 antibodies in human sera and BALB/c mice were also experimentally infected with PCV2 (14), suggesting that PCV may infect across species and thus pose a potential concern in xenotransplantation with porcine products. In this study, we found that one of the 2 lots of porcine-derived commercial pepsins was positive for both PCV1 and PCV2 DNA by PCR. The complete genomic sequences of contaminating PCV1 and PCV2 in this pepsin product were determined. Sequence analysis showed that the pepsin-derived PCV1 and PCV2 shared high nucleotide sequence identity with those published PCV1 and PCV2. Phylogenetic analysis showed that the pepsin-derived PCV2 is closely related to North American PCV2 isolates, and the pepsin-derived PCV1 clustered within the PCV1 major branch.

To determine if the contaminating PCVs in pepsin still retain infectivity *in vitro* and *in vivo*, we biologically and experimentally characterized the contaminating PCVs.

We were unable to infect PK-15 cells with the contaminated pepsin, suggesting that contaminated PCV1 and PCV2 viruses in pepsin were inactivated and lacked infectivity *in vitro*. Since we previously showed that the naked PCV genomic DNA is infectious when directly injected intramuscularly into pigs (6, 9), an *in vivo* animal study was conducted to further evaluate the infectivity of the contaminating viruses detected by PCR in pepsin. SPF piglets experimentally inoculated with PCV contaminated pepsin did not become viremic to PCV1 or PCV2, nor did they seroconvert to PCV2, and PCV2 antigen was not detected in the lymphoid tissues of the pepsin inoculated pigs. Compared to the PCV2-inoculated positive control group, the negative control pigs and the contaminated pepsin-inoculated pigs had no significant gross or microscopic lesions characteristic of PCV2 infection.

The lack of PCV infectivity *in vitro* and *in vivo* of the contaminated pepsin is likely due to the manufacturing process, which effectively inactivated the PCVs and degraded their genomic DNA. However, other porcine-derived products such as factor VIII from pig plasma may contain high titers of infectious PCVs and the manufacturing process may not completely inactivate the viruses. Porcine parvovirus (PPV) (25) and porcine endogenous retrovirus (27) were detected in porcine-derived porcine factor VIII. Therefore, it is important to test other porcine derived medical and research products such as factor VIII, heparin and insulin for potential contamination by PCVs.

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REFERENCES

1. **Allan, G.M., McNeilly, F., Cassidy, J.P., Reilly, G.A., Adair, B., Ellis, W.A., & McNulty, M.S. 1995.** Pathogenesis of porcine circovirus; experimental infections of colostrum deprived piglets and examination of pig foetal material. *Vet Microbiol* **44**, 49-64.
2. **Allan, G.M., McNeilly, F., Kennedy, S., Daft, B., Clark, E.G., Ellis, J.A., & Haines, D.M. 1998.** Isolation of porcine circovirus-like viruses from pigs with a wasting disease in the USA and Europe. J Vet Diagn Invest **10**, 3-10.
3. **Bassami, M.R., Berryman, D., Wilcox, G.E., & Raidal, S.R. 1998.** Psittacine beak and feather disease virus nucleotide sequence analysis and its relationship to porcine circovirus, plant circoviruses, and chicken anaemia virus. Virology **249**, 453-459.
4. **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.
5. **Edwards, S. & Sands, J.J. 1994.** Evidence of circovirus infection in British pigs. Vet Rec **134**, 680-681.
6. **Fenaux M, Halbur, P.G., Haqshenas, G., Royer, R., Thomas, P., Nawagitgul, P., Gill, M., Toth, T.E., & Meng, X.J. 2002.** Cloned genomic DNA of type 2 porcine circovirus is infectious when injected directly into the liver and lymph nodes of pigs: characterization of clinical disease, virus distribution, and pathological lesions. J Virol **76**, 541-551.

