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Original article

A comparison of immunohistochemistry and *in situ* hybridization for the detection of porcine circovirus type 2 in pigs

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Abstract

The aim of this study was to develop and to optimize an immunohistochemistry (IHC) method for PCV2 identification and to compare it with an *in situ* hybridization (ISH) technique. The results demonstrated that both ISH and IHC successfully detected PCV2 viral antigens or nucleic acid in the examined tissues. Most of the slides identified previously in ISH as PCV2-positive were also positive in IHC. In the case of nearly half of the slides the results of IHC examination revealed an increase in the intensity of staining. IHC presented higher sensitivity and specificity than ISH. No negative impact of the time of paraffin block storage on ISH detection results was observed. In addition, IHC results were easier to interpret due to better image quality after staining. Overall results confirmed IHC was a reliable and useful technique for PMWS diagnosis.

Key words: immunohistochemistry, *in situ* hybridization, pigs, PCV2

Introduction

Porcine circovirus type 2 (PCV2) is a small, nonenveloped, single-stranded DNA virus with a circular genome (Tischer et al. 1982) and it is classified in the family *Circoviridae*. It is now considered one of the most important virus pathogens of swine (Opriessnig et al. 2007). PCV2 is an etiological agent of postweaning multisystemic wasting syndrome (PMWS) (Allan et al. 1998). The disease is characterized mainly by growth retardation in weaned pigs, skin pallor, dyspnea and occasionally jaundice (Allan and Ellis 2000). The virus is also involved in several clinical conditions, known as “porcine circovirus-associated disease” (PCVD, PCVAD), including PCV2-asso-

ciated pneumonia, PCV2-associated enteritis, PCV2-associated reproductive failure and PCV2-associated porcine dermatitis and nephropathy syndrome (Opriessnig et al. 2007, Gillespie et al. 2009). Diagnosis of PCVD, and PMWS in particular, is still a controversial issue, because PCV2 is a ubiquitous agent and its presence in the development of the disease is essential, but not sufficient (Allan and Ellis 2000). For this reason, to confirm PMWS the following criteria must be fulfilled: 1. finding characteristic symptoms of the disease (wasting, weight loss, respiratory disorders); 2. presence of the hallmark PCV2-associated microscopic lesions; 3. detection of PCV2 antigen or nucleic acid associated with the microscopic lesions by immunohistochemistry (IHC)

or *in situ* hybridization (ISH), respectively (Sorden 2000). Laboratory methods commonly used for the detection of viral infections, such as PCR or ELISA, are of limited value regarding PCVD diagnosis. The aim of this study was to develop and to optimize the IHC method for PCV2 identification and to compare it with the ISH method.

Materials and Methods

Tissue samples

The study was conducted on formalin-fixed, paraffin embedded samples of internal organs that had been collected from 2008 to 2010 from wasted swine suspected of PMWS. The samples were submitted for PMWS diagnosis – either already formalin-fixed, or collected from carcasses during necropsy in our laboratory. The fixation was performed as soon as possible and never later than 48 hours after collection. The tissue processing was performed according to the standard protocol.

In total, 44 sections (including 38 lymph nodes, 5 intestines and 1 thymus) previously analyzed by ISH, were selected and tested by IHC for the presence of PCV2. These samples represented the full range of ISH stainings, from negative (-), through doubtful (+/-), weakly positive (+), moderately positive (++) to highly positive (+++).

As a negative control, sections of lymph nodes from clinically healthy pigs were used.

Immunohistochemistry

To detect PCV2 antigen in the collected material, 3 µm thick sections of tissue were cut onto SuperFrost Plus slides (Menzel-Glasser, Germany). They were then deparaffinized in xylene, rehydrated through graded alcohols, and air-dried. Endogenous peroxidase was blocked by incubation with 3% hydrogen peroxide for 30 min followed by 2 washes in Tris-buffered saline (TBS), pH 7.5-7.6. All sections were then subjected to proteolytic enzyme digestion using proteinase K (DAKO Denmark A/S, Copenhagen, Denmark), at a concentration of 20 µl/ml, at room temperature for 1, 2, 3, 5, 10 or 15 min. After proteolytic digestion, the sections were washed with TBS and blocked by 2 hours of incubation with TBS-bovine albumin (SIGMA, St. Louis, MO, U.S.A.) solution, 20 g/l, at room temperature. PCV2 specific monoclonal antibody 36A9 (Ingenasa, Madrid, Spain), diluted in TBS-bovine albumin, was then applied and incubated overnight at 4°C. Two-fold serial dilutions of the mon-

oclonal antibody were tested, from 1:100 up to 1:10 000. All stages of incubations and staining were conducted in Shandon immunostaining coverplates placed in Shandon Sequenza racks (Thermo Shandon, Cheshire, United Kingdom).

