RESEARCH REPORT

SWINE HEALTH

Title: Is Porcine Circovirus Vertically Transmitted - **NPB #98-202**

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I. Abstract: In order to determine if vertically transmitted porcine circovirus (PCV2) plays a role in reproductive failure in pigs, frozen and fixed tissues were examined by polymerase chain reaction (PCR), immunohistochemistry and virus isolation. Tissues tested were routine cases submitted between 1995-1999 from 30 high health herds in the provinces of Alberta and Saskatchewan comprising a total of 38 individual submissions. PCV1 was not detected by PCR in any submissions. PCV2 was detected by PCR in two submissions involving several stillborn piglets and non-viable neonates presenting with severe diffuse myocarditis, cardiac hypertrophy and evidence of chronic passive congestion. The two positive submissions were the same farm in Alberta, but occurred at two different times. The presence of PCV2 in the hearts and other tissues of affected piglets was confirmed by immunohistochemistry and virus isolation. The effect of extended formalin fixation on the detection of PCV2 by PCR was assessed and tissues fixed for up to one week had no gross effect on sensitivity of detection using this PCR technique. Failure to detect porcine circoviruses in cases of reproductive failure prior to1999 in areas of endemic infections, suggests that these cases may represent a new disease presentation of PCV2 infection and that vertical transmission may not have been the primary mechanism of initial dissemination in the pig population.

II. Introduction: Porcine circovirus 2 (PCV2) was first associated with postweaning multisystemic wasting syndrome (PMWS) in 1996 in western Canada. This virus was found to be genetically and antigenically distinct from PCV1 which was first recognized in the1970's as a common "contaminant" of porcine kidney cell lines. Studies to date suggest that PCV1 is apparently nonpathogenic in pigs. Subsequent to its consistent association with PMWS, PCV2 has been associated with other disease syndromes in pigs, including, most recently, myocarditis in stillborn piglets. Although the primary means of transmission of PCV2 remains to be determined, the presence of PCV2 in neonatal piglets suggested that vertical transmission may be an important means of viral transmission. This mode of transmission may be related not only to reproductive failure, but also to the development of multisystemic disease later in life.

III. Objectives: The purpose of this retrospective study was twofold: to determine if previously undetected PCV2 (and PCV1) has been vertically transmitted in pork producing areas where PMWS, and by extension PCV2 infection, has been endemic for at least several years, and to determine if PCV2 could be implicated as a pathogen in diagnosed and undiagnosed cases of reproductive failure.

IV. Procedures:

Case selection

Thirty eight submissions involving reproductive failure, in which archival fixed tissues from piglets were available were received in the diagnostic laboratory at the Western College of Veterinary Medicine (WCVM) between March 1995 and March 1999 from a total of 30 high health herds in Alberta and Saskatchewan. Five of these farms had diagnosed cases of PMWS. Twenty-seven of the thirty-eight submissions (71%) were classified as abortions; five of these (13%) also involved at least one mummified fetus. Of the remaining 10 cases: 5 involved stillborn piglets along with nonviable piglets (13%); 2 with stillborn and one or more mummified feti (5%); 2 with only stillborn piglets (5%); and one with only mummified feti (2.5%). Routine diagnostics for pathogens other than circovirus revealed 4 cases (11%) in which the etiology was determined to be porcine parvovirus and 2 cases (5%) in which the etiology was determined to be of bacterial origin. The duty pathologist at the WCVM or submitting clinical veterinarians performed gross necropsies. In the former cases, tissues were collected and fixed in buffered formalin (fixation time 24-72 hrs) and, in most cases, fresh tissues were also submitted for routine microbiological evaluation. In the latter cases, tissues may have remained in fixative for up to 1 week. None of the cases included in this study had been previously tested for PCV2.

Types of tissues tested

Individual blocks from each case containing particular tissue combinations were chosen based on the presence of lesions as indicated by the pathologist's report at the time of original examination. A range of organ tissues was tested by PCR in cases in which no histological lesions were identified. The tissue type in each block was identified based on the histology slides of each case. Of the tissues tested lung was present in 30 of the 38 submissions (79%) followed by; liver (63%), kidney (50%), thymus (26%), heart (21%), spleen (18%), placenta (18%), intestine (11%), brain (11%), and skeletal muscle (5%).

Polymerase chain reaction for PCV

The PCR technique used for the detection of PCV1 and PCV2 was performed as previously described (1). Briefly, primers were designed that allowed the amplification of a PCV1-specific, 347 bp fragment; PCV1-For, (5'- GCGCCATCTGTAACGGTTTC-3') and PCV1-Rev, (5'-TCCAAACCTTCCTCTCCGC-3'). As well as a PCV2-specific, 481 bp fragment; PCV2-1443, (5'-CGGATATTGTAGTCCTGGTCG-3') and PCV2-150, (5'-ACTGTCAAGGCTACCACAGTCA-3'). DNA was extracted from 10, 10 micron sections of paraffin-embedded tissue using the QIA Amp Tissue Kit according to the manufacturers instructions (Qiagen Inc., Mississauga, Ontario, Canada). The reaction mixture contained 200 μM dNTPs (Gibco-BRL, Burlington, Ontario, Canada), 1.5mM $MgCl_2$ (Gibco-BRL, Burlington, Ontario, Canada), 50 pmoles of each primer, 1x Taq buffer (Gibco-BRL, Burlington, Ontario, Canada), 1.25 units Taq polymerase (Gibco-BRL, Burlington, Ontario, Canada), and 100 ng of sample DNA in a final volume of 50 μl. Reaction conditions were 94°C for 1 min. (1 cycle), followed by 35 cycles of 94°C 1 min, 55°C 1 min, and 72°C 1min, and a final cycle at 72°C for 10 min. The identity of the amplified fragments was determined by DNA sequencing.