7. **Fenaux M, Halbur, P.G., Gill, M., Toth, T.E., & Meng, X.J. 2000.** Genetic characterization of type 2 porcine circovirus (PCV-2) from pigs with postweaning multisystemic wasting syndrome in different geographic regions of North America and development of a differential PCR-restriction fragment length polymorphism assay to detect and differentiate between infections with PCV-1 and PCV-2. J Clin Microbiol **38**, 2494-2503.
8. **Fenaux M., Opriessnig, T., Halbur, P.G., Elvinger, F., & Meng, X.J. 2004.** A chimeric porcine circovirus (PCV) with the immunogenic capsid gene of the pathogenic PCV2 cloned into the genomic backbone of the non-pathogenic PCV1 induces protective immunity against PCV2 infection in pigs. J Virol, In Press.
9. **Fenaux M., Opriessnig, T., Halbur, P.G., & Meng, X.J. 2003.** Immunogenicity and pathogenicity of the chimeric infectious DNA clones between pathogenic type 2 porcine circovirus (PCV2) and non-pathogenic PCV1 in weaning pigs. J Virol **77**, 11232-11243.
10. **Halbur P.G., Paul, P.S., Frey, M.L., Landgraf, J. Eernisse, K., Meng, X.J., Lum, M.A., Andrews, J.J., & Rathje, J.A. 1995.** Comparison of the pathogenicity of two U.S. porcine reproductive and respiratory syndrome virus isolates with that of the Lelystad virus. Vet Pathol **32,** 648-660
11. **Harding, J.C. & Clark, E.G. 1997.** Recognizing and diagnosing post-weaning multisystemic wasting syndrome (PMWS). Swine Health Prod **5**, 201-203
12. **Harms P.A., Sorden, S.D., Halbur, P.G., Bolin, S.R., Lager, K.M., Morozov, L., & Paul, P.S. 2001**. Experimental reproduction of severe disease in CD/CD pigs

concurrently infected with type 2 porcine circovirus and porcine reproductive and respiratory syndrome virus. Vet Pathol **38**, 528-539.

13. **Joo H.S., Donaldson-Wood, C.R., & Johnson, R.H. 1976.** A standardized haemagglutination inhibition test for porcine parvovirus antibody. Aust Vet J **52**, 422-424.

14. **Kuipel, M., Stevenson, G.W., Choi, J., Latimer, K.S., Kanitz, C.L., & Mittal., S.K. 2001.** Viral replication and lesions in BALB/c mice experimentally inoculated with porcine circovirus isolated from a pig with postweaning multisystemic wasting disease. Vet Pathol **38**, 74-82

15. **Ladekjaer-Mikkelsen, A.S., Nielsen, J., Stadejek, T., Storgaard, T., Krakowka, S., Ellis, J., McNeilly, F., Allan, G., & Botner, 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.

16. **Mankertz A., Hattermann, K., Ehlers, B., & Soike, B. 2000.** Cloning and sequencing of columbid circovirus (CoCV), a new circovirus from pigeons. Arch Virol **145**, 2469-2479

17. Meng, X.J. **2003**. Swine hepatitis E virus: cross-species infection and risk in xenotransplantation. Curr. Top. Microbiol. Immunol. **278**:185-216.

18. **Nawagitgul, P., Harms, P.A., Morozov, I., Thacker, B.J., Sorden, S.D., Lekcharoensuk, C., & Paul, P.S. 2002**. Modified indirect porcine circovirus (PCV) type 2-based and recombinant capsid protein (ORF2)-based ELISA for the detection of antibodies to PCV. Clin Diagn Lab Immunol **9**, 33-40.

19. **Nishizawa, T., Okamoto, H., Konishi, K., Yoshizawa, H., Miyakawa, Y., & Mayumi, M. 1997.** A novel DNA virus (TTV) associated with elevated transaminase levels in posttransfusion hepatitis of unknown etiology. Biochem Biophys Res Commun **241**, 92-97.

20. **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

21. **Phenix, K.V., Weston, J.H., Ypelaar, I., Lavazza, A., Smyth, J.A. Todd, D., Wilcox, G.E. & Raidal, S.R. 2001**. Nucleotide sequence analysis of a novel circovirus of canaries and its relationship to other members of the genus circovirus of the family Circoviridae. J Gen Virol **82**, 2805-2809.