The next day, the sections were washed with TBS, and incubated for 1 hour at room temperature with biotinylated polyclonal goat-anti-mouse immunoglobulins (DAKO Denmark A/S, Copenhagen, Denmark), at a dilution of 1:200. The sections were then washed with TBS and incubated with avidin-biotin peroxidase staining solution (Pierce Protein Research Product, Thermo Scientific, Dreieich, Germany) according to the manufacturer's recommendations. After a further washing step with TBS, the sections were incubated with aminoethylcarbazole (AEC) substrate chromogen, (DAKO Denmark A/S, Copenhagen, Denmark) for 10 min. After washing in distilled water the sections were counterstained with Mayer's hematoxylin for 3 s at room temperature. The tissue sections were then washed in running tap water and mounted with glycergel mounting medium (DAKO Denmark A/S, Copenhagen, Denmark).

After that the slides were submitted for microscopic examination for the presence of a cell-associated red staining within the tissue. The evaluation of results included a staining intensity gradation system, similar to the one used for ISH (-, +/-, +, ++, +++). Those with only a low number of stained cells were described as doubtful (+/-). Samples with foci of stained cells, restricted to lymphoid follicles, were described as weakly positive (+). Moderately positive (++) were those sections with numerous stained cells dispersed across the section. Tissues with abundant stained cells were described as highly positive (+++).

In situ hybridization and hematoxylin-eosin staining

In parallel, ISH was performed on corresponding slides as previously described (Rosell et al. 1999, Stadejek et al. 2006). Briefly, 3 µm thick sections of tissue on ProbeOn (Fisher Scientific, Germany), dewaxed in xylene and rehydrated in graded alcohols to Automation Buffer (Biomeda Corp., CA, U.S.A.), were digested with pepsin (SIGMA, St. Louis, MO, U.S.A.) in Automation Buffer for 10 min at 37°C, and then at 105°C for 8 min. They were then incubated with 100% formamide (SIGMA, St. Louis, MO, U.S.A.) for 105°C for 5 min. The sections were then hybridized to a PCV2-specific oligonucleotide DNA probe labelled with digoxigenin (5'-5DigN/CAG TAA ATA CGA CCA GCA CCA GGA CTA CAA TAT CCG TGT AAC CAT G-3'), and washed with saline sodium

