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COMPARISON OF SOME DIFFERENT METHODS FOR IDENTIFICATION OF *LAWSONIA INTRACELLULARIS* INFECTION IN PIGS

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Abstract

To compare different histopathological methods for diagnosis of Lawsonia intracellularis infection in pigs were taken in study 25 samples of ileum with specific lesions of intestinal adenomatosis. In order to perform slides were used Kinyoun, Green-Methyl-Pironine, Masson-Fontana, Schmitz, Diff-Quick methods and immunohistochemistry. The results showed that Green-Methyl-Pironine method has no value for diagnosis of porcine proliferative enteropathy, while Kinyoun coloration is capable to identify the bacteria only in 28% of samples. The argentic impregnation and Diff-Quick are able to highlight the aetiological agent in 44%, respectively 40% of the studied samples, so this methods have enlarge value of diagnosis. Immunohistochemistry demonstrated a high sensitivity and specificity and it was capable to emphasize the causative agent of intestinal adenomatosis in all 25 studied samples with proliferative ileitis.

Key word*: intestinal adenomatosi, Lawsonia intracellularis, porcine proliferative enteropathy*

INTRODUCTION

Infection of *Lawsonia intracellularis*, the causative agent of proliferative enteropathy, occurs all over the world, in different types of production systems, affecting young breeding and growing-finishing pigs. The disease occurs in two major clinical forms including a chronic form, called porcine intestinal adenomatosis (PIA), and an acute form, named proliferative hemorrhagic enteropathy (PHE) (Gyles et al, 2010; McOrist and Gebhart, 2006; Moga Mânzat, 2001).

The economic impact of proliferative enteropathy on the swine industry is estimated to be very high. It was considered the most common problem in grower-finisher pigs in the 2000 National Animal Health Monitoring System survey, occurring on more than a third of all sites and reported on 75% of large sites (Guedes, 2004). The economic damage due the evolution of this morbid entity could not be stopped, as long as the aetiopathogenesis is unclear, as the earlier diagnosis methods of outbreaks are not established, it is impossible to determine appropriate measures against the disease and to control it.

MATERIALS AND METHODS

A number of 25 samples of ileum, with specific lesions of intestinal adenomatosis, were submitted to microscopic examination, using Kinyoun, Green-Methyl-Pironine, Masson-Fontana, Schmitz, Diff-Quick methods and immunohistochemistry.

Protocol for slides stained include few step (Şincai, 2003):

- Samples were paraffined, after keeping them for 7 days in 80^{o} alcohol solution.
- The paraffin block was cut at 5 μm.
- Dewaxing involved 3 successive baths of toluene, 3-5 minutes each one.
- Dehydration in decrease concentration of alcohol (absolutely, 96^{o} and 80^{o}) was followed by hydration with distilled water for one minute.
- The slide were stained, noting that the staining technique depends by chosen method. In the present study we used Kinyoun, Green-Methyl-Pironine, Masson-Fontana, Schmitz, Diff-Quick methods.
- Before clearing with toluene (1 bath) and mounting, the samples were dehydrated with increase concentration of alcohol (80^{o}, 90^{o}, absolutely).

For immunohistochemical technique (IHC), initially, samples were subject to inclusion in

paraffin technique, sectioning, dewaxing and rehydrating, according to the above mentioned protocol. This method involves antigenic exposure and immunostaining. Antigenic exposure was performed by exposing of dewaxed and rehydrated sections to heat, into a sodium citrate solution at pH 6, for 30 minutes. To block endogenous peroxidase was used hydrogen peroxide 3% (Lin et al., 2011). Immunostaining involved use of work system NovoLink Max Polymer Detection (Novocastra, Newcastle UponTyne, UK). All steps were made using DakoCytomation Autostainer immunohistochemistry machine. Chromogen used consisted of 3.3-diaminobenzidine and for counter-stain was applied Lille haematoxylin. All samples were double staining using alcian blue coloration.
Microscopic evaluation was realized using Nikon Eclipse E 600 microscope and images were captured with LUCIA G system.

RESULTS AND DISCUSSIONS

Microscopic examination of intestinal fragments seems to be capable for highlight caracteristic lesions and causal agent of porcine proliferative enteropathy, depending of the chosen methods.
Comparison of different histopathologic methods results for diagnosis of porcine proliferative enteropathy, obtained in our study, are shown in table number 1.

Tabel no. 1
Comparison of some diagnostic methods of porcine proliferative enteropathy

Histopathologic methods	No. of examined samples	No. of positive samples	Diagnostic Value
Green-Methyl-Pironine	25	0	No value
Diff-Quick	25	10	Orientative
Masson-Fontana	25	11	Orientative
Schmitz	25	11	Orientative
Kinyoun	25	7	Orientative
IHC	25	25	Routine

Using Green-Methyl-Pironine method it was observed that epithelial proliferation of ileal mucosa associated goblet cell depletion alternate with epithelial desquamation (figure 1) and with lake of lesions areas. Characterization of inflammatory infiltrate it was not possible and also this method has not capacity to highlight the bacteria.

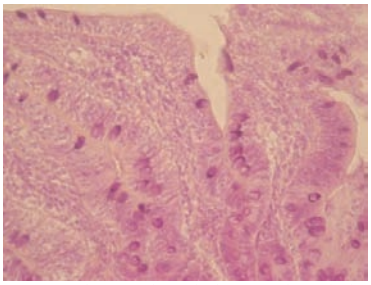

Fig. 1. Proliferated epithelium and epithelial desquamation (Green-Methyl-Pyronine, x400)

Diff-Quick coloration is a method capable to expose all characteristic lesions of porcine proliferative enteropathy, but not always the present of the bacteria, which was observed in 10 samples, that means 40%. It was observed areas with epithelial proliferation of ileal mucosa, goblet cell depletion, epithelial desquamation and inflammatory infiltrate in lamina propria of the mucosa characterized by mobilization of macrophages, lymphocytes and eosinophils (figure 2).

