Singular PCV2a or PCV2b infection results in apoptosis of hepatocytes in clinically affected gnotobiotic pigs

Avanti Sinha, Shayleen Schalk, Kelly M. Lager, Chong Wang, Tanja Opriessnig

A R T I C L E   I N F O

Article history:
Received 7 August 2010
Accepted 14 October 2010

Keywords:
Apoptosis
Cleaved caspase-3
Gnotobiotes
Porcine circovirus type 2
Terminal transferase dUTP nick end labeling assay

A B S T R A C T

Porcine circovirus type 2 (PCV2) is clinically associated with respiratory disease, failure-to-thrive, hepatitis, and diarrhea; however, the precise pathogenesis of PCV2-associated disease and in particular its involvement in apoptosis is still controversial. The objectives of this study were (1) to determine whether PCV2 is associated with apoptosis by examining and comparing hepatic tissues from clinically affected or unaffected gnotobiotic pigs that were experimentally infected with PCV2, (2) to determine if there are differences between PCV2a and PCV2b in inducing hepatocyte apoptosis, and (3) to determine if there are differences between apoptosis detection systems. Forty-eight gnotobiotic pigs were separated into five groups based on inoculation status and development of clinical disease: (1) sham-inoculated, clinically-unaffected (\( n = 4 \)), (2) inoculated with PCV2a, clinically-unaffected (\( n = 10 \)), (3) inoculated with PCV2a, clinically-affected (\( n = 6 \)), (4) inoculated with PCV2b, clinically-affected, (\( n = 13 \) and (5) inoculated with PCV2b, clinically-affected (\( n = 15 \)). Formalin-fixed, paraffin-embedded sections of liver from all pigs were analyzed for signs of apoptosis (presence of single strand DNA breaks in the nucleus by the terminal transferase dUTP nick end labeling (TUNEL) assay or presence of intra-nuclear cleaved caspase 3 (CCasp3) demonstrated by CCasp3 immunohistochemistry (IHC)). In addition, the liver tissues were also tested for presence of cytoplasmic and intra-nuclear PCV2 antigen by an IHC assay. Specific CCasp3 and TUNEL labeling was detected in the nucleus of hepatocytes in PCV2a and PCV2b infected pigs with significantly (\( P < 0.05 \)) higher levels of apoptotic cells in clinically-affected pigs. Regardless of PCV2 subtype (PCV2a; PCV2b), there were higher levels of PCV2 antigen in clinically-affected pigs compared to clinically-unaffected pigs. There was no significant difference in detection rate of apoptotic cells between the TUNEL assay and CCasp3 IHC. When high amounts of PCV2 antigen were present, the incidence of CCasp3 and TUNEL staining also increased regardless of the PCV2 genotype. This suggests that PCV2-induced apoptosis of hepatocytes is important in the pathogenesis of PCV2-associated lesions and disease.

1. Introduction

Porcine circovirus (PCV) is a small, non-enveloped, circular, single stranded DNA virus. There are two main types of PCV defined as PCV1 and PCV2 (Meehan et al., 1998). Phylogenetic analysis of PCV2 isolates has shown that PCV2 can be further divided into two main genotypes, PCV2a and PCV2b, which are both distributed worldwide (Gagnon et al., 2007; Olvera et al., 2007).

Initially, PCV2 was identified in cases of post-weaning multisystemic wasting syndrome (PMWS) in Canada in 1991 (Ellis et al., 1998; Harding and Clark, 1997). PCV2 is associated with several disease manifestations in pigs commonly summarized as porcine circovirus associated disease (PCVAD) (Opriessnig et al., 2007). PCVAD includes systemic infection in nursery age pigs commonly characterized by wasting and general failure to thrive (Chae, 2004), hepatic disease (Rosell et al., 2000a), respiratory disease (Harms et al., 2002), enteric disease (Kim et al., 2004), reproductive failure (Ladekjær-Mikkelsen et al., 2001; O’Connor et al., 2001) and porcine dermatitis and nephropathy syndrome (PDNS) (Rosell et al., 2000b). PCVAD has since emerged globally (Segalés et al., 2005) and now has become one of the most economically important diseases throughout pig producing countries (Opriessnig et al., 2007).