22. **Royer R.L., Nawagitgul, P., Halbur, P.G., & Paul. P.S. 2001.** Susceptibility of porcine circovirus type 2 to commercial and laboratory disinfectants. Swine Health Prod **9**, 281-284

23. **Sibila, M., Calsamiglia, M., Segales, J., Jestin, A., & Domingo, M. 2001.** Detection of porcine circovirus type 2 genome in nasal swabs and serum samples from naturally infected pigs using polymerase chain reaction. Proc of the ssDNA viruses of Plant, Birds, Pigs and Primates 115.

24. **Sorden S.D., Harms, P.A., Nawagitgul, P., Cavanaugh, D., & Paul, P.S. 1999.** Development of a polyclonal-antibody-based immunohistochemical method for the detection of type 2 porcine circovirus in formalin-fixed, paraffin-embedded tissue. J Vet Diagn Invest **11**, 528-530.

25. **Soucie J.M., Erdman, D.D., Evatt, B.L., Anderson, L.J., Torok, T.J., El-Jamil, M., Barnhart, E., Tepper, M., Burril, H.N., Pickett, A.M., & Mengeling, W.L. 2000.** Investigation of porcine parvovirus among persons with hemophilia receiving Hyate:C porcine factor VIII concentrate. Transfusion. **40**, 708-711.

26. **Takahashi, K., Iwasa, Y., Hijikata, M., & Mishiro, S. 2000.** Identification of a new human DNA virus (TTV-like mini virus, TLMV) intermediately related to TT virus and chicken anemia virus. Arch Virol **145**, 979-93.

27. **Takefman, D.M., Wong, S., Maudru, T., Peden, K., & Wilson, C.A. 2001.** Detection and characterization of porcine endogenous retrovirus in porcine plasma and porcine factor VIII. J Virol **75**, 4551-4557.

28. **Tischer, I., Mields, W., Wolff, D., Vagt, M., & Griem, W. 1986.** Studies on epidemiology and pathogenicity of porcine circovirus. Arch Virol **91**, 271-276.

29. **Tischer, I., Rasch, R., & Tochtermann, G. 1974.** Characterization of papovavirus and picornavirus-like particles in permanent pig kidney cell lines. Zentralbl Bakteriol Hyg A **226**, 153-167.

30. **Todd, D., Weston, J.H., Soike, D., & Smyth, J.A. 2001.** Genome sequence determinations and analyses of novel circoviruses from goose and pigeon. Virology **286**, 354-362

31. **Wilson, L.E., Umemeru, T., Astembroski, J., Ray, S.C., Alter, H.J., Strathdee, S.A., Vlahov, D., & Thomas, D.L. 2001.** Dynamics of SEN virus infection among injection drug users. J Infect Dis **184**, 1315-1319.