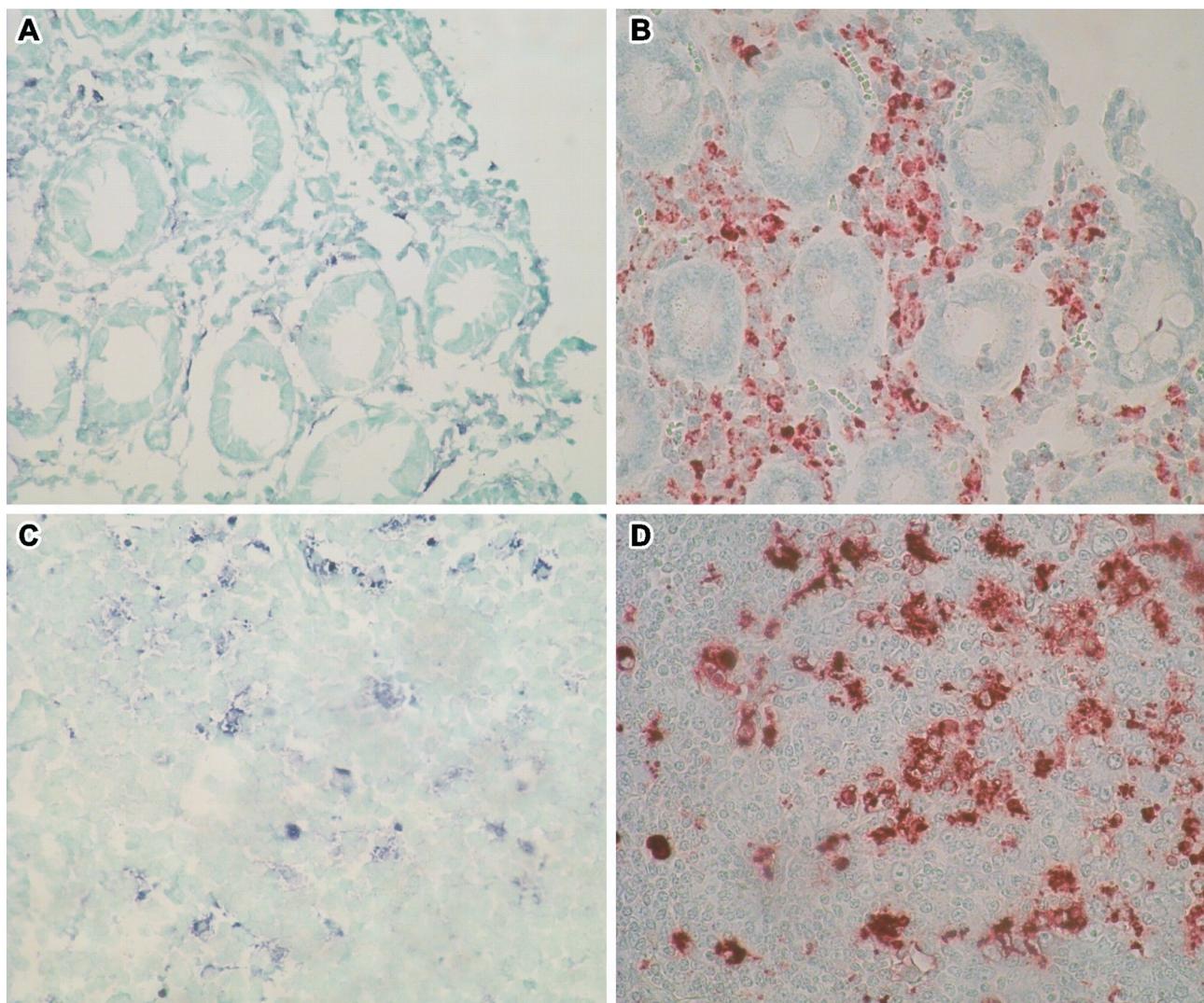


Fig. 1. Detection of PCV2 by *in situ* hybridization (ISH) (A, C) and immunohistochemistry (IHC) (B, D), in corresponding sections of lymph nodes (A, B) and intestines (C, D). PCV2-positive staining is present in ISH and IHC slides in a germinal centre, in cortex-like tissue of a lymph node (A and B) as well as in an intestinal lamina propria (C and D). More intensive staining is visible in immunohistochemistry. x200.

citrate buffers. An anti-digoxigenin antibody conjugated to alkaline phosphatase (Roche Diagnostics, Indianapolis, IN, U.S.A.) was then applied. Color reaction was developed using nitroblue tetrazolium dye (Roche Diagnostics, Indianapolis, IN, U.S.A.). Counterstaining was performed with Fast Green (SIGMA, St. Louis, MO, U.S.A.).

All the samples were also hematoxylin-eosin (HE) stained to assess the microscopic structure of the analyzed tissue.

Statistical analysis

The relative sensitivity and specificity of IHC were calculated in relation to ISH, according to OIE

Terrestrial Manual 2010 guidelines (<http://www.oie.int/international-standard-setting/terrestrial-manual/access-online/>) and PN-EN ISO 16140.

Results

Immunohistochemistry and *in situ* hybridization

Two parameters crucial for IHC, specificity and sensitivity, were optimized. Generally, increased time of proteolytic treatment resulted in more intense specific staining. Three minutes of proteinase K treatment was selected as the most favorable to allow optimal antigenic epitope exposure and was used for further analysis. Another parameter optimized in the

Table 1. Results of *in situ* hybridization (ISH) and immunohistochemistry (IHC) for PCV2 nucleic acid and antigen detection and microscopic examination of hematoxylin-eosin (HE) stained sections of tissues from pigs. Interpretation of ISH/IHC results.

