RESEARCH REPORT
SWINE HEALTH

Title: Molecular Epidemiology of Porcine Circovirus in the US Swine Herd, **NPB #07-221**

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Abstract

To better understand the epidemiology of porcine circovirus in the U.S. swine herd, ELISA and PCR tests were developed and characterized to specifically differentiate and quantitatively assess the status of PCV1 and PCV2 infection in serum samples collected from finishing pigs in 2006 as part of the NAHMS swine health survey. A quantitative Tetra-Nucleotide Discrimination (TND) Assay was developed to measure simultaneously the amount of PCV1 and PCV2 in a test sample. Approximately 80% of serum samples were positive for PCV2 virus, whereas only 2.5% were positive for PCV1 virus. The mean titer was about 10^4 copies/ml serum. Forty-one percent of samples were only PCV2b, 31% were PCV2a and 10% were mixed infections of PCV2a and PCV2b. To establish baseline serological data for exposure to PCV, more than 6,200 serum samples from 187 farms throughout the U.S. were evaluated for serological response to PCV1 and PCV2 using amino-terminal deleted capsid proteins expressed in bacteria. About 80% of samples were positive for PCV2. More than 50% of animals sampled on nearly all farms were seropositive; on only one farm were all samples test-negative. PCV1 antibodies were detected in about 20% of serum samples. The findings to date indicate that PCV1 is only rarely present in finishing pigs. PCV2 is widespread in individual pigs, indicative of a viremic, active infection. Interestingly, in 81% of cases the serological and virological status of a sample were concordant for PCV2,

either both positive and negative, and were discordant 19% of the time. While not a specific finding of this study, the data also suggest that the practice of serum inoculation might result in extensive dissemination of PCV2 within a herd.

Introduction

Porcine circoviruses (PCVs) PCV1 and PCV2 belong to the family Circoviridae (Lukert et al., 1995), and are small, icosahedral, non-enveloped viruses with a single-stranded DNA genome (Meehan et al., 1997). PCV1 and PCV2 are morphologically similar but genetically distinct (Allan et al., 2000). PCVs contain two major open reading frames (ORFs), ORF1 in both PCV1 and PCV2 encodes two replication associated proteins *Rep* and *Rep'* (Cheung, 2003; Mankertz and Hillenbrand, 2001), while ORF2 encodes a viral capsid protein, which is the major immunogenic protein of PCV2 (Nawagitgul et al., 2002). A smaller ORF3 was identified in PCV2 and may be involved in apoptosis (Liu et al., 2005).

PCV1 was discovered in 1974 as a non-pathogenic contaminant of the permanent pig kidney cell line PK-15 (Tischer et al., 1974), and is believed to be widespread in swine (Boisseson et al, 2004). PCV2 drew swine veterinarians' attention, after the description of its presence in animals with lesions of PMWS in 1997 or earlier (Segales, 2004, McIntosh et al., 2006). The significant clinical expression of PCV2 infection is PMWS, a worldwide disease with outbreaks in swine herds of North and South America, Europe, and Asia (Gagnon et al., 2007). Its presence also is associated with other syndromes, including porcine dermatitis and nephropathy syndrome (PDNS), reproductive failure, porcine respiratory disease complex, granulomatous enteritis, necrotizing lymphadenitis, exudative epidermitis, and congenital tremor, all of which are referred to as Porcine Circovirus Associated Disease (PCVAD) (Gagnon et al, 2007). To the present, the relationship between PCVAD and PCV2 is poorly understood (Segales, et al., 2004), and various experimental models including porcine parvovirus or porcine reproductive and respiratory syndrome virus (PRRSV) have been developed in order to explain the variable presence of PCV2 (Carman, et al., 2008; Pittman, 2008).

PCV2 has two strains formerly designated as PCV2a or RFLP 422, isolated in France, United Kingdom, and Asia; and PCV2b or RFLP 321, isolated in North America and Canada (Gagnon et al, 2007; Cheung et al,

2007). The relationship between severity and genotype is not clear; however, recent studies reported the presence of specific lesions associated with PCV2-RFLP 321 (Carman et al, 2008; Grau-Roma et al, 2007).

The disease has resulted in significant health challenges and economic damage to the swine industry (Puvanendiran et al., 2008). PMWS is considered to have the most severe impact in swine production among all the PCVAD. It has been estimated that PCVAD costs around 600 million Euros per year to the European Union (Armstrong and Bishop, 2004). The potential cost of a severe chronic form of PCV2 infection causing a 7% increase in mortality could be as much as 16.19 Euros/pig or US$ 21.37 (Burch, 2007).

