Quantitative, Competitive PCR Analysis of Porcine Circovirus DNA in Serum from Pigs with Postweaning Multisystemic Wasting Syndrome

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Received 24 February 2000/Returned for modification 3 May 2000/Accepted 22 June 2000

Postweaning multisystemic wasting syndrome (PMWS) was first observed in western Canada in 1991 and in many other countries later on (2, 3, 6, 8, 11, 14, 16–18). Clinical signs of the disease include progressive weight loss, dyspnea, diarrhea, pallor, and jaundice. It was histologically recognized by interstitial pneumonia, lymphoid depletion, and histiocytosis in lymphoid tissues and, less frequently, hepatitis and nephritis. Although the causal agent has not been identified explicitly, a new strain of porcine circovirus (PCV), PCV type 2 (PCV2), has been shown to be the causal agent in many cases 

A competitive PCR (cPCR) assay was developed for monitoring porcine circovirus (PCV) DNA in serum samples from piglets. The cPCR was based on competitive coamplification of a 502- or 506-bp region of the PCV type 1 (PCV1) or PCV2 ORF2, respectively, with a known concentration of competitor DNA, which produced a 761- or 765-bp fragment, respectively. The cPCR was validated by quantification of a known amount of PCV wild-type plasmids. We also used this technique to determine PCV genome copy numbers in infected cells. Furthermore, we measured PCV DNA loads in clinical samples. More than 50% of clinically healthy piglets could harbor both types of PCV. While PCV1 was detected in only 3 of 16 pigs with postweaning multisystemic wasting syndrome (PMWS), all the sick piglets contained PCV2. A comparison of the PCV2 DNA loads of healthy and sick animals revealed a significant difference, indicating that the development of PMWS may require a certain amount of PCV2.

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cPCR was carried out with a 50-μl reaction mixture containing 20 mM Tris-HCl (pH 8.3), 50 mM KCl, 5 mM MgCl₂, 200 μM (each) deoxynucleoside triphosphate, 500 nM (each) primer, and 2.5 U of platinum Taq polymerase (Gibco-BRL) with a fixed amount of wild-type DNA and various quantities of competitor DNA. Following incubation at 94°C for 2 min, PCR was performed for 30 cycles, with each cycle consisting of 94°C for 30 s, 57°C for 30 s, and 72°C for 1 min, with a final extension at 72°C for 7 min in a GeneAmp PCR System 2400 instrument (Perkin-Elmer). Ten microliters of the amplification products was analyzed on a 1% agarose gel (Ultraspure agarose; Gibco-BRL) in 1× Tris-acetate-EDTA electrophoresis buffer and 0.2 μg of ethidium bromide per ml. The equivalence points, at which the 502- and 506-bp bands and the 761- and 765-bp bands had equal intensities, were determined by visual inspection and densitometric scanning in an AlphaImager 2200 instrument (Alpha Innotech Corporation). Figure 1B shows a progressive competition between PCV2 wild-type DNA at two fixed amounts (1 ng or 1.8 × 10⁷ molecules and 1 pg or 1.8 × 10⁵ molecules) and 10-fold dilutions ranging from 100 ng to 1 pg or 100 pg to 1 fg of competitor DNA, indicating a broad range of sensitivity of the cPCR. A similar competition was also observed between PCV1 wild-type and competitor DNAs (data not shown).

We then applied this cPCR method to evaluate whether there were any changes in the amount of viral DNA after D-glucosamine treatment of PK-15 cells or PCV2-infected cells. About 10⁵ PK-15 cells or PCV2-infected PCV-free PK-15 cells (at a multiplicity of infection of ~1) were incubated with 300 mM D-glucosamine for 30 min, and 24 h later total DNA was extracted with a QIAamp kit and eluted into 200 μl of H₂O. Five microliters of the 10-fold-diluted (PCV1) or undiluted (PCV2) DNA was subjected to cPCR with various known amounts of competitor DNA. It turned out that the PCV1 DNA content in PK-15 cells was about 10³-fold the PCV2 DNA content in PCV2-infected cells under our experimental conditions. As illustrated in Fig. 2, D-glucosamine did not cause any increase in viral DNA levels in PCV-infected cells. These findings were compatible with those of previous studies in which D-glucosamine promoted the entry of viral DNA into the nucleus prior to mitosis but the number of viral inclusion bodies per infected cell was not increased after D-glucosamine induction (23, 25).

Subsequently, we measured the viral DNA loads in serum samples from piglets with various clinical signs and lesions. Serum samples were collected from two herds in Canada. Total DNA was extracted from 200 μl of each serum sample and was eluted into 200 μl of H₂O. A standard, noncompetitive PCR was carried out with type-specific primers to detect PCV before the quantitative cPCR was performed. Figure 3A and B show two examples of the results of the cPCR experiments. The specificity of the cPCR assay was evaluated by sequencing several randomly selected PCR products (two for PCV1 and five for PCV2). For this purpose, the samples were subjected to a standard PCR and were cloned into a pbKSSI(+) vector via BamHI, which was incorporated into the 5’ ends of the primers. The inserts were sequenced with an automated DNA sequencer.

