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PCR Detection and Evidence of Shedding of Porcine Circovirus Type 2 in Boar Semen

RENÉE LAROCHELLE,[1]* ANDRZEJ BIELANSKI,[2] PETER MÜLLER,[1] AND RONALD MAGAR[1]

Laboratoire d'Hygiène Vétérinaire et Alimentaire, Agence Canadienne d'Inspection des Aliments, Saint-Hyacinthe, Québec,[1] and Animal Diseases Research Institute, Canadian Food Inspection Agency, Nepean, Ontario,[2] Canada

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An experimental study was conducted to evaluate the potential presence of porcine circovirus type 2 (PCV2) in the semen of infected boars. Four mature boars were inoculated intranasally with PCV2 isolate LHVA-V53 propagated on PK15 cells. Two boars inoculated with the supernatant of noninfected PK15 cells were kept as controls. Serum samples were collected from all boars at 4, 7, 11, 13, 18, 21, 25, 28, 35, and 55 days postinoculation (dpi) and from the four PCV2-infected boars at 90 dpi. Samples were tested for the presence of antibodies to PCV2 by an indirect immunofluorescence assay and for the presence of PCV2 DNA by PCR and nested PCR. Semen samples were collected from all six boars at 5, 8, 11, 13, 18, 21, 25, 28, 33, and 47 dpi and tested for the presence of PCV2 DNA by a nested PCR assay. Antibodies to PCV2 could be detected as early as 11 dpi in one boar, and all four infected boars were found positive for PCV2 antibodies by 18 dpi. Thereafter all infected boars remained positive for antibodies to PCV2 until 90 dpi. Analysis of serum samples by nested PCR demonstrated the presence of PCV2 DNA as early as 4 dpi in three of four infected boars. Serum samples from all infected boars were positive for PCV2 DNA from 11 dpi until 35 dpi but were negative at 90 dpi. PCV2 DNA was detected as soon as 5 dpi in the semen of two infected boars and intermittently thereafter in the semen of all four infected boars. The semen of two infected boars was positive for PCV2 DNA at 47 dpi. Following infection, PCV2 DNA can be detected in semen concurrently with the presence of PCV2 DNA and antibodies in the serum. The present study suggests that PCV2 may be shed intermittently in the semen of infected boars.

Porcine circovirus (PCV) is a small, nonenveloped circular single-stranded DNA virus classified in the *Circoviridae* family (22) and was originally identified and described as a contaminant of a porcine kidney cell line (25). When experimentally transmitted to pigs, PCV was not found to cause disease or lesions (1, 26). Serological studies performed by various investigators have demonstrated that PCV was quite prevalent in swine herds (5, 6, 13, 26). Recently, PCV has been identified as being associated with a new condition in pigs, the postweaning multisystemic wasting syndrome (PMWS), first described in Western Canada (7, 12). This syndrome is characterized clinically by progressive weight loss, dyspnea, and jaundice and pathologically by lymphadenopathy, interstitial pneumonia, hepatitis, and nephritis. Similar syndromes have also been recently reported in the United States, Europe, and Asia (2, 17, 23, 24). The PMWS-associated PCV, called PCV type 2 (PCV2), is antigenically and genomically different from the previously known PCV now referred to as PCV1 (2, 7, 10, 21). Mild to moderate lesions associated with PMWS have been reproduced experimentally using PCV2 but the specific pathogenic role and modes of transmission of PCV2 are still unclear. PCV2 has an affinity for cells of the mononuclear-phagocyte system and can be detected in many tissues and organs, such as lungs, tonsils, lymph nodes, thymus, spleen, intestine, kidney, liver, serum, salivary glands, and testes following experimental infections (8, 14, 18). PCV2 has also been detected in nasal swabs following experimental infection, and transmission through nasal secretions has been suggested as a potential mode of horizontal spread (18). Recently, evidence for vertical transmission of PCV2 and associated reproductive failure has been reported (27). Also, in a recent field study PCV2 nucleic acid was detected in 2 of 34 randomly tested semen samples from healthy boars (11). Concerns have arisen in the swine industry with regard to this potentially newly emerging porcine virus and disease as well as towards the uncertainties surrounding the modes of viral transmission and the introduction of the virus in herds. Since artificial insemination is being used increasingly, not only for introducing new genetics in swine herds but also for better control of product quality with regard to transmission of diseases, it is of interest to determine if the semen of infected boars may represent a potential source of PCV2 introduction in a herd. In the present study we experimentally infected mature boars and evaluated boar semen over time for the presence of PCV2 DNA by PCR.