Currently there are contradicting reports about whether or not PCV2 plays a vital role in inducing apoptosis in various pig tissues. Examples of viruses that induce apoptosis include: adenovirus, human immunodeficiency virus (HIV-1), porcine reproductive and respiratory syndrome virus (PRRSV), influenza virus A/B, reovirus,
classical swine fever and many others (Roulston et al., 1999). Microscopically, apoptosis can be demonstrated by dUTP labeling of single strand DNA breaks in the nucleus (Krakowka et al., 2004) or by demonstrating the presence of cleaved caspase 3 (CCasp3) by immunohistochemistry (IHC) staining (Resendes et al., 2004a,b). A novel open reading frame (ORF3) was identified in PCV2 infected PK15 cells in 2005. It was discovered that the ORF3 protein is not essential for viral replication but is involved in activating caspase-8 and caspase-3 pathways which in turn induce apoptosis (Liu et al., 2005).

A study using naturally infected conventional pigs found that within lymphoid tissues, apoptosis of lymphocytes had occurred, which was determined by the use of the terminal transferase dUTP nick end labeling (TUNEL) assay (Shibahara et al., 2000). Within the same study PCV2 was stained via IHC and it was found that PCV2 was present in the nuclei of macrophages and apoptotic lymphocytes, as well as apoptotic bodies phagocytosed by macrophages, providing evidence that PCV2 was the cause of B lymphocyte depletion (Shibahara et al., 2000). Similarly in BALB/c mice experimentally inoculated with PCV2, it was found that PCV2 replicated in the spleen, lymph nodes and Peyer’s patches of infected mice and that the replication of PCV2 within these tissues was associated with apoptosis which was confirmed by the TUNEL assay (Kluep et al., 2005). It was also noted that upregulation of caspase 8 and 3 in the spleens of infected mice might be the mechanism through which PCV2 induces apoptosis in mice (Kluep et al., 2005).

In contrast, a recent study using pigs naturally affected by PCVAD noted that there was an inverse relationship between amounts of PCV2 antigen and apoptosis in the thymus and most peripheral lymphoid tissues (Resendes et al., 2004b). It was also found that pigs with severe lymphoid depletion had less apoptosis (Resendes et al., 2004b). In Italy, pigs with varying clinical presentations of PMWS were classified into three classes based on the severity of the disease presentation (Mandrioli et al., 2004). Apoptosis in lymphoid tissues was analyzed and there was a significant decrease in apoptosis in lymphoid tissues when control pigs were compared to pigs at the initial stage of infection; however, no significant differences were found in pigs in the intermediate and final stages of the disease (Mandrioli et al., 2004).

PCV2 has been found to replicate in a number of different tissues and the induction of severe immunosuppression using cyclosporine in PCV2 infected gnotobiotic piglets increased the replication of PCV2 within hepatocytes (Krakowka et al., 2002). Livers from 100 pigs with naturally occurring PMWS were examined for lymphohistiocytic hepatitis, and apoptotic bodies; of these 100 livers 88% were found to have apoptosis and 12% had no apoptosis (Resendes et al., 2004). Similarly another study using 15 pigs from five different farms with previous confirmation of PMWS found PCV2 antigen and DNA in hepatocytes of the livers from all pigs that were PCV2 positive (Rosell et al., 2000a). Similarly another study using 15 pigs from five different farms with previous confirmation of PMWS found PCV2 antigen and DNA in hepatocytes of the livers from all pigs that were PCV2 positive (Rosell et al., 1999).

The objectives of this study were to determine (1) whether PCV2 is associated with apoptosis of hepatocytes by comparing clinically affected or unaffacted gnotobiotic pigs that were experimentally infected with PCV2, (2) if there are differences between PCV2a and PCV2b in inducing hepatocyte apoptosis, and (3) if there are differences between apoptosis detection systems.

