RESEARCH REPORT
SWINE HEALTH

**Title:** Effects of high and low virulent PCV2 on activated PBMC populations. – **NPB #07-138**

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**Industry Summary:**
Porcine circovirus type 2 (PCV2) associated diseases (PCVAD) have been an important cause of mortality and economic loss to the swine industry. The importance of virulence between different virus genotypes known as PCV2a and PCV2b is poorly characterized. The purpose of this study was to compare *in vitro,* differences in the virus replication rate and induction of lymphocyte death due to apoptosis caused PCV2a and PCV2b *in vitro.* Previous work in our laboratory demonstrated that stimulated lymphocytes had increased PCV2 viral replication and apoptosis compared to non-stimulated cells. This study investigated the differences in these parameters in cells infected with either PCV2a or PCV2b. We found that infection with PCV2b, reported to be more virulent, resulted in increased replication and apoptosis early following infection of cells at 24 hours post infection (HPI), independent of stimulation, compared to PCV2a. Depending on the mitogens used, the increased viral replication of the PCV2b isolate varied compared to the PCV2a virus. However, by 120 HPI, the replication rate of the PCV2a isolate was greater than the rate of the PCV2b in all cases. These results suggest that the PCV2b virus replicates quickly in stimulated lymphocytes which may make control by the pig's immune system less effective in controlling PCV2b virus levels compared to PCV2a. An increased level of cell death or apoptosis of lymphocytes infected with PCV2b was observed at 72 HPI in PCV2b cells stimulated with concanavalin A (ConA), while pokeweed mitogen (PWM) increased PCV2b apoptosis at 120 HPI. PCV2b may have been due to a reduced number of susceptible cells due to In addition, we demonstrated that there was an increased rate of cell death in CD8+ cytotoxic T lymphocytes compared to the other populations. These findings are significant as this is the population of cells that recognizes and destroys virally infected cells and may be a mechanism by which the virus for persists in the pig.Reduced viral replication observed at the later time periods may have been due to fewer cells due to apoptosis. The differences in rate of viral replication and cell death in infected lymphoid cells may provide a potential explanation for the increased disease associated with PCV2b infection compared the level of disease observed with PCV2a. The findings of this study suggest that the type of stimulation to which the lymphoid cells are exposed as well as the genotype of the virus may be important in determining the disease severity by individual animal. Further work needs to be performed to confirm these results *in vivo*.
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**Scientific Abstract:**

The objective of this study was to investigate the potential differences in viral replication and apoptosis induced by two porcine circovirus type 2 (PCV2) isolates of different genotype and potential virulence. The PCV2 replication rate was determined using real-time polymerase chain reaction and reverse transcription polymerase chain reaction (RT-PCR) assays to detect viral DNA levels and a viral replication product, spliced Cap mRNA, respectively. The apoptotic index was measured using flow cytometric analysis which allowed the determination of the relationship between viral replication and lymphoid apoptosis based on lymphocyte subset. The studies were conducted *in vitro* and concanavalin A (ConA) or pokeweed mitogen (PWM) were used to stimulate peripheral blood mononuclear cells (PBMCs). We found that the high virulent PCV2b isolate, KSDVL 06-06274, replicated at a significantly greater rate, and had a higher apoptotic rate of T lymphocytes stimulated with mitogens ($P<0.05$) at 24 and 72 hours post infection (HPI) in PWM stimulated cells compared to the low virulent PCV2a isolate, 4838. Viral replication in the ConA stimulated cells was increased at the first time point, but no differences were observed at 72 or 120 HPI. By 5 days post infection, the PCV2a isolate, 4838, had a higher replication rate than the high virulent isolate with PWM stimulation. Increased apoptosis was observed at 72 HPI with the PCV2b isolate in ConA stimulated cells, while PWM stimulation resulted in increased apoptosis at 120 HPI. Different subpopulations of T lymphocytes exhibited different apoptotic rates, with CD8+ cells showing significantly greater apoptosis rate than any other population following stimulation with ConA at 72 HPI and at all times with PWM stimlation and infection with PCV2b. These results suggest that viral replication rate and apoptosis in activated immune cells may play a role in the virulence of PCV2 isolates and therefore lymphoid depletion and PCVAD. In addition, the induced apoptosis of CD8+ T cells may play a role in the ability of PCV2 to persist in the pig.

**Introduction**

Porcine Circovirus type 2 (PCV2), a member of the *Circoviridae* family, has been linked to a number of disease syndromes known as PCV2 associated disease (PCVAD), which includes postweaning multisystemic wasting syndrome (PMWS), porcine dermatitis and nephropathy syndrome (PDNS), porcine respiratory disease complex (PRDC), enteritis, reproductive failure, and congenital tremors type AII. Pigs affected by PCVAD are typically characterized by progressive weight loss, generalized enlargement of lymph nodes, dyspnea, and in some cases, pallor, jaundice and diarrhea. The primary pathological changes associated with PCV2 infection are lymphocyte depletion and granulomatous changes in lymphoid tissues.