Six boars originating from a specific-pathogen-free herd seronegative for PCV1 and PCV2 were raised and trained for semen collection. At approximately 7 months of age, four boars (boars 3, 4, 5, and 6) were inoculated intranasally with 5 ml of PCV2 isolate LHVA-V53 at a concentration of 10^4 50% tissue culture-infective doses ($TCID_{50}$)/ml for boars 3 and 4 and at a concentration of 10^6 $TCID_{50}$/ml for boars 5 and 6. The PCV2 isolate LHVA-V53 originated from a case of PMWS in Québec and was isolated from homogenates of lungs and lymph nodes on PCV-free PK15 cells (16). Two control boars (boars 1 and 2) were inoculated with 5 ml of the supernatant of noninfected PK15 cells. All six boars were housed in separate cubicles. Semen was collected from each boar at 5, 8, 11, 13, 18, 21, 25, 28, 33, and 47 days postinoculation (dpi), and serum samples were collected at 4, 7, 11, 13, 18, 21, 25, 28, 35, and 55 dpi. Serum samples were also collected from the four infected boars at 90 dpi. Preinoculation semen and serum samples were collected from each boar. The serum samples were tested for

* Corresponding author. Mailing address: Laboratoire d'Hygiène Vétérinaire et Alimentaire, Agence Canadienne d'Inspection des Aliments, 3400 Casavant Ouest, Saint-Hyacinthe, Québec, Canada J2S 8E3. Phone: (450) 773-7730. Fax: (450) 773-8152. E-mail: larocheller@em.agr.ca.

TABLE 1. Detection of PCV2 antibodies by IFA and PCV2 nucleic acid by PCR and nested PCR in serum and semen samples from experimentally infected boars

Boar no. and test method	Detection at postinoculation days:[a]											
	P/P	4/5	7/8	11/11	13/13	18/18	21/21	25/25	28/28	35/33	55/47	90/NA
1												
IFA serum	−	−	−	−	−	−	−	−	−	−	−	NA
PCR serum	−	−	−	−	−	−	−	−	−	−	−	NA
nPCR serum	−	−	−	−	−	−	−	−	−	−	−	NA
nPCR semen	−	−	−	−	−	−	−	−	−	−	−	NA
2												
IFA serum	−	−	−	−	−	−	−	−	−	−	−	NA
PCR serum	−	−	−	−	−	−	−	−	−	−	−	NA
nPCR serum	−	−	−	−	−	−	−	−	−	−	−	NA
nPCR semen	−	−	−	−	−	−	−	−	−	−	−	NA
3												
IFA serum	−	−	−	+	+	+	+	+	+	+	+	+
PCR serum	−	−	+	+	−	−	+	+	+	−	−	−
nPCR serum	−	+	+	+	+	+	+	+	+	+	+	−
nPCR semen	−	−	+	+	+	+	+	−	−	+	−	NA
4												
IFA serum	−	−	−	−	−	+	+	+	+	+	+	+
PCR serum	−	−	−	−	−	−	+	−	+	−	−	−
nPCR serum	−	+	−	+	+	+	+	+	+	+	−	−
nPCR semen	−	−	−	+	−	−	−	−	+	−	+	NA
5												
IFA serum	−	−	−	−	−	+	+	+	+	+	+	+
PCR serum	−	−	−	+	+	+	+	+	+	+	+	−
nPCR serum	−	−	+	+	+	+	+	+	+	+	+	−
nPCR semen	−	+	−	−	+	−	+	+	+	+	+	NA
6												
IFA serum	−	−	−	−	−	+	+	+	+	+	+	+
PCR serum	−	−	−	−	+	+	+	−	−	−	−	−
nPCR serum	−	+	−	+	+	+	+	+	+	+	−	−
nPCR semen	−	+	+	+	−	−	+	−	+	−	−	NA

[a] Days postinoculation are shown as days postinoculation for serum/days postinoculation for semen. P, preinoculation; NA, not available; nPCR, nested PCR. +, detected; −, not detected.

antibodies to PCV2 by an indirect immunofluorescence assay (IFA) using 96-well plates containing acetone-fixed infected and noninfected PK15 cells (18, 19). Serum samples were tested at a dilution of 1:20 in phosphate-buffered saline.