2. Materials and methods

2.1. Pig source and housing

Forty-eight gnotobiotic piglets acquired surgically from crossbred sows free of PCV2 were used. Directly after surgical removal pigs were placed into sterile stainless steel isolators which were mounted with polyvinyl canopies and maintained under positive pressure. The isolator units were cleaned with sodium hypochlorite and water before sterilization with ethylene oxide prior to the start of the experimental study. Isolators were sustained at a temperature of 32 °C and the temperature was progressively decreased to a subsistence temperature of 27 °C with HEPA filtration of the intake and exhaust air. The pigs were initially fed a pasteurized milk diet from a commercially available source (Esbilac™ Kansas City, Kansas, USA) three times per day, with increasing quantities and a reduction to feeding twice a day to a maximum of 370 ml per feeding. All animal manipulations were approved by the Institutional Animal Care and Use Committee (IACUC) according to the guidelines set by the National Animal Disease Center. The pigs were tested on a weekly basis and found to be negative for aerobic and anaerobic bacteria from rectal swabs by routine culture methods. In addition, all pigs tested negative for porcine parvovirus (PPV), PRRSV, swine hepatitis E virus, and bovine viral diarrhea virus (BVDV) as determined by routine PCRs on serum collected on the day of necropsy.

2.2. Inoculation

The pigs were inoculated intranasally between seven and 10 days of age. Control pigs (n = 4) were inoculated with 1 ml PCV2-free cell culture medium. The remaining pigs were either inoculated with 1 ml of PCV2a strain DQ629114 (Cheung et al., 2007) at a dose of 10^2–10^4 TCID50/ml (PCV2a, n = 16) or with 1 ml of PCV2b strain DQ629115 (Cheung et al., 2007) at a dose of 10^2–10^4 TCID50/ml (PCV2b, n = 28). The infectious dose of the inocula was determined by IHC as previously described (Cheung, 2003).

2.3. Study design

For the purpose of this study, the pigs were separated based on inoculation status and development of clinical disease resulting in five groups: (1) Sham-inoculated, clinically-unaffected (n = 4), (2) PCV2a, clinically-unaffected (n = 10), (3) PCV2a, clinically-affected (n = 6), (4) PCV2b, clinically-unaffected, (n = 13) and (5) PCV2b, clinically-affected (n = 15). Liver tissue from all 48 pigs was collected at the time of necropsy between 23 and 41 days post inoculation, evaluated for microscopic lesions, and scored for presence of apoptotic cells as determined by the TUNEL assay or CCasp3 IHC stain and for presence of PCV2 antigen by IHC.

2.4. Tissue preparation

At necropsy several sections of different liver lobes were collected and immediately immersed in 10% buffered formalin. After the tissues were fixed for 24 h, the tissues were dehydrated, paraffin-embedded, sectioned at 4 μm and mounted on a glass slide followed by routine hematoxylin/eosin staining as described (Krakowka et al., 2004).

2.5. Histopathology

Microscopic lesions were evaluated by a veterinary pathologist (TO) blinded to treatment groups. The sections of liver were evaluated for the presence of lymphohistiocytic inflammation (0 = none; 3 = severe), suppurrative hepatitis (0 = none; 3 = severe) and presence of hepatocyte degeneration (0 = none, 3 = diffuse, severe) and areas of necrosis (0 = none, 3 = diffuse, severe). The individual lesion scores were summed and the lesion sum scores were compared between groups.
2.6. Detection of PCV2 antigen

IHC for detection of PCV2-specific antigen was performed using a rabbit polyclonal antiserum as described previously (Sorden et al., 1999). PCV2-antigen scoring was done by a veterinary pathologist (TO) blinded to treatment groups. The following scoring protocol was used: 0 = no signal or no visible stain, 1 = staining was observed in individual inflammatory cells, 2 = staining was observed in moderate numbers of inflammatory cells and hepatocytes, 3 = abundant staining in inflammatory cells and hepatocytes. Although this system is subjective, the same veterinary pathologist scored treatment groups in an earlier study in which a high correlation was found with digital computer image analysis (Opriessnig et al., 2004).

