Original article

Immunohistochemical detection of *Lawsonia intracellularis* in tissue sections from pigs

A. Szczotka[1], T. Stadejek[1], J. Żmudzki[1], A. Nowak[1], Z. Osiński[2], Z. Pejsak[1]

[1] Department of Swine Diseases
[2] Department of Hygiene of Animal Feedingstuffs National Veterinary Research Institute
Partyzantow 57, 24-100 Pulawy, Poland

Abstract

The aim of the present study was to develop an immunohistochemical method (IHC) for detection of *Lawsonia intracellularis* (*L. intracellularis*) in formalin-fixed, paraffin embedded sections of intestines from pigs and to implement this method in differential diagnosis of swine diseases with diarrhea in postweaning pigs. The study was conducted on 165 sections of intestines (ileum, caecum and colon) collected from 76 pigs, representing 42 Polish pig farms. The animals included in the analysis suffered from diarrhea, with bloody or grey to brown feces, and were suspected of porcine proliferative enteropathy (PPE). Sections of intestines were analyzed for the presence of *L. intracellularis* by polymerase chain reaction (PCR) and IHC. Among 165 intestinal samples from pigs with diarrhea, *L. intracellularis* DNA was detected by PCR in 33 (20.0%) samples. In this group, 30 samples (18.2% of all the samples tested) were also found positive in IHC, while only 3 (1.8%) were IHC-negative. One hundred thirty-two (80.0%) samples were negative in both tests. The PCR- and IHC-positive samples originated from 11 pigs, 4- to 20-week old, from 8 farms. *L. intracellularis* antigen was visualized by IHC mostly in intestinal crypts and/or in mononuclear cells of the lamina propria). The positive signal in epithelial cells was observed close to the luminal borders, creating typical specifically stained rims around the crypt lumina.ąThe results of the present study further confirm the usefulness of IHC in the detection of *L. intracellularis* antigen in the intestinal tissues.

Key words: immunohistochemistry, *Lawsonia intracellularis*, PPE, diarrhea, pigs

Introduction

Lawsonia intracellularis (L. intracellularis), an obligate intracellular bacterium, is known as an etiological agent of porcine proliferative enteropathy (PPE). The disease is widespread in pig farms worldwide and affects mainly growing and finishing pigs. The clinical symptoms comprise diarrhea, growth retardation and death in more severe, hemorrhagic forms. At necropsy significant lesions are found mainly in the ileum, but also in the colon and caecum, in form of thickening and ridges of the intestinal mucosa as the most prominent findings (Lawson and Gebhart 2000).

Diarrhea in postweaning pigs is still a serious problem causing about 50% of morbidity in piglets and over 10% of mortality (Laine et al. 2008). In weaned piglets diarrhea can be caused by several pathogens, like *L. intracellularis*, *Brachyspira hyodysenteriae* and *Brachyspira pilosicoli*, *Salmonella sp.*, *Escherichia coli*, *Clostridium perfringens* type C,

Correspondence to: A. Szczotka, e-mail: anna.szczotka@piwet.pulawy.pl, tel.: +48 81 889 30 46

Cryptosporidium spp., *Isospora suis*, *Oesophagostomum duodenatum*, *Trichuris suis*, *Yersinia sp.*, as well as rotaviruses and coronaviruses (transmissible gastroenteritis virus, porcine epidemic diarrhea virus). Therefore proper identification of the pathogen is a key to success in treatment of the disease.

PPE remains a significant problem in Polish swine production. According to the previous studies, *L. intracellularis* infections are widespread in the Polish swine population. The disease was diagnosed in 46% medium-sized farms and in 72.5% large-sized farms (Pejsak et al. 2007).

The identification of *L. intracellularis* is difficult. The bacterium does not grow on standard media and *in vitro* growth of *L. intracellularis* is possible only in cell cultures (Huerta et al. 2003). Since this technique is sophisticated and expensive, it has been introduced in few laboratories in the world.

L. intracellularis can also be detected by polymerase chain reaction (PCR). This technique is characterized by high sensitivity and specificity, but may also detect shedding or recovering pigs and therefore provide misleading results that are not related to clinical manifestation of the disease in a herd.

Similar limitations are related to serology. The detection of specific anti-*L. intracellularis* antibodies cannot be used for confirmation of PPE and may only serve as a tool for the identification of the age of seroconversion (Jacobson et al. 2010).

The preliminary diagnosis of PPE can be made based on clinical symptoms and gross and microscopic lesions. However, they are not very specific and can also be found in weaned pigs suffering from porcine circovirus type 2 (PCV2) related enteritis (Jensen et al. 2006, Zlotowski et al. 2008, Szczotka et al. 2010, Opriessnig et al. 2011). As both pathogens may also cause mild or subclinical infections, and develop very similar clinical symptoms and necropsy lesions, the correct diagnosis is possible only using laboratory methods that provide specific detection in tissue sections (Jensen et al. 2006).