Fig. 1

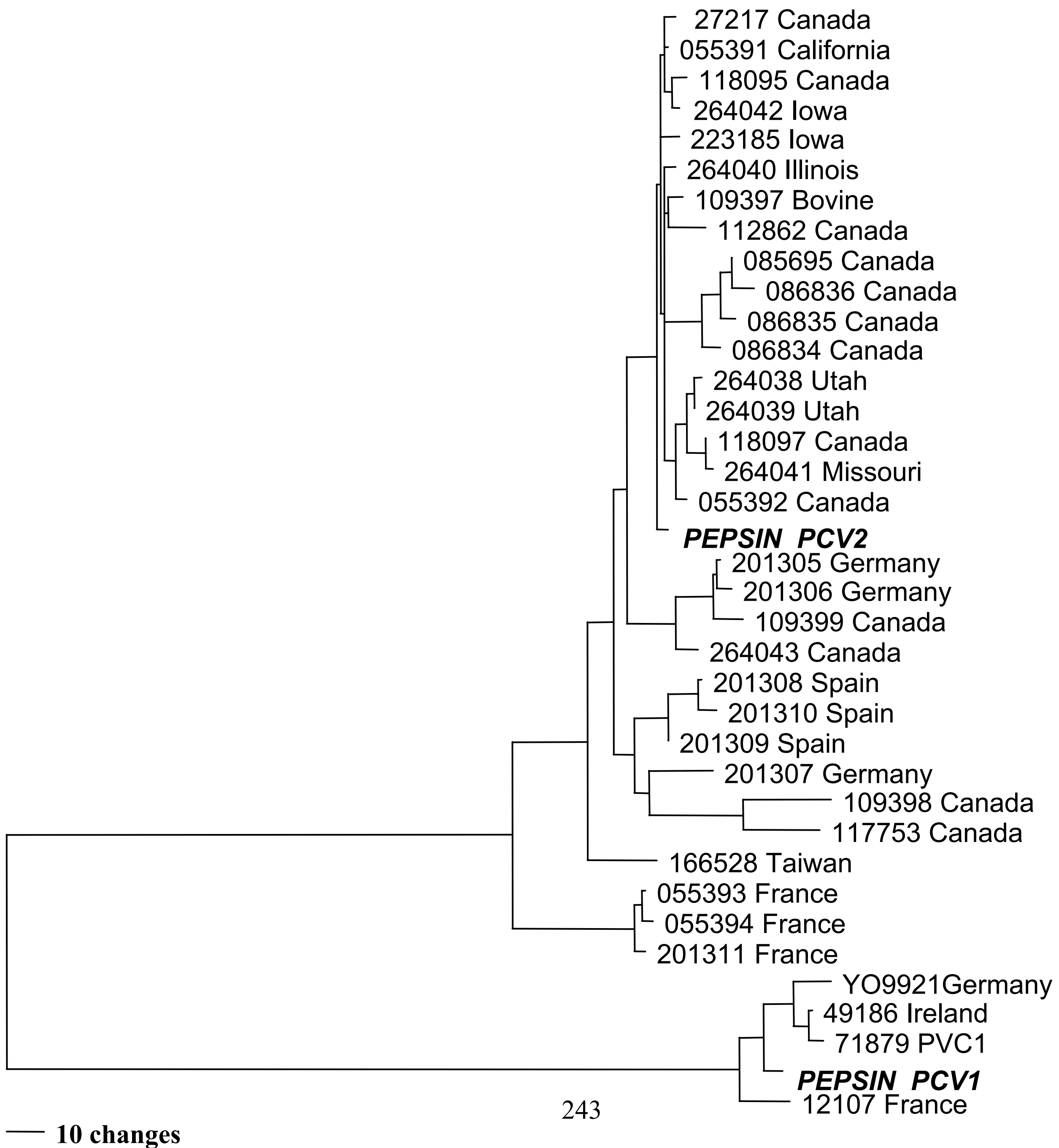

Fig. 1. A phylogenetic tree based on the complete genomic sequences of 37 PCV1 and PCV2 isolates available in GenBank. The GenBank accession numbers followed by the geographic origin of each isolate were used to designate the PCV isolate. The tree was constructed with the aid of the PAUP program. Heuristic search and midpoint rooting options were used to produce a consensus tree. The scale bar representing the numbers of character state changes is shown. Branch lengths are proportional to the numbers of character state changes.

Table 1. Primers used to amplify and sequence the complete pepsin-derived PCV1 and PCV2 genomes