2.7. TUNEL assay

In brief, liver tissue sections were dewaxed, rehydrated in graded alcohol, incubated in 3% hydrogen peroxide for 10 min at 22 °C and rinsed twice with phosphate buffered saline (PBS). This was followed by treatment with Proteinase K (Ambion, Inc., Foster City, USA) for 20 min at 30 °C. After two washing steps with PBS, the tissues were incubated in 10% sheep serum (Sigma, St. Louis, Missouri, USA) for 20 min at 22 °C and rinsed twice with the PBS. TUNEL staining was performed using a commercially available kit (in situ cell death detection kit, POD; Roche Diagnostics, Indianapolis, Indiana, USA). The manufacturers’ instructions were followed and DAB chromogen (Dako North America Inc., Carpentaria, California, USA) was applied for 5 min and then counterstained with hematoxylin.

2.8. Cleaved Caspase-3 (CCasp3) immunohistochemistry (IHC)

The tissue sections were dewaxed, rehydrated in graded alcohol and incubated in 3% hydrogen peroxide for 10 min at 22 °C, rinsed three times in Tris buffered saline (TBS) treated with 0.05% protease (Sigma Aldrich) for 2 min and washed three times with TBS buffer. A CCasp3 IHC (Cell Signaling Technology, Danvers, Massachusetts, USA) was applied at a concentration of 1:100 for 1 h at 22 °C. Further processing was done by using a streptavidin–biotin detection kit (Dako North America Inc.).

2.9. Apoptosis controls

Known negative and positive controls were included in each TUNEL and CCasp3 run. As a positive control, sections of murine intestines from a mouse experimentally inoculated with live mycobacterium paratuberculosis followed by dextran sulfate sodium treatment were used (Courtesy of Dr. C. Johnson). This procedure produced colitis in the mouse which resulted in epithelial cell apoptosis as described previously (Vetuschi et al., 2002).

2.10. Apoptotic cell scoring

Apoptotic cell counts were performed by scoring 10 random hepatic areas using a 25× lens and a 10× eyepiece, yielding a final magnification of 250× with an Olympus® microscope (Leeds Precision Instrument, Inc; North Minneapolis, Minnesota, USA). The apoptotic cell score ranged from 0 to 3 (0 = average of 0.0–0.9 apoptotic cells/10 fields, 1 = average of 1–4.9 apoptotic cells/10 fields; 2 = average of 5–14.9 apoptotic cells/10 fields; 3 = average of 15 or more apoptotic cells/10 fields).

2.11. Statistical analysis

The statistical analysis on the data was performed using the SAS software version 9.2, SAS system for windows (SAS Institute Inc., Cary, North Carolina, USA). A Kruskal–Wallis non-parametric analysis of variance (ANOVA) was used to determine differences in median scores between groups. A P value of 0.05 or less was considered statistically significant. If significant, pairwise Wilcoxon tests were conducted to determine which groups were different. An association between categorical variables in paired situations was evaluated by using Fisher’s exact test.

3. Results

3.1. Microscopic lesions

Microscopic lesions in liver sections were observed in 88.6% (39/44) of the experimentally inoculated pigs, no microscopic lesions were observed in sham-inoculated pigs (Fig. 1). Hepatic lesions were characterized by infiltration with low-to-high numbers of inflammatory cells and hepatocytes.
Different superscripts indicate significant differences compared to sham-inoculated pigs (Table 1). The association was not shown). Regardless of clinical disease status and genotype, spleen, inguinal lymph node, tracheobronchiolar lymph node (data not shown). Regardless of clinical disease status and genotype, PCV2 inoculated pigs had significantly (P = 0.003) more severe lesions compared to sham-inoculated pigs (Table 1). The association between disease status (clinically-affected, unaffected) and lesion sum score was significant (P = 0.003) and clinically-affected pigs had significantly (P = 0.001) more severe lesions compared to clinically-unaffected pigs. No significant (P = 0.228) difference of lesion score was observed between pigs inoculated with PCV2a or PCV2b (Table 1).