The objective of this study is to assess the prevalence, level and relative incidence of PCV1 and PCV2 in the US swine population.

Objectives

(1) To survey the frequency of active infection by PCV1, PCV2, and co-infection in the US swine herd. The goal here is to develop a graphical picture of the rate of infection of pigs on farms for PCV1 alone, PCV2 and co-infection with both and to verify the unexpected preliminary observations that PCV1 is uncommon and PCV2 is abundant.The project is ahead of schedule due to the excellent work of laboratory personnel.

(2) To quantify the level of acute infection in commercial swine. The goal here is to assess the level of PCV1 and PCV2 active infection in pigs in 186 farms of the NAHMS survey.

(3) To quantitatively evaluate the PCV2a and PCV2b status of the US swine herd prior to or associated with PCV-AD outbreaks (collaboration with Kansas State University).

Materials & Methods

Approximately 6,300 serum samples from finishing swine were collected in 2006 as part of the NAHMS survey of swine health, prior to use of PCV2 vaccines.

Quantitative PCR. The Tetra-Nucleotide Discrimination (TND) Assay, was based on Fenaux et al. (2000). The primers 5’-TGGCCCGCAGTATTTTGATT-3’ and 5’-CAGCTGGGACAGCAGTTGAG-3’ amplify both PCV1 and PCV2. The PCV1 probe was 5’-VIC-CCAGCAATCAGGCCCCCCAGGAAT-TAMRA-3’ and the PCV2 probe was 5’-FAM-CCAGCAATCAGACCCCGTTGGAATG-TAMRA-3’. Reactions were performed in an ABI 7500 PCR machine and were carried out at 95°C for 10 min, then 95°C for

15 sec and 60°C for 1 min for 40 cycles. Cycle threshold values were converted to DNA copies by comparison to a standard curve of cloned target DNA. The test was validated on serial dilutions of known positive control samples, by comparison to other tests that independently measured PCV1 or PCV2, by analysis of test samples spiked with known amounts of PCV1 and PCV2, and by comparison to isolation of PCV1 and PCV2 on cultured PK-15 cells. A second SYBR green PCR assay quantitatively differentiating PCV2a and PCV2b was developed from a protocol kindly provided by Robert R.R. Rowland, Kansas State University.

DNA was extracted from 200 µl of serum with the QIAmp DNA Blood mini kit (Qiagen, Inc., Valencia, CA) according to the manufacturer's instructions. The resulting DNA was eluted in 50 µl of TE buffer.

Antigen Preparation. PCV1 and PCV2 capsid proteins were expressed in E. coli strain BL21 RP, purified by Ni-NTA agarose bead affinity chromatography (Qiagen), and refolded as described by Johnson et al (2007). The N-terminus of PCV1 and PCV2 were devoid of first 40 amino acids of native PCV1 and PCV2 capsid proteins to facilitate bacterial expression and because the amino terminal portion is highly conserved in both PCV1 and PCV2 (Fenaux et al, 2000). PCV1 and PCV2 truncated capsid proteins were used as antigens for indirect ELISA.

Indirect ELISA. Plates were coated with the antigens at 100 ng/ well in carbonate buffer and incubated at 4°C overnight. Wells were blocked with 5% nonfat dry milk (NFDM), washed, and test sera diluted 1:500 were added. Peroxidase-labeled goat anti-swine IgG (H+L) (KPL, Gaithersburg, MD) was diluted 1:5000 in 5% NFDM in PBST (pH 7.4) and 100 µl was added to each well and then incubated at room temperature for one hour. The plates were washed for times and equal volumes of TMB peroxidase substrate and peroxidase H_2O_2 (KPL) were added to each well. Reactions were stopped with phosphoric acid and were then read at 450 nm in a microplate reader (ThermoMax plate reader). Recombinant α1S subunit of porcine haptoglobin was used as a background control. Since absorbance values of test sera were between 0.1-0.2 with recombinant porcine hpatoglobin α1S subunit, the cut-off point for a positive test was determined to be ≥0.4.

Assay Repeatability. For intra-assay (within-plate) repeatability the samples and controls were run in duplicate. For inter-assay (between-run) repeatability there was one positive sample and one negative sample

run on every plate. These positive and negative serum samples were run several times and always produced similar OD values.