The specific identification of *L. intracellularis* is possible with the use of monoclonal antibodies in immunohistochemical staining of formalin-fixed, paraffin-embedded tissue sections (Boesen et al. 2005, Jensen et al. 2010). Also, detection in tissue can be made with *in situ* hybridization (ISH) using *L. intracellularis*-specific oligonucleotide probes (Weissenbock et al. 2007). The important advantage of both immunohistochemistry (IHC) and ISH is providing relation between the development of microscopic lesions and the presence of bacterium within these lesions. Moreover, these techniques enable to evaluate the accumulation of specific signal within the tissue and therefore they provide information concerning different stages of infection. ąFor all the above mentioned reasons IHC and ISH are considered "the gold standard" for diagnosing PE (Jensen et al. 2010, Pedersen et al. 2010).

The aim of the present study was to develop immunohistochemical method for detection of *L. intracellularis* in formalin-fixed, paraffin embedded sections of intestines from pigs and to implement this method in differential diagnosis of swine diseases with diarrhea in postweaning pigs.

Materials and Methods

The study was conducted on 165 sections of intestines (ileum, caecum and colon) collected from 76 pigs, representing 42 Polish pig farms. The animals included in the study suffered from diarrhea, with bloody or grey to brown feces, and were suspected of PPE. The clinically healthy pigs served as negative controls. The age of pigs varied from 4 to 21 weeks.

Sections of intestines were analyzed for the presence of *L. intracellularis* by PCR and IHC. DNA for PCR was isolated from intestine mucosa scrapings from fresh/unfrozen intestines (Pejsak et al. 2007). The corresponding, 3-5 cm long, segments of intestines were cut longitudinally and the intestinal content was removed by gentle flushing with 10% buffered formalin. Next, sections were fixed with formalin and embedded in paraffin. To identify *L. intracellularis* antigen in paraffin embedded intestinal tissues, an avidin-biotin-complex immunoperoxidase method was developed. Sections (3 μm thick) of tissue samples were cut onto SuperFrost Plus slides (Menzel-Glässer, Germany), deparaffinized in xylene, rehydrated through graded alcohols, and air-dried. Endogenous peroxidase was blocked by treating sections with 3% hydrogen peroxide solution for 30 min followed by 2 washes in Tris-buffered saline (TBS), pH 7.5-7.6. All slides were then subjected to proteolytic enzyme digestion using proteinase K (DAKO), at a concentration of 20 μl/ml, for 3 min at room temperature. After proteolytic digestion, sections were washed with TBS and blocked by 2 hours of incubation with TBS-bovine albumin (SIGMA) solution, 20 g/l, at room temperature. Then monoclonal mouse antibodies: antibody number 1 (kindly provided by Dr. T.K. Jensen, Technical University of Denmark, Copenhagen), and antibody number 2, purchased from EuroClone, Italy, both detecting a proteinase K resistant molecule of 21 kDa (Boesen et al. 2005), diluted in TBS with bovine albumin, were applied and incubated overnight at room temperature. For the antibody number 1, dilution 1:100 was used (Boesen et al. 2005), while for the antibody number 2 increasing 2-fold concentrations
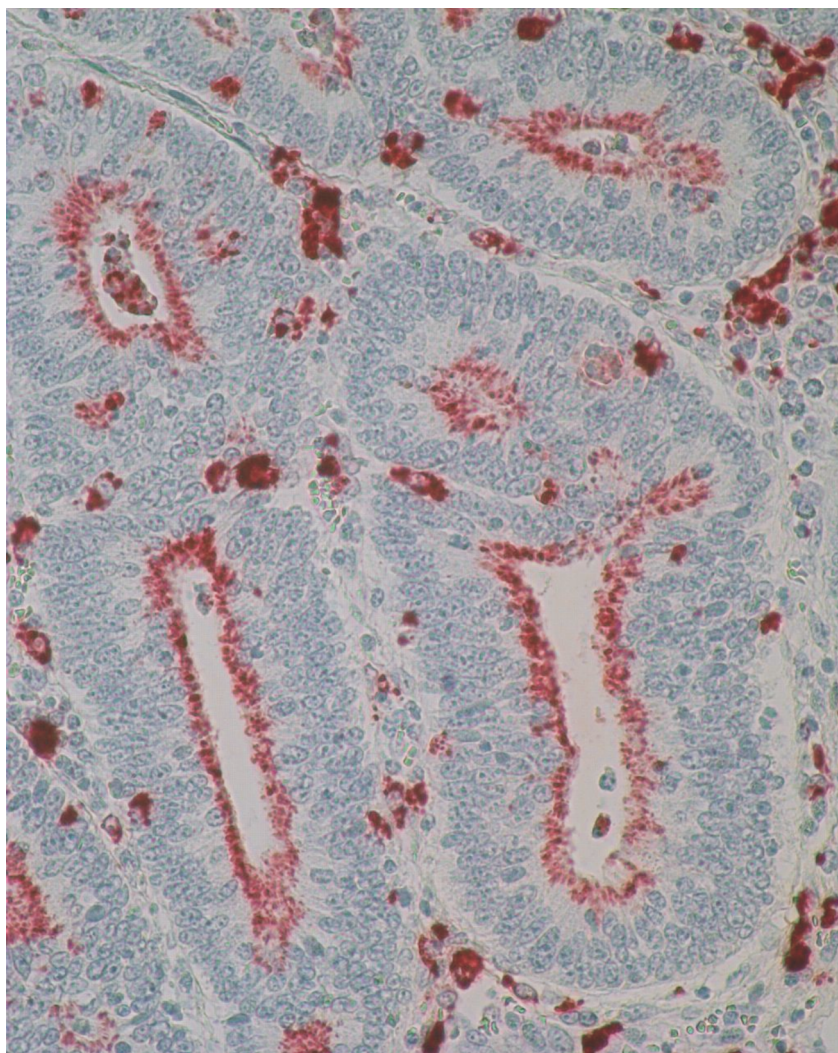
Fig. 1. Identification of *L. intracellularis* in the apical part of the crypt enterocytes (red staining). Ileum, positive control. IHC, x 200.