3.2. PCV2 antigen in liver

Low to high amounts of PCV2 antigen was detected in 24/48 pigs (Table 1; Fig. 1). There were significantly (P < 0.001) higher amounts of PCV2 antigen in clinically-affected pigs compared to clinically-unaffected pigs; however, there was no significant (P = 0.402) difference between pigs inoculated with PCV2a and PCV2b (Table 1). PCV2 antigen was detected in the cytoplasm of macrophage-like cells in areas of inflammation but also in the cytoplasm and nuclei of hepatocytes.

of mixed inflammatory cells (mainly lymphocytes, macrophages, and a few neutrophils). In addition, there was mild-to-severe vascular degeneration and loss of hepatocytes in 58.3% (28/48) of the pigs (Fig. 1). Affected pigs also had severe lymphoid depletion and a few neutrophils. In addition, there was mild-to-severe vacuolar degeneration and loss of hepatocytes in 58.3% (28/48) of the pigs (Fig. 1). There were significantly (P < 0.001) higher CCasp3 IHC scores compared to clinically-unaffected pigs, P = 0.003) more severe lesions compared to sham-inoculated pigs (Table 1). The association between disease status (clinically-affected, unaffected) and lesion sum score was significant (P = 0.003) and clinically-affected pigs had significantly (P = 0.001) more severe lesions compared to clinically-unaffected pigs. No significant (P = 0.228) difference of lesion score was observed between pigs inoculated with PCV2a or PCV2b (Table 1).

3.3. Detection of apoptotic cells by the TUNEL assay

The distribution of the final TUNEL scores in the different treatment groups is summarized in Table 2 and representative images are shown in Fig. 1. Twelve of 48 (25.0%) pigs had no detectable CCasp3 staining in sections of liver (score 0). 39.6% (19/48) of the pigs were found to have low amounts of CCasp3 staining (score 1), 18.8% (9/48) had moderate amounts of CCasp3 staining (score 2), and 16.7% (8/48) of the pigs had large amounts of CCasp3 staining in sections of liver (score 3). Clinically-affected pigs had significantly (P < 0.001) higher CCasp3 IHC scores compared to clinically-unaffected pigs. No significant (P = 0.228) difference of lesion score was observed between pigs inoculated with PCV2a or PCV2b (Table 1).

3.4. Detection of apoptotic cells by CCasp3 immunohistochemistry

The distribution of the final scores in the different treatment groups is summarized in Table 2 and representative images are shown in Fig. 1. Twelve of 48 (25.0%) pigs had no detectable CCasp3 staining in sections of liver (score 0). 39.6% (19/48) of the pigs were found to have low amounts of CCasp3 staining (score 1), 18.8% (9/48) had moderate amounts of CCasp3 staining (score 2), and 16.7% (8/48) of the pigs had large amounts of CCasp3 staining in sections of liver (score 3). Clinically-affected pigs had significantly (P < 0.001) higher CCasp3 IHC scores compared to clinically-unaffected pigs. No significant (P = 0.228) difference of lesion score was observed between pigs inoculated with PCV2a or PCV2b (Table 1).

Table 1

<table>
<thead>
<tr>
<th>Clinical Status</th>
<th>Inoculation</th>
<th>PCV2 IHC</th>
<th>CCasp3</th>
<th>TUNEL</th>
<th>Microscopic lesions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-Affected</td>
<td>Sham (n = 4)</td>
<td>0 (0, 0)</td>
<td>0 (0, 0)</td>
<td>0 (0, 0)</td>
<td>0 (0, 0)</td>
</tr>
<tr>
<td></td>
<td>PCV2a (n = 10)</td>
<td>0 (0, 2)</td>
<td>1 (0, 2)</td>
<td>0.5 (0, 2)</td>
<td>3 (0, 4)</td>
</tr>
<tr>
<td></td>
<td>PCV2b (n = 13)</td>
<td>0 (0, 1)</td>
<td>0 (0, 1)</td>
<td>0 (0, 1)</td>
<td>1 (0, 4)</td>
</tr>
<tr>
<td>Affected</td>
<td>PCV2a (n = 6)</td>
<td>3 (0, 3)</td>
<td>2.5 (1, 3)</td>
<td>2 (0, 3)</td>
<td>4 (3, 8)</td>
</tr>
<tr>
<td></td>
<td>PCV2b (n = 15)</td>
<td>2 (0, 3)</td>
<td>2 (0, 3)</td>
<td>2 (0, 3)</td>
<td>3 (1, 5)</td>
</tr>
</tbody>
</table>