Recently, it was demonstrated that different PCV2 isolates with high genomic nucleotide sequence homology differ significantly in their serum antibody profiles, level of PCV2 viremia, virus levels in lymphoid tissues and the severity of PCV2-associated macroscopic and microscopic lesions using an experimental *in vivo* model (Opriessnig et al. 2006). These viruses have been labeled PCV2a and PCV2b depending on their genetic make up. Recent PCVAD outbreaks in field situations have suggested that viruses characterized as PCV2b have been associated with an increased incidence of disease. This suggests that minor alterations in genomic sequences in PCV2 isolates play a role in the pathogenicity of PCV2 isolates.

Recent research reported that stimulation of the immune system increases PCV2 replication *in vitro* (Yu et al. 2007). In earlier studies, we demonstrated that *in vitro* the various populations of peripheral blood mononuclear cells (PBMCs) infected with PCV2 and stimulated with the mitogen, Concanavalin A (ConA), had significantly increased virus replication compared to non-stimulated cells (Yu et al. 2007). We also demonstrated that infection with PCV2-40895, a PCV2a virus, did not impact the ability of PBMCs to proliferate in response to either ConA or pokeweed mitogen (PWM) stimulation. In addition, we found an increase in apoptosis of PBMCs in the presence of PWM, but not ConA (Yu et al. unpublished data). However, similar studies on the replication ability of a PCV2b isolate and its effects on the immune cells had not been determined. In this study, we hypothesized that PCV2 isolates vary in virulence due to differences in replication rates in activated immune cells altering the level of apoptosis and therefore lymphoid depletion.

We tested the hypothesis by measuring virus replication and apoptosis frequency in PBMCs infected with either PCV2a or a PCV2b in the presence or absence of the mitogens, ConA or PWM *in vitro*. ConA and PWM have

been reported to stimulate different subpopulations of T lymphocytes. They both stimulate CD4+CD8+, CD4-CD8+ alpha (hi), however, only ConA stimulates CD4-CD8+ alpha (lo) and gamma delta TCR+ cells (Dorn et al. 2002).
A real-time polymerase chain reaction assay was used to assess PCV2 DNA levels. A real-time reverse transcription polymerase chain reaction (RT-PCR) assay was used to detect spliced Cap mRNA of PCV2 as a measure of PCV2 replication. These assays allowed us to quantify viral replication. Additionally, the apoptotic index was assessed by flow cytometric analysis, which enabled us to assess cell populations impacted by the viruses. We found that PCV2b isolate, KSDVL 06-06274, caused significantly higher replication rate than PCV2a isolate, 4838, during the initial stages of infection period. As a result, a higher apoptotic rate of T lymphocytes that corresponded to viral replication was observed when PBMCs were stimulated with mitogens. However, later, the PCV2a isolate had a higher replication rate than PCV2b, KSDVL 06-06274. This study also found that different subpopulations of T lymphocytes had different apoptotic rates upon the stimulation of mitogens after infection with the two PCV2 isolates.

**Objectives:**

1. Measure and correlate PCV2 replication and PBMC proliferation in response to mitogen stimulation in cells infected with the two PCV2 isolates. This will enable us to determine if the two PCV2 isolates vary in their ability to replicate or impact PBMC proliferation as a role in PCVAD.

2. Evaluate cell apoptosis in PBMCs infected with each of the two PCV2 isolates in response to stimulation with the different mitogen and determine the correlation between virulence of PCV2 and the rate of apoptosis. This will provide further information on the impact of

**Materials and Methods:**

In this study, two isolates of PCV2 were used. The strains used in this study were PCV2 isolate 4838 (GenBank Accession No. DQ397521) a PCV2a isolate and PCV2 isolate, KSDVL 0606274, a PCV2b isolate. The isolate, PCV2-4838, was recovered from a 45 kg pig that died from respiratory disease in 2003 on an Iowa farm (Opriessnig, 2006) (1-5) and is considered of low pathogenicity and has been characterized as a PCV2a isolate. Isolate PCV2-KSDVL 06-07724, was recovered in Kansas in 2005 and is considered a highly pathogen PCV2b isolate.
Blood samples were collected in sodium heparin collection tubes from four healthy and PCV2-free crossbred pigs and the PBMCs were separated by density gradient centrifugation (Yu, 2007). After isolation, cells were suspended in complete RPMI 1640 medium. Isolated PBMCs ($4\times10^5$ cells/sample) were infected with either PCV2a isolate, PCV2-48095, or PCV2b isolate, PCV2-4838, at a multiplicity of infection (MOI) of approximately 0.1, or mock infected with media (harvested from uninfected PK15 cell lysate supernatant) for 1 h followed by washing with phosphate-buffered saline (PBS) and resuspended in complete medium. PBMCs were incubated at 37 C with either ConA at 5 μg/ml (Sigma, St. Louis, MO), PWM stimulated at 10 μg/ml (Sigma, St. Louis, MO), or media as controls. All treatments were performed in triplicate.
The real-time PCR assay was performed on cells from each well that were harvested at 1, 24, 72 and 120 h post-infection (HPI). Cells at each time point were counted and viable cells suspended in 200 μL PBS for DNA isolation, with an additional 1 X 105 cells placed in 100 μL RNAlater for total RNA isolation. The apoptosis index assay was performed on cells harvested at 72 and 120 HPI. Cells collected at each time point were washed three times with PBS to remove free PCV2. The cells were counted after the addition of a non-enzymatic cell dissociation solution and cell viability was determined using trypan blue exclusion. Harvested cells were labeled with CD3, CD21, or CD4 and CD8 to identify different cell sub-populations.
Viral DNA was extracted from each cell sample using the QIAamp DNA Mini kit according to the manufacture's instruction. The DNA extracts were kept at -20 C until analyzed. Total RNA was extracted using the RNAqueous kit according to the manufacture's instruction. The RNA extracts were stored at -80 C until assayed.