Primer	Primer sequence
PCV1	
PCV1.2B[a]	'5-CTCGGAAGGATTATTAAGGGTG-'3
PCV1.2B.N	'5-TTATGGGGTTGCGGGC-'3
PCV1.2A	'5-TCGACATTGGTGTGGGTATTT-'3
PCV1.2A.N	'5-AAATGGAGCCACAGCTGG-'3
PCV1.3B	'5-TTCTACTAGGCTTCCAGTAGGTGT-'3
PCV1.3B.N	'5-CCTAGGCTCAGCAAAATTACG-'3
PCV1.3A	'5-CCAGCCCACTTCGGC-'3
PCV1.3A.N	'5-AGCACCTCGGCAGCG-'3
PCV1.4B	'5-CCACACCATAAGGCAGCC-'3
PCV1.4B.N	'5-TTCACCCCCAAACCTGAG-'3
PCV1.4A	'5-CAGCAGCGTGATTGGAAG-'3
PCV1.4A.N	'5-GCTGTACACGTCATAGTGGGC-'3
PCV1.5B	'5-CCTCCGATAGAGAGCTTCTACAG-'3
PCV1.5B.N	'5-TGGGACAGCAGTTGAGGAGTA-'3
PCV1.5A	'5-CGGGGTCTTTGGTGACTGTA-'3
PCV1.5A.N	'5-CCGAGCAGTTCCCTGTAACG-'3
PCV1.6B	'5-GCAAACAAAATAATCAAAAAGGG-'3
PCV1.6B.N	'5-ATTGGAAGCTCCCCGTATTTG-'3
PCV1.6A	'5-GGCTGGAACCAATCAATTGT-'3
PCV1.6A.N	'5-TTGGTCCAGCTCAGGTTTG-'3
PCV2	
PCV2.2B	'5-TCCGAAGACGAGCGCA-'3
PCV2.2B.N	'5-GAGCTCCCAATCTCCCTATT-'3
PCV2.2A	'5-GAAGTAATCCTCCGATAGAGAGC-'3
PCV2.2A.N	'5-TTCTACAGCTGGGACAGCAG-'3
PCV2.3B	'5-GTTACAAAGTTATCATCTAGAATAACAGC-'3
PCV2.3B.N	'5-CCCACTCCCCTATCACCC-'3
PCV2.3A	'5-ATTAGCGAACCCCTGGAG-'3
PCV2.3A.N	'5-GTGAGGTGTTCGTCCTTCC-'3
PCV2.4B	'5-AGAGACTAAAGGTGGAACTGTACC-'3
PCV2.4B.N	'5-GGCCCGCAGTATTCTGATTA-'3
PCV2.4A	'5-AGGGGGGACCAACAAAAT-'3
PCV2.4A.N	'5-CAAAATCTCTATACCCAAAGAATACTAC-'3
PCV2.6B	'5-GGGCCACCTGGGTGTG-'3
PCV2.6B.N	'5-AATGGGCTGCTAATTTTGC-'3
PCV2.6A	'5-CATATGGAAATTCAGGGCATG-'3
PCV2.6A.N	'5-GGGGAAAGGGTGACGAAC-'3
PCV2.7B	'5-CATTTAGGGTTTAAGTGGGGG-'3
PCV2.7B.N	'5-TAAATTCTCTGAATTGTACATACATGG-'3
PCV2.7A	'5-CCGCCACCGCTACCG-'3
PCV2.7A.N	'5-TTGGAGTAGGAAAAATGGCAT-'3
PCV2.8B	'5-ACCAGCGCACTTCGGC-'3
PCV2.8B.N	'5-CACCTCGGCAGCACCTC-'3
PCV2.8A	'5-CCGTTGTCCTTGAGATCGAG-'3
PCV2.8A.N	'5-TCCACATTCAATAAGTAAGTTGCC-'3

[a]B, forward primer; A, reverse primer, N, nested primer

Table 2. Detection of viremia by real-time PCR in sera of inoculated and control pigs

Group	Inoculum	**No. of pigs positive/no. tested** Days post-inoculation							
		-1	7	14	21	28	35	42	Total
1	Control	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
2	PCV-contaminated Pepsin	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6
3	PCV2	0/5	4/5	4/5	4/5	3/5	3/5	1/5	4/5

Table 3. Seroconversion to PCV2 antibodies of inoculated and control pigs

Group	Inoculum	No. of pigs positive/no. tested Days post inoculation						
		-1	7	14	21	28	35	42
1	Control	5/5[a]	1/5	1/5	0/5	0/5	0/5	0/5
2	PCV contaminated Pepsin	2/6	0/6	0/6	0/6	0/6	0/6	0/6
3	PCV2	1/5	0/5	0/5	1/5	4/5	5/5	5/5

[a] Maternal antibodies specific to PCV2 were detected at –1 days post inoculation (DPI) but waned in all groups by 14 DPI.