Results

Standard curves were developed with cloned target sequences for PCV1 and PCV2. Seven replicates gave results with a correlation coefficient of 0.95 for PCV2 in the range of 10^3 to 10^{10} copies per ml, and of 0.98 for PCV1 in the range of 10^2 to 10^9 copies per ml. Approximately 3,800 serum samples from 187 farms were processed for the TND assay. Approximately 80 percent of serum samples were positive or suspect positive for PCV2 nucleic acids. Only one farm showed uniformly negative results. Thus, in the majority of farms the majority of pigs were actively infected as defined by virus circulating in blood. The levels of infection were substantial, commonly in the range of 3 to 5 logs of virus per ml. By contrast, PCV1 infection was essentially absent in finishing swine. Occasionally high values were obtained in PCR screening, but the data were most accurately modeled as a single population of negative animals.

The differential quantitative PCR to discriminate PCV2a and PCV2b was redesigned and optimized and 1000 samples, 10 from each of 100 farms, were processed. About 30% of pigs were positive for PCV2a only, 41% of pigs were positive for PCV2b, 9% of pigs had a mixed infection, and 20% were negative. The findings also validate the previous results in that two independent PCR assays show that 75 to 80% of finisher pigs are PCV2 positive. The PCV2b genotype is more common than the PCV2a genotype, and both genotypes were frequently observed on the same farm, but rarely in the same pig.

For the ELISA, amino-terminal deleted capsid proteins expressed in bacteria were sensitive and specific antigens. About 80 percent of 6,200 samples were positive for PCV2, 4% were suspect positive, and 16% were negative. More than 50% of animals sampled on farms were seropositive in most cases. PCV1 antibodies were detected in 23% of serum samples and an additional 9% were suspect positives. Only about 5% of farms had more than 50% of samples test-positive.

Discussion

The findings revealed that PCV1 is essentially not present in finishing swine, but PCV2 is nearly universally distributed as an acute, viremic infection, with approximately 80% of pigs being positive. We

showed that both PCV2 genotypes were widely distributed in the USA in 2006. The absence of PCV1 could be due to an immune response that cleared PCV1 infection from the blood. However, most pigs were also seronegative for PCV1. In contrast to previous reports, this study indicates that PCV1 is not prevalent in swine populations in the U.S. In most cases, PCV2-viremic pigs also were seropositive, suggesting that the immune response does not resolve infection in a timely manner. Thus, it appears that the natural immune response to PCV2 infection does not control the infection. In contrast, current vaccines based on recombinant capsid proteins or inactivated viral preparations appears to provide solid protection against PCV-AD. The data do not explain why PCV-AD occurs sporadically even though nearly all herds are infected with PCV2. Anti-PCV1 immunity is not produced to cross-protect against PCV2 since the prevalence of PCV1 is negligible. A shift from possibly avirulent PCV2a to virulent PCV2b seems unlikely since both genotypes were widely distributed before the appearance of pronounced PCV-AD in the USA. In conclusion, the results of our virological and serological assessment of finishing pigs in the U.S. suggest that PCV1 is rare. By contrast, infection by genetically diverse PCV2 is widespread and nearly universal among herds and occurs in the presence of a humoral immune response.

References:

Allan, G. M., Ellis, J. A. (2000). Porcine circoviruses: a review. J Vet Diag Invest. 12:3-14

Armstrong, D. and Bishop, SC. (2004). Does genetics or litter effect influence mortality in PMWS. In: Proc Pig Vet Soc Congr, p. 809.

Carman, S., Cay, H., DeLay, J., Youssef, S., McEwen, B., Gagnon, C., Tremblay, D., Hazlett, M., Lusis, P., Fairles, J., Alexander, H., Dreumel, T. (2008). The emergence of a new strain of porcine circovirus-2 in Ontario and Quebec swine and its association with severe porcine circovirus associated disease — 2004–2006. Can J Vet Res. 72: 259-268.