were tested: from 1:20 (recommended by the producer) to 1:160.

Next day, the sections were washed with TBS, and incubated with secondary antibody: polyclonal goat-anti-mouse biotinylated immunoglobulins (DAKO), at a dilution of 1:200, for 1 h, at room temperature. The sections were then washed with TBS and incubated with avidin-biotin peroxidase staining kit (Pierce), at room temperature, according to manufacturer's recommendations. After a further wash with TBS, the sections were incubated with the enzyme substrate aminoethylcarbazole (AEC), (DAKO) for 10 min. After washing in distilled water, the slides 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).

After that the slides were submitted for microscopic examination. Determination of a positive IHC result required the presence of a red to brownish cell-associated staining signal within the tissue in question with no similar signal present on any negative control from the same run. Positive controls for IHC were kindly provided by Dr. T.K. Jensen (DTU-VET) and Dr. Dinko Novosel (Croatian Veterinary Institute, Zagreb, Croatia). Sections of intestines from clinically healthy pigs served as negative controls. The other set of slides was additionally hematoxylin-eosin (HE) stained.

Statistical analysis

Pigs identified as PCR-positive for *L. intracellularis* were considered infected. This method was
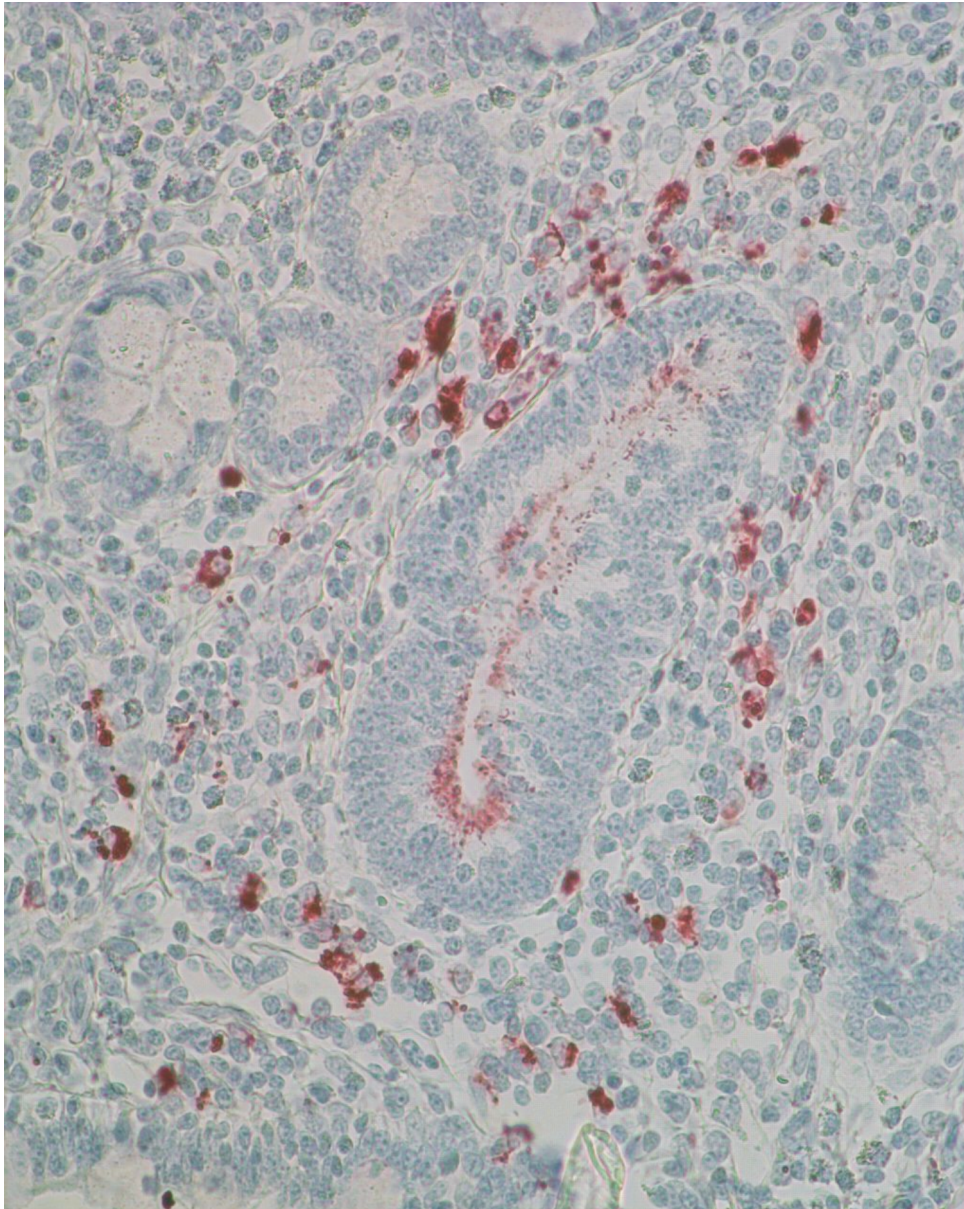
Fig. 2. Identification of *L. intracellularis* in the porcine colonic mucosa. Proliferation of immature epithelial cells with *L. intracellularis* located within the apical cytoplasm. Ileum, field case. IHC, x 400.