Serum and semen (seminal fraction: sperm cells and seminal plasma) samples were tested by nested PCR for the detection of PCV2 nucleic acid. DNA was extracted from volumes of 200 μl of semen and serum samples using a commercial kit (DNeasy tissue kit; Qiagen Inc., Mississauga, Ontario, Canada). The DNA was recovered with 50 μl of elution buffer and kept at −70°C. The outer primers were designed to amplify both PCV1 and PCV2 following the comparison of DNA sequences of PCV1 (20) and the PCV2 associated with PMWS (10), while the inner primers were designed to amplify PCV2 only (15). The outer sense and antisense primers were 5′-CAACTGCTGTCCCAGCTGTAG-3′ (nucleotides 844 to 864) and 5′-AGGAGGCGTTACCGCAGAAG-3′ (nucleotides 1704 to 1723), amplifying an 894-nucleotide region mainly from open reading frame 2. The inner sense and antisense primers were 5′-TAGGTTAGGGCTGTGGCCTT-3′ (nucleotides 1323 to 1342) and 5′-CCGCACCTTCGGATATACTG-3′ (nucleotides 1567 to 1586), amplifying a 263-nucleotide region from open reading frame 2. For PCR, 5 μl of DNA was added to 45 μl of reaction mixture containing final concentrations of 1.25 mM $MgCl_2$, 1× PCR buffer, 0.2 mM each deoxynucleoside triphosphate, 1.00 μM each primer, and 2.5 U of *Taq* DNA polymerase (Canadian Life Technologies, Burlington, Ontario, Canada). Amplification of DNA was achieved by 35 cycles of 95°C for 1 min, 65°C for 1 min, and 72°C for 1 min. For nested PCR, 2 μl of the outer DNA product was added to a fresh tube containing a 48-μl volume of the reaction mixture described above, and this second round of amplification was achieved by 20 cycles of 95°C for 30 s, 65°C for 30 s, and 72°C for 30 s. The specificity of the primers used in the nested PCR was demonstrated by sequencing and by testing other porcine viruses (15). To determine the lower limit of detection by PCR and nested PCR, 10-fold dilutions of PCV2 LHVA-V53 isolate (2×10^4 $TCID_{50}$/ml: titer determined by indirect immunofluorescence assay) in semen were used.

Serological analysis indicated that all boars were seronegative to PCV2 prior to the inoculation and that all control boars (boars 1 and 2) remained PCV2 seronegative throughout the experimental study period. Antibodies to PCV2 could be detected as early as 11 dpi in boar 3, and all four infected boars were found positive by 18 dpi (Table 1). Thereafter all infected boars remained seropositive to PCV2 until 55 dpi. Serum samples collected from the four PCV2-infected boars at 90 dpi were still positive for PCV2 antibodies.

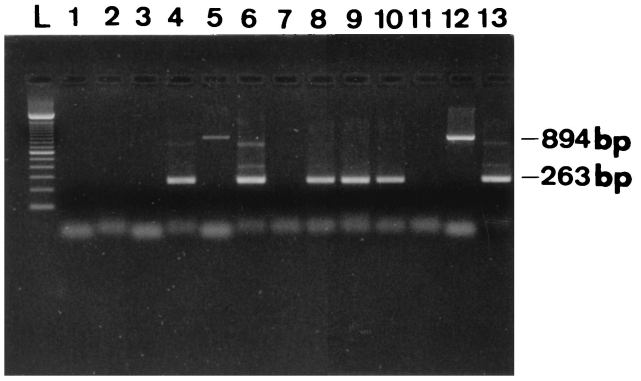

FIG. 1. Agarose gel electrophoresis of PCR and nested PCR (nPCR) products of PCV2 detected in serum and semen samples collected from experimentally infected boars. Lane 1, PCR on serum 0 dpi; lane 2, nPCR on serum 0 dpi; lane 3, PCR on serum 4 dpi; lane 4, nPCR on serum 4 dpi; lane 5, PCR on serum 28 dpi; lane 6, nPCR on serum 28 dpi; lane 7, nPCR on semen 0 dpi; lane 8, nPCR on semen 5 dpi; lane 9, nPCR on semen 21 dpi; lane 10, nPCR on semen 47 dpi; lane 11, negative control semen from an uninfected boar; lane 12, PCR product of PCV2 LHVA-V53 isolate (2×10^{-1} $TCID_{50}$/ml) diluted in semen from an uninfected boar; lane 13, nPCR product of a PCV2 LHVA-V53 isolate (2×10^{-3} $TCID_{50}$/ml) diluted in semen from an uninfected boar; L, 100-bp DNA ladder.