The majority of clinically healthy piglets had both PCV1 and PCV2. The DNA loads ranged from 1.8 × 10⁷ to 1 × 10⁹ copies/ml of serum for PCV1 (n = 11) or 1.8 × 10⁷ to 1 × 10⁴ copies/ml of serum for PCV2 (n = 25), with PCV2 DNA loads (mean, 1.89 × 10⁵) being approximately 1 log higher than the PCV1 DNA loads (mean, 2.68 × 10⁴) (Fig. 3C). In contrast, 3 of 16 piglets with PMWS had detectable PCV1, whereas all 16 had PCV2, suggesting that PCV2 might play a role in the development of PMWS and PCV1 might not be involved. It was noteworthy that the mean PCV2 DNA content (4.61 × 10⁵ copies/ml) obtained for 25
healthy animals ($P = 0.0273$ in a two-tailed $t$ test) (Fig. 3C), indicating that there might be a threshold of the PCV2 amount which was needed to trigger the disease.

Furthermore, we wanted to examine the relationship between the PCV2 DNA content and the clinical expression of disease in different phases of PMWS. Two piglets with poor growth conditions suggestive of possible early signs of PMWS were sampled at 10-day intervals until they were diagnosed with clinical acute PMWS. One animal showed a continuous increase in PCV2 content from $10^5$ to $10^7$ copies/ml of serum during the development of PMWS, whereas the PCV2 load of the other piglet remained at a stable (and high) level ($10^6$) during this process. In another series of experiment, a follow-up sample was collected from six piglets 10 days after the onset of the acute phase of PMWS since they were found to be recovering from PMWS, as judged clinically. At the acute phase of PMWS, the piglets had $10^6$ to $10^7$ copies/ml of serum. The cPCR assay with the follow-up samples did not reveal any change in the viral DNA content, indicating that PCV2 had not been effectively eliminated in this 10-day period, even though our preliminary data demonstrated that the animals mounted an antibody response (data not shown). The observation of the apparent irrelevance of the PCV2 DNA content (at a certain level, though) to the alleviation and aggravation of clinical PMWS was in line with the findings that PCV2 is not the only infectious agent required for the production of PMWS (7, 10, 12).

In conclusion, the viral DNA levels in serum carried by the piglets in the acute (and recovering) phases of PMWS were significantly higher than those in clinically healthy piglets. This observation suggested that the development of PMWS might require a certain amount of PCV2. Although all the piglets with PMWS tested in this study had high PCV2 genome contents (above the threshold), some animals carrying the same...
amount of PCV2 remained healthy, leading us to speculate that PCV2 is required, but may not be sufficient, to cause PMWS. The ultimate occurrence of PMWS may probably rely on a secondary pathogen and/or the conditions of individual piglets. It is also intriguing that the clinically healthy piglets could harbor both types of PCV, whereas the majority of the clinically PMWS-positive piglets tested in this study contained only PCV2. The fairly high incidence of PCV1 and PCV2 in healthy piglets and the rather low incidence of PCV1 in piglets with PMWS are in agreement with several recent serological surveys (9, 13, 19). The biological significance of the coexistence of the nonpathogenic and apparently pathogenic strains of PCV in healthy piglets and the almost entire exclusion of the nonpathogenic PCV strain from the piglets with PMWS requires further studies. Also compatible with a previous study (13) is the observation of the apparent predominance of PCV2 in all the samples tested, suggesting that PCV2 might have developed a much more efficient way of transmission.

The type-specific primers used in this study were designed on the basis of the sequences of one PCV1 strain and one PCV2 strain, respectively. Compared to all four complete PCV1 sequences in GenBank, the forward primer for detecting PCV1 had an entire match to three isolates and a one-nucleotide mismatch to the French isolate, whereas the backward primer had an one-nucleotide mismatch to the French and the U.S. isolates. In the case of PCV2-specific primers, the backward primer had full homology to all 26 PCV2 sequences in GenBank, while the forward primer had an one-nucleotide mismatch to the two isolates reported from Taiwan. Because of the mismatches of the primers, we could not exclude the possibility of missing certain genetically divergent PCV strains, albeit it seemed somewhat unlikely since the one-nucleotide mismatches were located internally and not at the extreme 3’ ends of the primers, which would not affect the primer-template binding dramatically (21).

In summary, we have described the development of a cPCR and the monitoring of PCR DNA levels in serum samples, which makes it possible to analyze the correlation between the amount of circulating viruses and clinical parameters in different groups of piglets. Moreover, the cPCR method will also benefit studies of PMWS as well as PCV, e.g., the spread of virus within tissues of swine.

This research was supported by Boehringer-Ingelheim and the Natural Science and Research Council of Canada.

The technical assistance of Betty Chow, Elaine Van Moolehem, and Tammy Karkut is greatly appreciated.

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