The Cap mRNA reverse transcriptase (RT) real-time PCR assay was performed as previously described (Yu et al., 2007)(7). A RNA standard was prepared as described in the previous study (Yu et al., 2007)(7). All standard dilutions and unknown samples were tested in duplicate. Quantification of PCV2 Cap mRNA were assessed by comparing the threshold cycle (CT) value of the input sample RNA with the CT value of different dilutions of the standard RNA. Standard curves were accepted when the coefficients of correlation (R2) were >0.99.
Each assay was performed at least twice. To evaluate intra-assay variability and the inter-assay reproducibility, 10-fold serial dilutions of the Cap mRNA standard were tested in triplicate in three separate experiments.
The PCV2 real-time assay was performed as previously described (Opriessnig, et al., 2003)(6) with some modifications. Viral DNA was quantified by comparing the CT value of the input sample DNA with the CT value of the standard template DNA. To minimize in-assay variability, all unknown samples and different dilutions of PCV2 plasmid DNA were assessed twice as described above.
Apoptosis was measured using Annexin V and PI staining in addition to stain cell surface markers described above using three-color flow cytometry. The AN-/PI- and AN+/PI- populations correspond to live cells and early apoptotic cells (Takahashi et al., 2001). The percentage of apoptotic cell populations were calculated as follows: (% of AN+ cells)/ ((% of AN+ cells) + (% of AN- cells)) *100% when cells were gated by PI negative staining.
Statistical analysis was calculated for all groups to assess the overall quality of the data. Analysis of variance (ANOVA) was used to analyze the data. P values less than 0.05 were considered to be statistically significant for all test procedures. To obtain and normalize the copy numbers of PCV2 DNA and viral cap mRNA, non-parametric correlations test and Spearman's Rho in Multivariate Methods were applied using JMP 5.1 (SAS Institute Inc., Cary, NC).

**Results:**
The high pathogenic PCV2b isolate had a significantly higher rate of replication in the ConA and PWM stimulated PBMCs at 24 HPI. But there were no differences between the two PCV2 isolates in replication in cells stimulated with ConA at 72 and 120 HPI. The PBMCs stimulated with PWM and infected with PCV2b maintained a higher rate of replication at 72 HPI compared to the PCV2a virus. In contrast to the ConA stimulated cells, the cells infected with the highly pathogenic virus had reduced replication at 120 HPI.
The highly pathogenic PCV2b isolate had a higher rate of apoptosis in the ConA stimulated CD3+ cells at 72 HPI; however by 120 HPI, there was no difference in the apoptotic rate between the isolates in cells stimulated with ConA. In the PWM stimulated PBMCs, no difference in apoptosis induced by PCV2 was observed at 72 HPI. However, by 120 HPI, the PCV2b isolate had a higher rate of apoptosis. No difference in any of the other cell populations undergoing apoptosis was observed except for CD4-CD8+ cells which had a higher rate of apoptosis at 72 HPI in the ConA stimulated cells. In contrast, the PWM stimulated CD4-CD8+ cells had a higher rate of apoptosis at both time intervals.

**Conclusions:**
The results of this study further confirm our earlier findings that stimulated cells enhance viral replication and apoptosis in lymphoid cells. However, this effect is dependent on the mitogen used for stimulation, suggesting that viral replication and apoptosis may be impacted by the type of lyphocytes stimulated during infection. Based on the results of this *in vitro* study, it appears that the PCV2b isolate used in this study replicated at a higher rate in the initial stages of infection, independent of the mitogen used for stimulation; however, over time, the replication rate decreased. Stimulated cells also tended to show increased apoptosis rates, again the level and timing depended on the mitogen used to stimulate the cells. The impact of PCV2 infection on lymphocytes has been demonstrated in earlier studies. Differences between pigs and in the stimulation of cells may be important in determining whether pigs remain asymptomatically infected or progress to clinical PCVAD. More research needs to be performed to assess differences between isolates in their ability to induce disease, replicate virus and induce apoptosis in infected cells *in vivo* to determine what role this may play in the pathogenesis of PCVAD The differences in pathogenicity of the two isolates used in this study were based on their genetic make up, thus their labels of high and low pathogenicity was not confirmed by us in pigs.

Ultimately, the differences between viral replication and cell apoptosis induced by these two isolates and their role in inducing PCVAD needs to be compared and confirmed in *in vivo* studies in pigs.

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