Table 4. Distribution of histopathological lesions in different tissues and organs from inoculated and control pigs

Group	Inocula [a]	DPI	No. of pigs positive / no. of pigs tested [b]									
			Lung	Lymph nodes		Tonsil		Spleen		liver	Kidney	Heart
				LD	HR	LD	HR	LD	HR			
1	No inoculum	42	3/5(0.6)	0/5(0.0)	0/5(0.0)	0/5(0.0)	0/5(0.0)	0/5(0.0)	0/5(0.0)	1/5(0.2)	1/5	0/5
2	Pepsin	42	5/6(0.8)	1/6(0.2)	1/6(0.2)	0/6(0.0)	0/6(0.0)	2/6(0.3)	0/6(0.0)	1/6(0.2)	0/6	0/6
3	PCV2	42	5/5(1.4)	5/5(1.4)	3/5(0.8)	0/5(0.0)	1/5(0.2)	4/5(1.0)	3/5(0.6)	3/6(0.6)	1/5	2/5

[a] Contaminated pepsin and PCV2 virus stock as inocula.
[b] Value in parentheses are the mean histological scores for interstitial pneumonia (0, normal; through 6, severe) and for interstitial hepatitis and lymphoid depletion (LD) and histioctyic replacement (HR) for lymph nodes, tonsils and spleen.

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3. **1st** place agriculture and animal science category, *19th Annual Research Symposium of Virginia Tech*, Blacksburg, VA, March 28, 2003
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5. **1**st place agriculture and animal science category, *18th Annual Research Symposium of Virginia Tech*, Blacksburg, VA, April 2, 2002
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8. **2nd** place in the *clinical* science category, *12th Annual VMRCVM Research Symposium*. Blacksburg, VA, June7-9,2000.

PATENTS PENDING

1. Co-inventors: **Martijn Fenaux**, P.G. Halbur, X.J. Meng. USPTO application no. 60/340,775 filed on Dec. 12, 2001. Title: Chimeric infectious DNA clones, chimeric porcine circoviruses and uses thereof.
2. Co-inventors: **Martijn Fenaux**, X.J. Meng. U.S. Patent application (AM100732, filed on June 29, 2001 in USPTO). Title: Differential PCR-RFLP assay for detecting and distinguishing between non-pathogenic PCV1 and pathogenic PCV2.

REFEREED PUBLICATIONS

1. **Fenaux M**., T. Opriessnig, P.G. Halbur and X.J. Meng. Detection but lack of viral infectivity, both *in vitro* and *in vivo,* of contaminating porcine circoviruses in porcine-derived commercial pepsin product.2004. Submitted to the *Journal of General Virology*.
2. **Fenaux M.,** T. Opriessnig, P.G. Halbur and X.J. Meng. Identification of Genetic Markers that are Responsible for PCV2 Replication and/or Virulence. 2004. Submitted to the *Journal of Virology*.
3. **Fenaux M**., T. Opriessnig, P.G. Halbur and X.J. Meng. A Chimeric Porcine Circovirus (PCV) with the Immunogenic Capsid gene of the Pathogenic PCV2 Cloned into the Backbone of the Nonpathogenic PCV1 Induces Protective Immunity against PCV2 Infection.2004. In press for *Journal of Virology*.
4. McKeown N.E., **M. Fenaux**, P.G. Halbur, and X.J. Meng. Molecular characterization of an orphan virus, porcine TT virus, from pigs in 6 different countries.2003. submitted to *Veterinary Microbiology.*
5. **Fenaux M**., T. Opriessnig, P.G. Halbur and X.J. Meng. Immunogenicity and pathogenicity of the chimeric infectious DNA clones between pathogenic type2 porcine circovirus (PCV2) and non-pathogenic PCV1 in weaning pigs. *Journal of Virology*. 2003; **77**(20):11232-43
6. Opriessnig, T., **M. Fenaux**, S. Yu, R.B. Evans, D. Cavanaugh, J.M. Gallup, , F.J. Pallares, E.L. Thacker, K.M. Lagger, X.J. Meng and P.G. Halbur. Effect of