Effect of formalin fixation on PCR sensitivity

The effect of formalin fixation on the sensitivity of detection of PCV by PCR was determined by using fresh frozen tissues from four weanling piglets with a confirmed diagnosis of PCV2 infection by immunohistochemistry. Samples were taken from a variety of tissues (lung, liver, kidney, bronchial lymph node) and fixed in 10% buffered formalin for either 5 hours, 1 day, 2 days, 3days, or 7 days. PCR was then performed to determine if band intensity was dependent on the fixation time of each tissue.

Immunohistochemistry (IHC)

Immunohistochemical identification of PCV2 in tissues was performed as previously described (1). Briefly, sections cut from blocks of embedded tissue were reacted with either rabbit anti-PCV antiserum, porcine immune serum, or a monoclonal antibody specific for PCV2. Following reaction with the primary antibody, tissues were incubated with appropriate secondary antisera before visualizing the reaction product using an avidin-biotin complex technique as previously described (1). Negative controls included serial sections of each block stained with the omission of primary antisera and with the substitution of primary antisera with irrelevant polyclonal antisera from the appropriate species. Positive control tissue from a pig with naturally acquired PMWS was also stained.

Virus isolation

Virus isolation of PCV2 from fresh frozen tissues was performed as previously described (1). Briefly, tissues were homogenized, sonicated and clarified by centrifugation. The remaining supernatant was extracted with freon, reclarified, and the aqueous layer inoculated onto a glucosamine treated PCV-free pig kidney cell line (PK-15, Dr. A, Afshar, Animal Diseases Research Institute, Nepean, Ontario). Cell cultures were then stained immunohistochemically using an avidin-biotin immunoperoxidase technique as previously described.

V. Results: PCV1 was not detected by PCR in any submissions comprising reproductive failure from 1995-1998. PCV2 was detected by PCR in three different submissions, two of which originated from the same multi-site pork production unit in Alberta on two separate occasions in the spring of 1999. . The first submission originated in Saskatchewan and 1 aborted piglet had severe cardiac and hepatic lesions associated with PCV2 antigen and DNA. No other abortogenic agents were implicated in the reproductive failure in this herd. The second of these submissions comprised a litter of piglets with gross evidence of myocarditis, cardiac hypertrophy, and chronic passive congestion. Only formalin fixed paraffin embedded cardiac tissue was submitted. Immunohistochemical staining for PCV2 was positive in hearts from all six of the submitted piglets that were submitted, while 4 of 6 were positive by PCV2 PCR .

The third submission was from the same farm as the second and consisted of a litter of four piglets in which 2 were stillborn and 2 others died shortly after birth. All four piglets also had gross evidence of a severe, diffuse myocarditis, cardiac hypertrophy, and chronic passive congestion. Only fresh frozen heart, and pooled lung/spleen tissues were submitted for analysis. PCV2 PCR was positive in the hearts of 2 of 4 piglets and in the pooled lung and splenic tissues of 4 of 4 piglets. Isolation of PCV2 from affected hearts and/or pooled lung and splenic tissue was positive in 2 of the 4 cases that were PCV2 positive by PCR. Based on serology and/or PCR, other agents associated with reproductive failure in swine, including porcine reproductive and respiratory syndrome virus and porcine parvovirus were apparently circulating in the breeding herd. However, these agents could not be shown to be associated with the severe cardiac (or other) lesions in the affected piglets.

PCV2 was not detected by PCR or IHC in any representative cases of reproductive failure submitted during 1995-1998. In order to rule out damage to DNA due to formalin fixation as a possibly adverse factor on the ability to detect PCV2 by PCR, PCR was performed on tissues collected from four weanling piglets with PMWS. PCV2 DNA was amplified in all fixed tissues tested, including; lung, liver, kidney and bronchial lymph node, from all four individuals. Moreover, the sensitivity of the PCR PCV2 was independent of the length of time that each tissue was fixed in formalin. Lengths of fixation of up to one week in 10% buffered formalin did not grossly affect the intensity of the PCV2 product obtained through PCR.

Genotypic and antigenic analyses of PCV2 isolates from aborted fetuses is currently underway.

VI. Importance of findings: This study for the first time demonstrated that PCV2 can be vertically transmitted and can be directly associated with severe multisystemic fetal disease and reproductive failure in naturally infected sows. These findings further indicate that PCV2 should be considered as a cause of abortion and reproductive failure in swine. The role of vertical transmission in the pathogenesis of wasting disease and in the overall epidemiology of PCV2 infection in the field remains to be determined.

VII. Publications resulting from, or reporting results of, this study:

1. West KH, et al. Myocarditis and abortion associated with intrauterine infection of sows with porcine circovirus –2. J Vet Diag Invest 1999; 11: 530-532.
2. Allan GM and Ellis JA. Porcine circoviruses: a review. J Vet Diagn Invest 2000; 12: 3-14.
3. Bogdan J, et al. Detection of porcine circovirus 2 in cases of reproductive failure in swine: a retrospective study 1995-1999. Can Vet J, submitted.