Burch, D. Costs of PCV2 Infection in Finishing Pigs and Potential Benefits of Vaccination. Pig Clinical Pharmacolgical Swine. http://www.octagon-services.co.uk/pigs.htm

Cheung, A. K. (2003). The essential and non essential transcription units for viral protein synthesis and DNA replication of porcine circovirus type 2. Virology. 313:452-459

De Boisseson, C., Beven, V., Bigarre, L., Thiery, R., Rose, N., Eveno, E., Francois, M., Jestin, A. (2004). Molecular characterization of porcine circovirus type 2 isolates from post weaning multisystemic wasting syndrome-affected and non-affected pigs. J Virol. 85:293-304

Gagnon, G. A., Tremblay, D., Tijssen, P., Venne, M. H., Houde, A., Elahi, S. M. (2007) The emergence of porcine circovirus 2g genotype (PCV2b) in swine in Canada. Can Vet J. 48:811-819

Grau-Roma, L., Crisci, E., Siblia, M., Lopez-Soria, S., Nofrarias, M., Cortey, M., Fraile, L., Olvera, A., Segales, J. (2007). A proposal on porcine circovirus type 2 (PCV2) genotype definitions and their relation with post weaning multisystemic wasting syndrome (PMWS) occurrence. Vet Micro. Doi:10.1016/j.vetmic.2007.09.007

Liu, J., Chen, I., Kwang, J. (2005). Characterization of a previously unidentified viral protein in Porcine Circovirus Type 2-Infected Cells and Its Role in Virus-Induced Apoptosis. J Virol. 79:8262-8274

Lukert P, de Boer GF, Dale JL, et al.: 1995, The Circoviridae. *In:* Virus taxonomy. Sixth report of the International Committee on Taxonomy of Viruses, ed. Murphy FA, Fauquet CM, Bishop DHL, et al., pp. 166–168. Springer-Verlag, New York, NY.

Mankertz, A., Hillenbrand, B. (2001). Replication of Porcine Circovirus Type 1 requires two proteins encoded by the viral rep gene. Virology. 279: 429-438

McIntosh K A., Harding J.C., Ellis J.A., Appleyard G.D. (2006). Detection of Porcine circovirus type 2 viremia and seroconversion in naturally infected pigs in a farrow-to-finish barn. Can J Vet Res. 70:58-61

Meehan BM., Creelan JL., McNulty MS., Todd D. 1997. Sequence of porcine circovirus DNA: affinities with plant circoviruses. J Gen Virol 78:221–227.

Nawagitgul, P., Harms, P. A., morozov, I., thacker, B. J., Sorden, S. D., Llekcharoensuk, C., Paul, P. S.(2002) Modified indirect Porcine Circovirus (PCV) Type-2 based and ecombinant Capsid Protein (ORF2)-based Enzyme-Linked Immunosorbent Assays for detection of Antibodies to PCV. Clin Diag Lab Immunol. 9:33-40

Pittman, S. Jeremy. 2008. Reproductive failure associated with Porcine Circovirus Type 2 in Gils. J Swine Hlth Prod. 16(3):144-148.

Puvanendiran, S., Stone, S., Wangin,Y., Johnson, C., Garcia L., Griggs, T., Fuentes, M., Murtaugh, M., Haley C., Wagner, B. 2008. Differentiating PCV1 and PCV2 Infection in Swine. Proceedings of the 39th Amer Assoc Swine Vet annual meeting. pp. 131-135.

Segales, J. Rosell, C. Domingo, M. 2004. Pathological findings associated with naturally acquired porcine circovirus type 2 associated disease. Vet Micro 98:137-149.

Tischer, I. Rasch, R. Tochtermann G. 1974. Characterization of Papovavirus- and Picornavirus-like particles in permanent pig kidney cell lines. Zentralbe Bakteriol Org A 226:153–167.

Lay Interpretation

Data from 187 finishing herds shows that PCV2 is nearly universally present as an active infection in finishing herds, often at moderate to high levels of viremia in the presence of anti-capsid antibodies. PCV2a and PCV2b genotypes were widely distributed, with one or the other usually in different pigs on the same site, but as a dual infection nearly 10% of the time. Average viral titer was the same in animals infected with PCV2a, or PCV2b or both genotypes. Unexpectedly, PCV1 was essentially absent from swine, contrary to statements often found in the literature. The findings suggest that all of the conditions attributed to PCV-AD, including virulent genotypes and high viral titers, were commonly observed in the U.S. swine herd in 2006, and that anti-PCV immune responses also were present. Thus, it is surprising that PCV-AD was not more prevalent until recent times, and that capsid-based vaccines provide effective protection against PCV-AD. It appears that PCV2 by itself does not cause PCV-AD, but that it plays a role that has not yet been figured out.

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