Tissue type	Year of origin	ISH	HE	IHC	Tissue type	Year of origin	ISH	HE	IHC
Lymph node 1-2, thymus 1	2009	-	normal structure	-	lymph node 20-21	2008	+	slight lymphocyte depletion and granulomatous inflammation; presence of giant cells	++
Lymph node 3, ileum 1	2008	+/-	slight granulomatous inflammation	-	lymph node 22	2008	+	slight lymphocyte depletion and granulomatous inflammation; hemosiderin deposits	++
Lymph node 4-6, ileum 2	2008	+/-	slight granulomatous inflammation	+/-	lymph node 23-27, ileum 4	2008	+	slight lymphocyte depletion and granulomatous inflammation; infiltration by histiocytes	++
Lymph node 7-10	2008	+/-	slight lymphocyte depletion; presence of giant cells	+	lymph node 28	2009	+	slight lymphocyte depletion and granulomatous inflammation; infiltration by histiocytes; presence of inclusion bodies	+++
Lymph node 11, ileum 3	2008	+/-	slight lymphocyte depletion	++	lymph node 29, ileum 5	2008	+	slight lymphocyte depletion and granulomatous inflammation; infiltration by histiocytes	+++
Lymph node 12-13	2008	+	slight lymphocyte depletion and granulomatous inflammation; infiltration by histiocytes	-	lymph node 30-32	2008	++	moderate lymphocyte depletion and granulomatous inflammation	+++
Lymph node 14	2008	+	slight lymphocyte depletion and granulomatous inflammation; infiltration by histiocytes	+/-	lymph node 33-34	2009	+++	severe lymphocyte depletion and granulomatous inflammation; reduction or absence of follicles	++
Lymph node 15	2008	+	slight lymphocyte depletion and granulomatous inflammation; infiltration by histiocytes; hemosiderin deposits	+	lymph node 35	2009	+++	severe lymphocyte depletion and granulomatous inflammation; hemosiderin deposits; reduction or absence of follicles	+++
Lymph node 16-19	2009	+	slight lymphocyte depletion and granulomatous inflammation; infiltration by histiocytes	+	lymph node 36-38	2010	+++	severe lymphocyte depletion and granulomatous inflammation; reduction or absence of follicles	+++

(-) negative samples; (+/-) – doubtful samples: containing few stained cells; (+) weakly positive samples: with foci of stained cells, restricted to lymphoid follicles; (++) moderately positive samples: with numerous stained cells dispersed across the section; (+++) highly positive samples: with abundant stained cells.

study was the dilution of the primary antibody. In all compared dilutions the staining was clearly visible. However, in order to avoid potential problems with background staining or with very weak positive samples, the dilution of the primary antibody of 1:1000 was selected as satisfactory for standard IHC protocol.

PCV2 antigen and nucleic acid were detected in most tissues subjected to the analysis. After IHC intensive red staining was observed in the cytoplasm

of macrophages and multinucleated giant cells in lymph nodes. In the small intestine the staining was observed in gut-associated lymphoid tissue and in villous epithelium.

All 4 sections identified by ISH as highly positive gave similar results by IHC. No specific IHC staining was observed in negative control slides. Also, all experimental samples negative in ISH showed no staining in IHC. However, there were differences in scoring of the sections after staining with the two com-

pared methods. In 21 slides (47.7%) stronger staining was found in IHC than in ISH. Of these, 6 slides that scored doubtful in ISH, were found clearly positive in HC (Fig. 1).

Of 12 sections that were weakly positive in ISH, 3 scored highly positive in IHC and 9 scored moderately positive. Five slides showed similar low positive staining. In the case of 7 (15.9%) slides less intensive staining was found in IHC than in ISH. In this group 2 sections (1 sample of lymph node and 1 of intestine), previously identified as doubtful in ISH, were negative in IHC and 1 section of lymph node, identified in ISH as weakly positive, scored negative in IHC. Only in 16 (36.4%) samples were the results of ISH and IHC identical. The detailed results are presented in Table 1 and Table 2.

Table 2. Comparison of *in situ* hybridization (ISH) and immunohistochemistry (IHC) results for PCV2 detection. Sections in medium grey indicate IHC and ISH results which are in agreement. Dark grey sections specify higher results of IHC compared to ISH. Weaker results of IHC compared with ISH are in light grey.

		ISH				
		-	+/-	+	++	+++
IHC	-	3	2	2	0	0
	+/-	0	4	1	0	0
	+	0	4	5	0	0
	++	0	2	9	0	2
	+++	0	0	3	3	4
TOTAL (44)		3	12	20	3	6

Microscopic lesions

The results of microscopic evaluation of the HE stained sections are shown in Table 1. All PCV2-positive slides exhibited histopathological lesions typical for PMWS (Ellis et al. 1998, Opriessnig et al. 2007).

Statistical analysis

The relative specificity of IHC was estimated as 100%, with a 100% confidence interval. The relative sensitivity of the method was 90.2%, with confidence intervals between 85.6% and 94.9%. The relative accuracy was 90.9%, with confidence intervals between 86.6% and 95.2%.

Discussion

In situ detection of pathogens (antigen, nucleic acid) is a powerful tool for diagnostic pathology

(Haines and Chelack 1991, Ramos-Vara et al. 1999). Providing reliable diagnosis of a disease is especially difficult when dealing with a virus that infects most of an animal population, but causes a disease in only a small proportion of it, such as PCV2. According to Sorden's criteria, IHC and ISH are the only laboratory methods fulfilling the requirements of PMWS diagnosis (Sorden 2000). During the course of PMWS massive PCV2 replication is detected along with the presence of different sorts and distribution of microscopic lymphoid lesions (Opriessnig et al. 2007, Rosell et al. 1999). Typical lesions and high viral load are characteristic for an acute stage of the disease, and during recovery they diminish. Diagnosis may also be hampered by clinical signs in a herd (wasting) unrelated to PCV2 infection, where a small amount of PCV2 can be present in tissues (Opriessnig et al. 2007). High viral loads can also be present in apparently healthy pigs.