ABC Different superscripts indicate significant (P < 0.05) differences between group medians within the same column.

* A = negative, 1 = low number, 2 = moderate numbers, 3 = large numbers.

** 0 = average of 0.0–0.9 apoptotic cells/10 fields, 1 = average of 1–4.9 apoptotic cells/10 fields; 2 = average of 5–14.9 apoptotic cells/10 fields; 3 = average of 15 or more apoptotic cells/10 fields.

*** The lesions sum score for microscopic lesions on liver tissues ranged from 0 = no lesions to 12 = severe lesions.

Table 2

<table>
<thead>
<tr>
<th>Score range</th>
<th>PCV2 IHC</th>
<th>CCasp3</th>
<th>TUNEL</th>
<th>Microscopic lesions</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>4/4</td>
<td>9/16</td>
<td>10/28</td>
<td>1/16 7/28 4/4 6/16 12/28</td>
</tr>
<tr>
<td>1</td>
<td>0/4</td>
<td>1/16</td>
<td>5/28</td>
<td>0/4   9/16 10/28 0/4 5/16 6/28</td>
</tr>
<tr>
<td>2</td>
<td>0/4</td>
<td>2/16</td>
<td>6/28</td>
<td>0/4   3/16 6/28 0/4 3/16 7/28</td>
</tr>
<tr>
<td>3</td>
<td>0/4</td>
<td>4/16</td>
<td>7/28</td>
<td>0/4   3/16 5/28 0/4 2/16 3/28</td>
</tr>
</tbody>
</table>

* 0 = negative, 1 = low number, 2 = moderate numbers, 3 = large numbers.

** 0 = average of 0.0–0.9 apoptotic cells/10 fields, 1 = average of 1–4.9 apoptotic cells/10 fields; 2 = average of 5–14.9 apoptotic cells/10 fields; 3 = average of 15 or more apoptotic cells/10 fields.

### Mean PCV2 IHC, CCasp3, and TUNEL scores by liver tissue levels

<table>
<thead>
<tr>
<th>PCV2 IHC scores</th>
<th>CCasp3</th>
<th>TUNEL</th>
<th>Microscopic lesion scores</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (n = 23)</td>
<td>0.6 ± 0.1</td>
<td>0.3 ± 0.1</td>
<td>1.7 ± 0.4</td>
</tr>
<tr>
<td>1 (n = 6)</td>
<td>1.0 ± 0.3</td>
<td>0.5 ± 0.3</td>
<td>1.8 ± 0.5</td>
</tr>
<tr>
<td>2 (n = 8)</td>
<td>1.6 ± 0.2</td>
<td>1.4 ± 0.4</td>
<td>2.4 ± 0.4</td>
</tr>
<tr>
<td>3 (n = 11)</td>
<td>2.6 ± 0.2</td>
<td>2.3 ± 0.2</td>
<td>5.3 ± 0.7</td>
</tr>
</tbody>
</table>

* 0 = negative, 1 = low number, 2 = moderate numbers, 3 = large numbers.

** 0 = average of 0.0–0.9 apoptotic cells/10 fields, 1 = average of 1–4.9 apoptotic cells/10 fields; 2 = average of 5–14.9 apoptotic cells/10 fields; 3 = average of 15 or more apoptotic cells/10 fields.