porcine parvovirus vaccination on the development of PMWS in segregated early weaned pigs coinfected with type 2 porcine circovirus and porcine parvovirus. *Veterinary Microbiology*.2003. **98**:209-220

7. Opriessnig, T., S. Yu, J.M. Gallup, R.B. Evans, **M. Fenaux**, F.J. Pallares, E.L. Thacker, C.W. Brockus, M.R. Ackerman, P. Thomas, X.J. Meng and P.G. Halbur. Effects of vaccination with selective bacterins on conventional pigs infected with wild type 2 porcine circovirus. *Veterinary Pathology*.2003.**40**.(5):521-9
8. **Fenaux, M**, P.G. Halbur, G. Haqshenas, R. Royer, P. Nawagitgul, M. Gill, T.E. Toth, and X.J. Meng. The cloned genomic DNA of the type-2 porcine circovirus (PCV-2) is infectious when injected into the liver and lymph nodes of SPF pigs: characterization of clinical course, virus distribution and pathological lesions. *Journal of Virology*. 2002.76:541-551
9. Haqshenas G., F.F. Huang, **M. Fenaux,** D.K. Guenette, F.W. Pierson, H.L. Shivaprasda, T.E. Toth and X.J. Meng. The putative capsid protein of the newly identified avian hepatitis E virus shares antigenic epitopes with that of swine and human hepatitis E viruses and the chicken big liver and spleen disease virus. *Journal of General Virology*.2002. 83:2201-2209.
10. **Fenaux M**, P.G. Halbur,M. Gill,T.E. Toth,X.J. Meng. Genetic characterization of type 2 porcine circovirus (PCV-2) from pigs with postweaning multisystemic wasting syndrome in different geographic regions of North America and development of a differential PCR-restriction fragment length polymorphism assay to detect and differentiate between infections with PCV-1 and PCV-2. *Journal of Clinical Microbiology*. 2000 Jul;38(7):2494-503
11. Loor J. **M. Fenaux**. X. Lin, and J.H. Herbein. Influence of dietary conjugated linoleic acid isomers and trans-vaccenic acid on body composition and fatty acid profiles in tissues of lactating mice. 2000. FASEB J 14:A252.

NON-REFEREED PUBLICATIONS

1. **Fenaux M.,** Y. Xu, X.J. Meng. Detection but lack of viral infectivity of porcine circoviruses (PCVs) in porcine derived commercial pepsin product. *Proceedings of the 15th annual VMRCVM research symposium*. June 11, 2003. Blacksburg VA
2. **Fenaux M**., T. Opriessnig, P.G. Halbur, and X.J. Meng. Immunogenicity and pathogenicity in pigs of chimeric infectious DNA clones between pathogenic type 2 porcine circovirus (PCV2) and non-pathogenic PCV1. *Proceedings of the 19th Annual graduate research symposium of Virginia Tech*. March 27, 2003. Blacksburg, VA
3. Opriessnig, P.G. Halbur, F. Pallares, **M. Fenaux**, P. Thomas, and X.J. Meng. Effect of age and vaccine induced immune stimulation on PCV2 infection. *Proceedings of international pig veterinary society*. June 22, Ames, Iowa.
4. Opriessnig T., P.G. Halbur, **M. Fenaux**, S. Yu, F.J. Pallares, and X.J. Meng. Effect of PPV-vaccination on the development of PMWS in PCV2 and PPV coinfected SPF pigs. *Proceedings of the 83rd conference of research workers in animal diseases*. Nov. 10-12, 2002. #178. St. Louis, MO.
5. Haqshenas G., F.F. Huang, **M. Fenaux**, D.K. Guenette, F.W. Pierson, C.T. Larson, H.L. Shivaprasad, T.E. Toth, and X.J. Meng. The puative capsid protein

of the newly identified avian hepatitis E virus shares antigenic epitopes with that of swine and human hepatitis E viruses and the chicken big liver and spleen disease viruses. *Proceedings of the 83rd conference of research workers in animal diseases*. Nov. 10-12, 2002. #173. St. Louis, MO.