used as the reference due to its higher sensitivity and specificity than any other alternative (McOrist et al. 1994, Ladinig et al. 2009). The relative diagnostic sensitivity and specificity of immunohistochemistry were calculated in relation to PCR, according to OIE Terrestrial Manual 2010 guidelines (http://www.oie.int/international-standard-setting/terrestrial-manual/access-online/) and PN-EN ISO 16140.

Results

No difference in reactivity was observed for both antibodies used for IHC. Analyzing the results of *L.*

intracellularis detection in positive control slides with the increasing dilutions of the antibody number 2, positive staining was visible even at the highest dilution applied, but with a tendency to fade. Therefore, to avoid potential false negative results in case of weakly positive tissues, a dilution of 1:40 was selected as optimal. For the antibody number 1, apparent and satisfactory results were obtained with the recommended dilution.

Among 165 intestinal samples from pigs with diarrhea, *L. intracellularis* DNA was detected by PCR in 33 (20.0%) samples. In this group, 30 samples (corresponding to18.2% of all the samples tested) were also found positive in IHC, while only 3 (1.8%) were
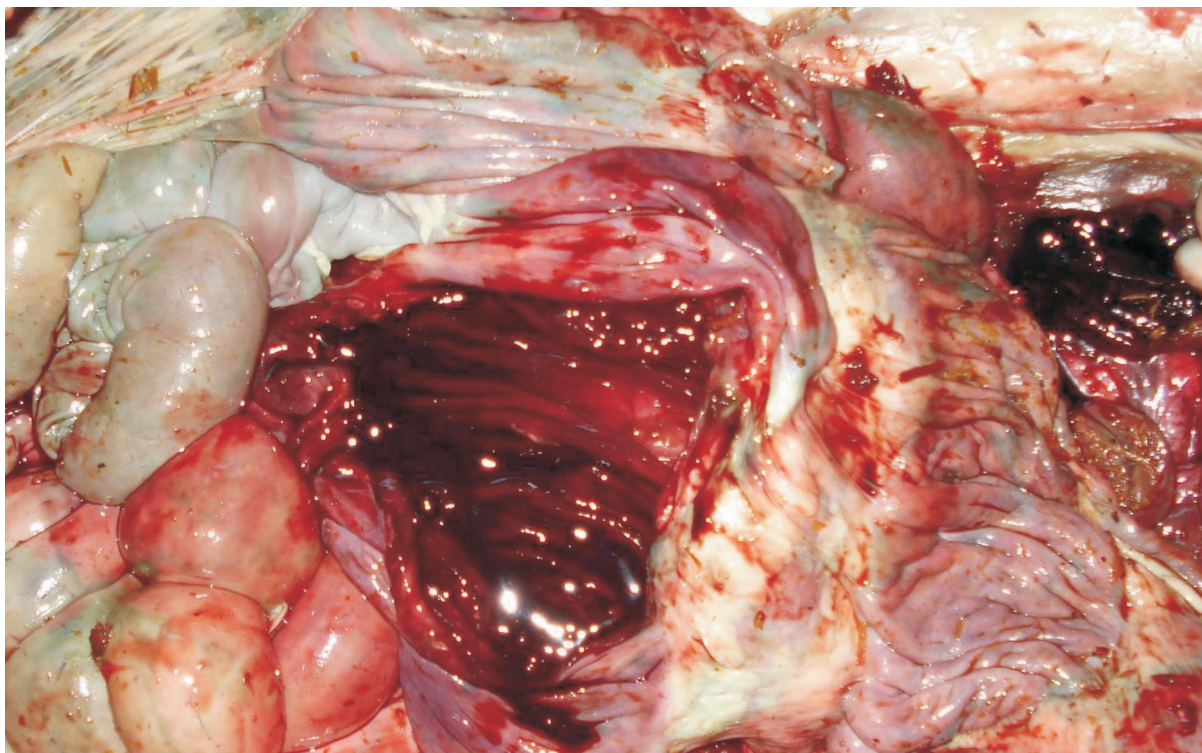
Fig. 3. Hemorrhagic enteritis due to *L. intracellularis* infection in 18-week old boar. Colon filled with blood; thickening of the intestinal walls.
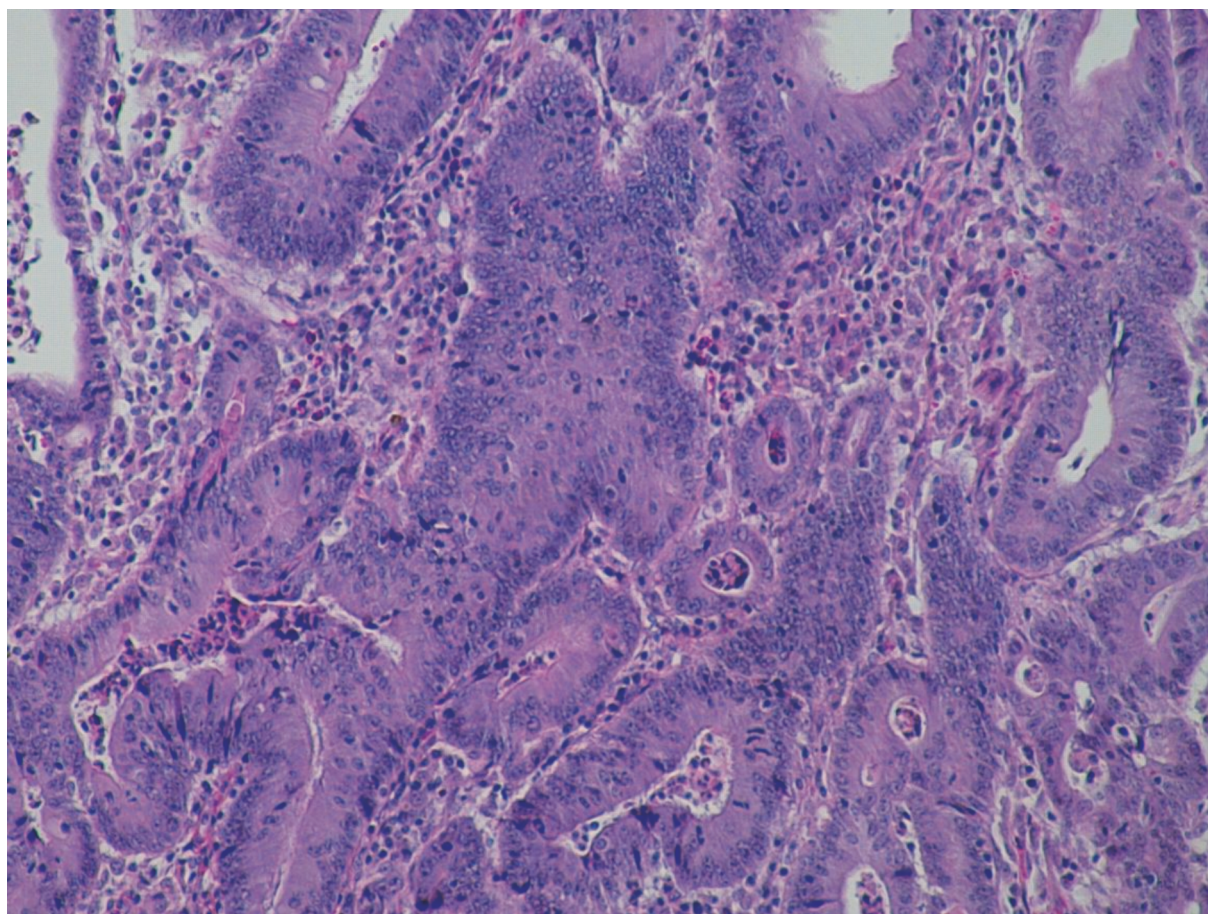