The detection limit determined using DNA extracted from 10-fold dilutions of the PCV2 LHVA-V53 isolate in semen was 2×10^{-3} $TCID_{50}$/ml (1,000-fold below the detection limit of virus infectivity in cell culture as determined by indirect immunofluorescence assay) for PCR and 2×10^{-4} $TCID_{50}$/ml for the nested PCR assay. Analysis by nested PCR of the presence of PCV2 DNA in the serum samples indicated that preinoculation sera were negative and that all serum samples from control boars remained negative throughout the experimental period (Table 1). Eighteen serum samples from PCV2 experimentally infected boars were found positive by PCR; however, the intensity of the band was generally weak. In comparison to the PCR, the nested PCR allowed the detection of PCV2 DNA in 35 of these serum samples with a stronger intensity of the band (Fig. 1). Serum samples positive for PCV2 nucleic acid by nested PCR could be detected as soon as 4 dpi in three of four infected boars, and all infected boars were positive from 11 dpi until 35 dpi (Table 1). The sera of two infected boars were positive at 55 dpi by nested PCR, and at 90 dpi serum samples from all four infected boars were negative. By nested PCR all preinoculation semen samples were negative and semen samples of control boars remained negative throughout the experimental period. PCV2 nucleic acid was detected in semen by nested PCR as early as 5 dpi in two infected boars and intermittently thereafter in all four infected boars (Table 1). The semen of two infected boars was positive for PCV2 nucleic acid by nested PCR at 47 dpi (Fig. 1). PCV2 nucleic acid was not detected in semen by a single round of PCR.

In the present study mature boars were successfully infected following intranasal inoculation with PCV2, as demonstrated by the appearance of PCV2 specific antibodies. All experimentally infected boars shed PCV2 DNA in semen intermittently following infection. PCV2 nucleic acid could be demonstrated in semen concurrently with the presence of PCV2 antibodies in serum, and the virus could still be detected in the semen of two boars at 47 dpi, suggesting viral persistence in spite of an immune response. Detection of PCV2 DNA in semen occurred 3 to 13 days prior to the detection of antibodies by IFA, indicating that the serological status of the boar may not be a good indicator of the shedding of PCV2 DNA in semen.

In this study, the amount of PCV2 DNA present in semen appears to be low since it could only be detected following nested PCR, in contrast to serum where DNA could be detected using only one round of PCR. It cannot be concluded that infectious PCV2 was present in the semen since no virus isolation or swine bioassays were performed. In addition, it was not determined if virus was free in seminal plasma or in association with sperm or nonsperm cells. Porcine reproductive and respiratory syndrome virus (PRRSV), another important swine pathogen, also possesses, like PCV2, an affinity for cells of the monocyte/macrophage lineage. In a recent study, PRRSV was identified in the nonsperm cell fraction of the semen, and it has been suggested that this virus most likely traffics from lymphoid tissues through peripheral blood to reproductive tissues or directly into semen (4). A similar mechanism could be considered for PCV2. In recent experimental transmission studies of PCV2 in piglets, PCV2 antigen was demonstrated in testes, in particular in infiltrated macrophages in the tunica albuginea, in interstitial macrophages, and in infiltrated macrophages in the epididymis as well as in germinal epithelial cells (14). A good correlation between a nested PCR assay and a swine bioassay has been demonstrated for PRRSV (3), and in addition, transmission of PRRSV to artificially inseminated sows has been demonstrated by using undiluted and extended semen from experimentally infected boars (9, 28). Similar studies need to be initiated for PCV2 to determine if the quantity of PCV2 present in the semen of an infected boar can induce infection in sows following natural or artificial insemination. At the present time, the results of this study suggest that PCV2 may be shed intermittently in semen following infection of boars by the virus.

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