6. **Fenaux M**., T. Opriessnig, P.G. Halbur, and X.J. Meng. Immunogenicity and pathogenicity in pigs of chimeric infectious DNA clones between pathogenic type 2 porcine circovirus and non-pathogenic PCV1. *Proceedings of 83rd Conference of research workers in animal diseases*. Nov. 10-12, 2002, #176. St. Louis, MO.
7. Opriessnig T., P.G. Halbur, F. Pallares, R. Evans, **M. Fenaux**, X. J. Meng. Vaccine induced immune stimulation and PCV2 infection in conventional pigs. *Proceedings of American association of veterinary laboratory diagnosticians.* October 16-25, 2002. St. Louis, MO.
8. **Fenaux M**., X.J. Meng Construction and in vitro characterization of chimeric viruses between non-pathogenic porcine circovirus type 1 (PCV1) and pathogenic PCV2. *Proceedings of the 14th annual VMRCVM research symposium*. June 6, 2002. Blacksburg, VA
9. **Fenaux M**, P.G. Halbur, G. Haqshenas, R. Royer, P. Nawagitgul, M. Gill, T.E. Toth, and XJ. Meng. The cloned genomic DNA of type –2 porcine circovirus is infectious when directly injected into the liver and lymph nodes of pigs. *Proceedings of the 18th Annual graduate research symposium of Virginia Tech*. April 3, 2002. Blacksburg, VA
10. **Fenaux M**, P.G. Halbur, G. Haqshenas, R. Royer, P. Thomas, P. Nawagitgul, M. Gill, T.E. Toth, and X.J. Meng. The cloned genomic DNA of the type-2 porcine circovirus (PCV2) is infectious when injected directly into the liver and lymph nodes of pigs: Characterization of clinical disease, virus distribution and pathologic lesions. *Proceedings of the 82nd annual meeting of the conference of research workers in animal diseases*. 125P. St. Louis MO, Nov. 2001.
11. **Fenaux M**, P.G. Halbur, R. Royer, M. Gill, T.E. Toth and X.J. Meng Infection of pigs by injections with cloned genomic DNA of a type-II porcine circovirus (PCV-2). *Proceedings of the 13th Annual VMRCVM Research Symposium*, June 7-8, 2001. Blacksburg, VA
12. **Fenaux M**, P.G. Halbur,M. Gill,T.E. Toth,X.J. Meng. Type-2 Porcine Circovirus (PCV-2) Associated with Postweaning Multisystemic Wasting Syndrome (PMWS) in Pigs: Genetic Characterization and Development of a Differential PCR-RFLP Diagnostic Assay. *Proceedings of the 17th annual graduate research symposium at Virginia Tech*. March 26, 2001. Blacksburg, VA
13. **Fenaux M**, P.G. Halbur,M. Gill,T.E. Toth,X.J. Meng. Genetic characterization of PCV-2 isolates from different geographic regions of North America and development of a universal PCR-RFLP diagnostic assay to detect and differentiate between infections caused by the pathogenic PCV-2 and the nonpathogenic PCV-1. *Proceedings of the 81st annual meeting of the conference of research workers in animal diseases.* November 12-14, 2000. Chicago, IL
14. **Fenaux M**, P.G. Halbur,M. Gill,T.E. Toth,X.J. Meng. Type-2 Porcine Circovirus (PCV-2) isolated from pigs with PMWS in different geographic regions of North America: genetic characterization and development of a PCR-RFLP test to detect and differentiate between infections with nonpathogenic PCV-1 and PMWS-

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