Fig. 4. Branched intestinal crypts with proliferation, cellular debris and absence of goblet cells. Ileum, field case. HE, x 100.
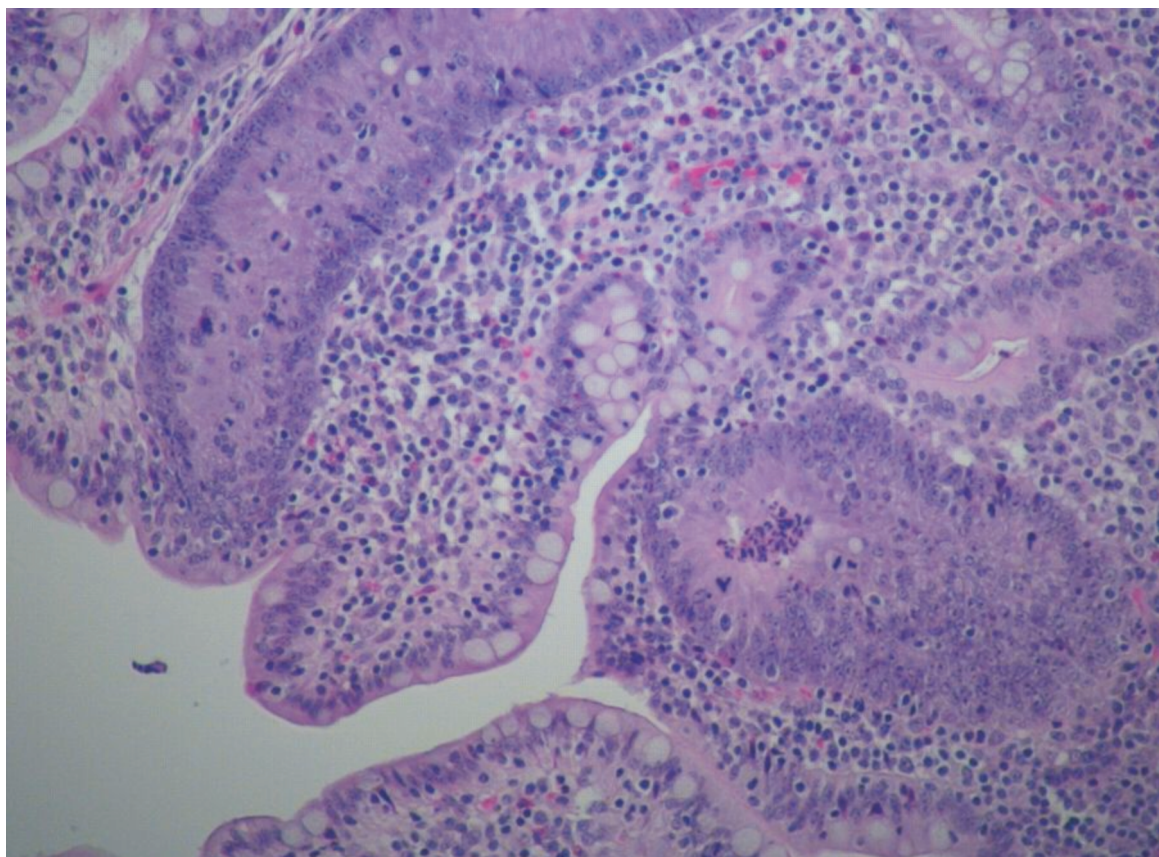
Fig. 5. Cross-section of the intestinal mucosa with marked proliferation of immature epithelial cells, reduced number of goblet cells, increased number of mitotic figures and cellular debris within crypts. Ileum, field case. HE, x 100.

IHC-negative. One hundred thirty-two (80.0%) samples were negative in both tests. The PCR- and IHC-positive samples originated from 11 pigs, 4- to 20-week old, from 8 farms.

L. intracellularis antigen was visualized by IHC mostly in intestinal crypts and/or in mononuclear cells of the lamina propria (Figs. 1, 2). The positive signal in epithelial cells was observed close to the luminal borders, creating typical, specifically stained rims around the crypt lumina (Figs. 1, 2). The distribution of antigen was mostly focal, or restricted to individual epithelial and mononuclear cells in the mucosa and lamina propria. The presence of *L. intracellularis* antigen was not correlated with significant crypt elongation or proliferation of crypt cells, indicating an acute/subacute course of the disease.

Only in tissue sections from 4 pigs (15- and 18-week old and two 12-week old), from 3 farms an abundant diffuse positive staining was observed. In tissue sections from these animals *L. intracellularis*-positive cells were present both in intestinal crypts and in the lamina propria. These findings were clearly in agreement with the clinical condition and necropsy findings in these animals, indicating an acute form of PPE, with hemorrhagic diarrhea and blood in both small and large intestines (Fig. 3). Crypt proliferation nd branching, as well as an accumulation of necrotic debris and an increased mitotic index were detected histopathologically (Figs. 4, 5).