The analysis was conducted on samples that were selected from material that had been collected for 3 years and represented a full range of virus load and microscopic lesions commonly observed in PMWS cases. As expected, most of the slides identified previously in ISH as PCV2-positive were also positive in IHC. Interestingly, in the case of nearly half of the slides the results of IHC examination revealed an increase in the intensity of staining which clearly indicated improved sensitivity of PCV2 detection based on antigen visualization. This is in agreement with observations by other authors (McNeilly et al. 1999, Rosell et al. 1999). A possible explanation could be the presence of nucleotide mismatches between the probe and the template which, in stringent hybridization conditions, could result in inefficient staining (Mc Neilly et al. 1999). However, this seems unlikely considering the very low genetic diversity of PCV2, especially in the probe binding region. A more plausible hypothesis could be DNA degradation during sample processing.

The weaker results of IHC compared to ISH observed in the case of 7 (15.9%) samples may indicate higher specificity of IHC compared to ISH. However, it may also be attributed to antigenic epitope destruction due to excessive enzymatic treatment and usage of monoclonal antibodies giving a narrower spectrum of detection (Kim et al. 2009). Also, as ISH and IHC detect PCV2 DNA and protein, respectively, which represent transcriptional and translational events in infected cells, the differences between ISH and IHC results may be associated with different quantities of PCV2 DNA and antigen, at different stages of cell infection (Kim et al. 2009).

In this study we did not observe any negative impact of the time of paraffin block storage on ISH

staining. The results of ISH performed shortly after submission of samples for routine diagnosis and repeated for the purpose of this study showed no significant differences (Haines and Chelack 1991).

The results of PCV2 identification by IHC and ISH were comparable. In 21 (47.7%) of cases significantly stronger staining was detected after IHC than ISH, indicating higher sensitivity of the former method. Moreover, an important advantage of IHC is relatively easier localization of an antigen in a tissue structure due to more contrasting image and better preservation of tissue microscopic structure compared to ISH. In ISH a tissue section is subjected to a DNA denaturation step at 105°C, which causes partial destruction of tissue structure (Fig 1C).

The correct interpretation of IHC results may be hampered by the presence of pigments, such as ferrous pigment, for example hemosiderin, which can reduce the signal-to noise ratio (Ramos-Vara 2005). To avoid confusion in the interpretation of such findings the recommended procedure is additional treatment of tissue with a 1% solution of dithionite in pH 5.0 acetate buffer or using a detection system producing a different colored precipitate (Ramos-Vara 2005). In IHC protocols using diaminobenzidine (DAB) as a chromogen, giving brown staining, the presence of iron deposits may be mistaken for positive staining. In this study golden-brown granules of hemosiderin were found in 3 slides of lymph nodes. They were easy to discriminate from PCV2 antigen staining due to the use of red color chromogen, AEC, which contrasts well with brownish deposits.

The most common organs submitted for PMWS diagnosis are lymph nodes. However, PCV2 related lesions can also be present in intestinal tissues, sometimes resembling proliferative enteropathy, so differential diagnosis with PCVD is recommended (Jensen et al. 2006). Therefore, in our study, PCV2 identification was also performed on intestinal samples. The presence of PCV2 was detected in most analyzed intestines, confirming the usefulness of this technique in differential diagnosis of antibiotic-nonresponsive diarrhea. Further studies on involvement of PCV2 in enteritis in pigs in Poland are required.

Use of IHC in virology has several advantages over other methods, such as relevance with tissue structure, so it gives better insight into the pathogenesis of a particular disease. In spite of its great potential, IHC is still relatively rarely used in Polish veterinary diagnostic laboratories. The few examples of its use are almost exclusively dedicated to oncological analysis (Dolka 2009, Sapieryński 2010). To the best of our knowledge, there are no publications on swine pathogens detection by IHC in Poland.

In this study IHC protocol was developed for de-

tection of PCV2 antigen in tissues from pigs with PMWS. The results demonstrate that both ISH and IHC successfully detected PCV2 viral antigens or nucleic acid in examined tissues. However, our results indicate that IHC has higher sensitivity and specificity than ISH. In addition, IHC results are easier to interpret due to the better image quality after staining. In summary, IHC is reliable and useful technique for PMWS diagnosis and is likely to substitute ISH in our diagnostic laboratory.

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