*** The lesions sum score for microscopic lesions ranged from 0 = no lesions to 12 = severe lesions.
clinically-unaffected pigs. There was also a significant ($P = 0.03$) association between lesion sum scores and CCasp3 IHC scores. Although CCasp3 staining was significantly ($P = 0.023$) higher in clinically-unaffected pigs inoculated with PCV2a compared to clinically unaffected pigs inoculated with PCV2b and sham-inoculated pigs, a difference in CCasp3 staining was not observed between clinically-affected pigs inoculated with PCV2a or PCV2b (Table 1). However, there was a significant ($P < 0.001$) association of CCasp3 IHC scores and PCV2 IHC scores (Table 3). Comparison of apoptotic rates as determined by CCasp3 IHC at different DPIs demonstrated that there was no age dependent difference in the severity of apoptosis (data not shown).

4. Discussion

PCV2 infection in gnotobiotic pigs has been found to result in liver failure associated with icterus which becomes the imminent cause of death in pigs with this manifestation of PCVAD (Krakowka et al., 2004). The current study used experimentally PCV2-inoculated gnotobiotic pigs with and without signs of clinical disease and hepatic failure. The severity of microscopic lesions in hepatic tissue was significantly ($P < 0.05$) higher in the clinically-affected pigs when compared to the clinically-unaffected and sham-inoculated control pigs (Fig. 1) which is in agreement with previous studies in conventional pigs (Rosell et al., 2000a), further emphasizing the association of PCV2 with hepatic lesions and clinical disease.

The main goal of this study was to determine if there is an association between PCV2 and apoptosis of hepatocytes. We found that the numbers of apoptotic cells increased with increased lesion severity and increased amount of PCV2 antigen. The pigs used were essentially germ-free gnotobiotic pigs and the presence of other pathogens or factors can be ruled out. This is in contrast to field studies, where association of specific microscopic changes such as apoptosis with a specific pathogen is difficult as pigs in the field are commonly infected with more than one pathogen. Therefore, results obtained on field cases need to be viewed with caution. Studies conducted by several research groups have identified apoptosis by hybridization (ISH) provided evidence to associate PCV2 with apoptosis (Liu et al., 2005, 2006, 2007), is located within the numbers of apoptotic cells within PCV2a or PCV2b inoculated pigs. PCV2a and PCV2b differ in a small region in ORF2 (Cheung et al., 2007) encoding the capsid protein (Nawagitgul et al., 2000), whereas ORF3 which has been demonstrated to be associated with apoptosis (Liu et al., 2005, 2006, 2007), is located within ORF1 and is more conserved among PCV2 isolates, which may explain the results of this study.

A recent study compared the differences between the TUNEL and the CCasp3 IHC assays on lymphoid tissues of healthy conventional pigs of different ages (Resendes et al., 2004a); however, to the authors' knowledge no experimental studies have been performed to compare these assays in hepatic tissues. Our study concluded that both methods detected significantly ($P < 0.05$) higher numbers of apoptotic cells in clinically-affected pigs compared to clinically-unaffected pigs and sham-inoculated control pigs. Nevertheless, when comparing the TUNEL assay directly with the CCasp3 assay we found no differences in detection rates of apoptotic cells (Fig 1) indicating that the same type of cell was labeled by both techniques as described by others using a double staining technique (Mirkes et al., 2001). However, in order to further confirm this observation, staining of serial sections would be necessary.

5. Conclusion

In summary, higher amounts of PCV2 antigen were demonstrated to be associated with more severe microscopic lesions and increased apoptosis in the hepatic tissues from PCV2a or PCV2b singularly-infected, clinically-affected gnotobiotic pigs. No differences were observed between PCV2a- or PCV2b-infected pigs. The TUNEL assay and Caasp-3 IHC appear to be equivalent in their ability to detect apoptotic cells in liver tissues from PCV2-infected gnotobiotic pigs.

Acknowledgements

We thank Dr. HuiGang Shen for critical review of the manuscript.

References


lesions of pigs with postweaning multisystemic wasting syndrome. Canadian Veterinary Journal 39, 44–51.