The relative diagnostic specificity of IHC for *L. intracellularis* identification was estimated as 100%, with a 100% confidence interval for p=95%. The relative diagnostic sensitivity of the method was 90.9%, with confidence intervals for p=95% ranging between 85.9% and 95.9%.

Discussion

Enteric disorders and diarrhea are still one of the major problems in swine herds, contributing to a significant increase in cost of pig production. The incorrect diagnosis of a given condition implies an excessive usage of antimicrobial agents, leading to an ineffective but still expensive therapy. It often results in a potential gradual increase in an antibiotic resistance of microorganisms present at a farm site. Such situation has already been identified for tylosin (Hidalgo

et al. 2009) and tiamulin (Rhode at al. 2004) – the antibiotics frequently applied in case of diarrhea in pigs and expected to be effective in case of PPE. So, precise recognition of an agent involved in a disease is of great importance for implementation of correct measures to control it.

The preliminary diagnosis of PPE is based on necropsy findings followed by laboratory methods (Dittmar et al. 2003). Several laboratory methods could be used for the detection of *L. intracellularis*. The most sensitive is the detection of bacterial genetic material by PCR. Unfortunately it cannot discriminate between animals with PPE from asymptomatic *L. intracellularis* carriers or carriers with diarrhea of different etiology. Also, serologic methods have limitations related to short period of specific antibodies detection (Guedes at al. 2002, Jakobson et al. 2010).

The gold standard for the diagnosis of PPE is the demonstration of proliferative intestinal lesions associated with the presence of intracellular curved bacteria (Pedersen et al. 2010). Several histological techniques have been described to visualize the lesions and the associated bacteria – HE staining, Warthin-Starry silver staining, modified Ziehl-Nielsen staining, ISH and IHC (Pedersen et al. 2010). It has been proved that diagnosis by PCR or IHC have higher sensitivity compared to traditional histopathology (Huerta et al. 2003). These techniques can be used even in cases where typical gross lesions are not present (Ladinig et al. 2009). Identification of carrier animals is important facing the fact that growth performance of pigs subclinically infected with *L. intracellularis* is poor and they shed the bacterium into the environment, resulting in infection of susceptible penmates (Jacobson et al. 2003).

The results of the present study further confirm the usefulness of IHC in the detection of *L. intracellularis* antigen in the intestinal tissues. From the localization of *L. intracellularis* within the lesions, the time of infection can be inferred (Brandt et al. 2010). For example, detection of bacteria in both the intestinal epithelium and intestinal lymph nodes indicates *L. intracellularis* infection not older than 3-4 weeks, while identification of *L. intracellularis* in local lymph nodes alone corresponds to longer lasting infection (Brandt et al. 2010).

In our study, in only 3 samples from diarrheic pigs the presence of *L. intracellularis* was detected by PCR and not confirmed by IHC. This finding can be explained by significantly higher sensitivity of PCR compared to IHC (Guedes et al. 2002, Huerta et al. 2003), that enables to detect also animals shedding bacteria or recovering from the disease. In such situation, bacteria may not be present in high number within a tissue, so cannot be detected using IHC. Also, at the above mentioned stages of disease even microscopic lesions may not be present. The interpretation of such discrepant results should be made with care and involve clinical situation in the farm. Also, such result may be due to improper selection of animals for sampling. Pigs at early stage of infection may have no typical for PPE microscopic lesions, nor detectable amount of *L. intracellularis* antigen in the intestinal tissue.

In conclusion, the present study data proved high specificity and sensitivity of IHC in detection of *L. intracellularis*. However, from the practical point of view, this method should not be recommended as the first choice for fast identification of *L. intracellularis* in a herd and, as mentioned before, for epidemiological studies PCR is still more advisable. On the other hand, IHC method seems better suited for detection of PPE – especially that similar macroscopic alterations may be observed in PCV2 related enteritis and only specific immunohistochemical detection of pathogens connected with microscopic lesions may differentiate these two diseases.

Acknowledgements

References

Boesen HT, Jensen TK, Jungersen G, Riber U, Boye M, Mzller K (**2005**) Development, characterization and diagnostic application of a monoclonal antibody specific for a proteinase K resistant *Lawsonia intracellularis* antigen. Vet Microbiol 105: 199-206.

Brandt D, Kaim U, Baumgartner W, Wendt M (**2010**) Evaluation of *Lawsonia intracellularis* infection in a group of pigs in a subclinically affected herd from weaning to slaughter. Vet Microbiol 146: 361-365.

Dittmar M, Hoelzle LE, Hoelzle K, Sydler T, Corboz L, Miserez R, Wittenbrink MM (**2003**) Diagnosis of porcine proliferative enteropathy: detection of *Lawsonia intracellularis* by pathological examinations, polymerase chain reaction and cell culture inoculation. J Vet Med B 50: 332-338.