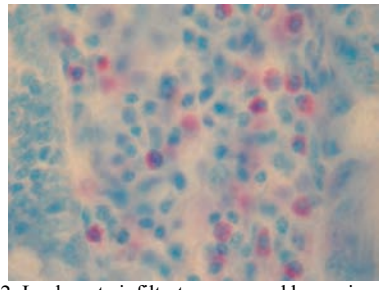

Fig. 2. Leukocyte infiltrate composed by eosinophils, lymphocytes and macrophage cells (Diff-Quick, x400)

The present of eosinophils as a cellular components, involved in antibacterial defense, characteristic of *Lawsonia intracellularis* infections, was first reported in this study, and may suggest an allergic reaction caused by the existence of protein LsaA in bacterial wall, a phenomenon that triggers edema as a

consequence of histaminic release by mast cells. On the other hand, eosinophils may play a role in bacterial neutralization, knowing the fact that they are attracted to the lipopolysaccharides from bacterial Gram-negative wall.

Argentic impregnation, Masson-Fontana and Schmitz, allowed emphasizing less the histological aspects, but, due agrophilic characteristic of *L.intracellularis* strains, the methods were able to highlight the presence of the bacteria (figure 3, figure 4) in 11 samples, which implies a rate of 44% positive samples. However, these methods are capable to exposure microscopic lesions of epithelial proliferation caused by multiplying immature enterocytes.

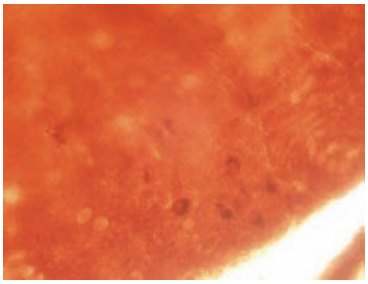

Fig. 3. Epithelial proliferation of intestinal mucosa with intracellular bacteria (Masson-Fontana, x1000)

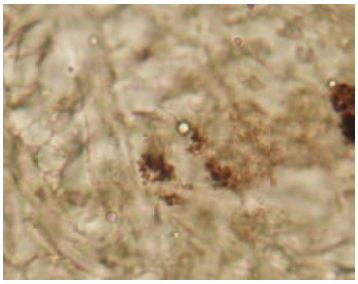

Fig. 4. Cluster of bacteria in cytoplasm of immature enterocytes from epithelial proliferated layer (Schmitz, x1000)

Concerning to Kinyoun coloration, 7 samples were positive, which implies a rate of 28% positive pigs. Bacteria could be highlighted in the cytoplasm of enterocytes from intestinal villi (figure 5), into enterocytes of the intestinal glands and in macrophages. Being an acid-fast stain, this technique is not capable to express microscopic lesions.

Fig. 5. Cluster of bacteria in cytoplasm of immature enterocytes from epithelial proliferated layer (Kinyoun, x1000)

Unlike all histological methods that we described, immunohistochemistry was able to identify the bacterial agent in all examined samples. Even it highlights only few microscopic lesions, mentioning depletion of goblet cell, epithelial desquamation, immature enterocytes proliferation (figure 6), this diagnostic method represents an important tools for postmortem diagnostic of porcine proliferative enteropathy.

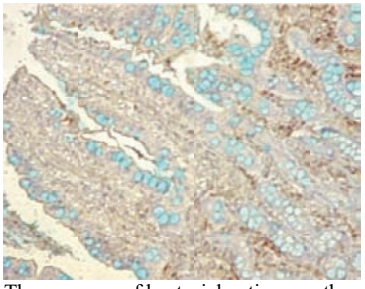

Fig. 6. The presence of bacterial antigen on the surface of intestinal villi and lamina propria between the intestinal glands (IHC – double staining with Alcian blue, x100)

Many studies were designed to compare some histopathological methods for diagnostic of swine proliferative enteropathy, but these were limited to H&E, Ziehl-Neelsen, Warthin–Starry technique and immunohistochemistry. Guedes et al. (2002) showed that all 14 pigs with microscopic lesions detectable by H&E staining were revealed the etiologic agent using Warthin-Starry methods, and of the 33 samples positive by IHC in only 19 specimens the bacteria was identified by silver impregnation (Guedes et al., 2002). Moreover, it seems that silver impregnation was able to highlight only a rate of 42% positive samples confirmed by PCR (Weissenbo et al., 2007). It seems that in

acute form of porcine proliferative enteropathy, Warthin-Starry and Ziehl-Neelsen stains are able to highlight the etiologic agent in all examined samples confirmed as positive by PCR (Dittmar et al., 2003). The low percentage of positive samples by Warthin-Starry and Ziehl-Neelsen stains which were obtained in our study may be due to the chronic form of this infectious disease.

Diagnosis of porcine proliferative enteropathy represents a problem faced by many researchers, but also by breeders. Earlier and low cost diagnosis remains a goal that seems to be difficult to achieve, as soon as there are still many questions about the etiopathogenesis of this disease.

CONCLUSIONS

Immunohistochemistry remains a precision diagnostic method of porcine proliferative enteropathy outbreaks.

Due to expedient technique and satisfactory results, Diff-Quick method can successfully replace the argentic impregnation. Poor results obtained in case of Green-methyl-pironine method recommend that these techniques are not used.

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