Guedes RM, Gebhart CJ, Winkelman NL, Mackie-Nuss RA, Marsteller TA, Deen J (**2002**) Comparison of different methods for diagnosis of porcine proliferative enteropathy. Can J Vet Res 66: 99-107.

Hidalgo A, Carvajal A, García-Feliz C, Osorio J, Rubio P (**2009**) Antimicrobial susceptibility testing of Spanish

field isolates of *Brachyspira hyodysenteriae*. Res Vet Sci 87: 7-12.

Huerta B, Arenas A, Carrasco L, Maldonado A, Tarradas C, Carbonero A, Perea A (**2003**) Comparison of diagnostic techniques for porcine proliferative enteropathy (*Lawsonia intracellularis* infection). J Comp Pathol 129: 179-185.

Jacobson M, Hård af Segertad C, Gunnarson A, Fellström C, de Verdier Klingenberg K, Wallgren P, Jensen-Waern M (**2003**) Diarrhoea in the growing pig – a comparison of clinical, morphological and microbial findings between animals from good and poor performance herds. Res Vet Sci 74: 163-169.

Jacobson M, Fellström C, Jensen-Waern M (**2010**) Porcine proliferative enteropathy: an important disease with questions remaining to be solved. Vet J 184: 264-268.

Jensen TK, Boesen HT, Vigre H, Boye M (**2010**) Detection of *Lawsonia intracellularis* in formalin-fixed porcine intestinal tissue samples: comparison of immunofluorescence and *in-situ* hybridization, and evaluation of the effects of controlled autolysis. J Comp Pathol 142: 1-8.

Jensen TK, Vigre H, Svensmark B, Bille-Hansen V (**2006**) Distinction between porcine circovirus type 2 enteritis and porcine proliferative enteropathy caused by *Lawsonia intracellularis*. J Comp Pathol 135: 176-182.

Ladinig A, Sommerfeld-Stur I, Weissenböck H (**2009**) Comparative evaluation of diagnostic methods for *Lawsonia intracellularis* infection in pigs, with emphasis on cases lacking characteristic lesions. J Comp Pathol 140: 140-148.

Laine TM, Lyytikäinen T, Yliaho M, Anttila M (**2008**) Risk factors for post-weaning diarrhoea on piglet producing farms in Finland. Acta Vet Scand 50: 21.

Lawson GH, Gebhart CJ (**2000**) Proliferative enteropathy. J Comp Pathol 122: 77-100.

McOrist S, Gebhart CJ, Lawson GH (**1994**) Polymerase chain reaction for diagnosis of porcine proliferative enteropathy. Vet Microbiol 41: 205-212.

Opriessnig T, Madson DM, Roof M, Layton SM, Ramamoorthy S, Meng XJ, Halbur PG (**2011**) Experimental reproduction of porcine circovirus type 2 (PCV2)-associated enteritis in pigs infected with PCV2 alone or concurrently with *Lawsonia intracellularis* or *Salmonella typhimurium*. J Comp Pathol 145, 261-270.

Pedersen KS, Holyoake P, Stege H, Nielsen JP (**2010**) Diagnostic performance of different fecal *Lawsonia intracellularis*-specific polymerase chain reaction assays as diagnostic tests for proliferative enteropathy in pigs: a review. J Vet Diagn Invest 22: 487-494.

Pejsak Z, Żmudzki J, Wasak M, Porowski M, Wojciechowski J, Mirt D (**2007**) Prevalence of *Brachyspira hyodysenteriae* and *Lawsonia intracellularis* infections in Polish swine population. Medycyna Wet 63: 994-996.

Rohde J, Kessler M, Baums CG, Amtsberg G (**2004**) Comparison of methods for antimicrobial susceptibility testing and MIC values for pleuromutilin drugs for *Brachyspira hyodysenteriae* isolated in Germany. Vet Microbiol 102: 25-32.

Szczotka A, Zmudzki J, Pejsak Z, Stadejek T (**2010**) Ocurrence of *porcine circovirus type 2* (*PCV-2*) in cases of antibiotic non-responsive diarrhoea in pigs. J Comp Pathol 143: 326.

Weissenböck H, Mrakovcic M, Ladinig A, Fragner K (**2007**) *In situ* hybridization for *Lawsonia intracellularis*-specific 16S rRNA sequence in parrafin-embedded tissue using a digoxigenin-labeled oligonucleotide probe. J Vet Diagn Invest 19: 282-285.

Zlotowski P, Correa AMR, Borba MR, Cruz CEF, Driemeier D (**2008**) Enteritis associated with *Lawsonia intracellularis* and *porcine circovirus type 2* infection in wild boars in Southern Brazil. Cikncia Rural, Santa Maria 38: 2540-2544.