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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

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Summary

Two methods, an immunofluorescence assay (IFA; with a *Lawsonia intracellularis*-specific monoclonal antibody) and fluorescent in-situ hybridization (FISH; with a specific oligonucleotide probe targeting 16S ribosomal RNA of the bacterium), were compared for their ability to detect *L. intracellularis* (the cause of porcine proliferative enteritis [PE]) in formalin-fixed samples of intestinal tissue. Of 69 intestinal samples with gross lesions of PE, 63 were positive by both FISH and IFA, but six were positive only by IFA. This indicated that the sensitivity of FISH was 91% that of IFA. However, both methods had a specificity of 100%. Fifty normal porcine intestines were negative by both tests. IFA was much less susceptible than FISH to the effects of autolysis. Thus, three of nine samples from pigs with PE were FISH-negative after being kept at 20°C for 4 days, and seven were FISH negative after 2 weeks; after 4 weeks at this temperature, however, six of the nine samples were still IFA positive. After being kept at 4°C for 12 weeks, the majority of samples (≥66%) were positive by both methods.

Keywords: bacterial infection; *Lawsonia intracellularis*; PE diagnosis; pig; proliferative enteropathy (PE)

Introduction

Proliferative enteropathy (PE) is a major cause of economic loss in intensive pig production worldwide, causing diarrhoea, retarded growth and sudden death among 'growing to finishing' pigs (Lawson and Gebhart, 2000; McOrist and Gebhart, 2006). The disease affects the aboral small intestine, in particular the ileum, but may also affect the caecum and colon. Macroscopically, the intestinal mucosa appears thickened and ridged. Microscopically, PE is characterized by adenomatous proliferation of immature epithelial cells infected by *Lawsonia intracellularis*, an obligate intracellular gram-negative bacterium (McOrist *et al.*, 1995; Lawson and Gebhart, 2000; McOrist and Gebhart, 2006). So far, in-vitro growth of the bacterium has proved possible only in cell cultures (McOrist *et al.*, 1995), a method unsuitable for routine diagnosis. A presumptive diagnosis of PE may be made on the basis of gross pathological findings and on the demonstration, in Wartin–Starry silver-stained sections, of adenomatous crypts lined by large immature enterocytes containing small, comma-shaped bacteria in the apical cytoplasm (Lawson and Gebhart, 2000). Diagnostic methods based on the polymerase chain reaction (PCR) or immunohistochemistry appear to be more sensitive than traditional routine histopathology (Jones *et al.*, 1993; Guedes *et al.*, 2002; Huerta *et al.*, 2003), especially when intestinal specimens are autolysed or lack typical gross lesions (Jensen *et al.*, 1997). Other methods for detection of the bacterium in tissue samples include in-situ hybridization

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targeting DNA (Gebhart *et al.*, 1994), and fluorescent in-situ hybridization (FISH) with an oligonucleotide probe targeting ribosomal RNA (Boye *et al.*, 1998).

Immunohistochemistry (IHC), which is suitable for routine diagnostic purposes, has been applied in numerous studies with a monoclonal antibody that recognizes an epitope in the outer leaflet of the bacterial membrane (McOrist *et al.*, 1987). Recently, a further monoclonal antibody against *L. intracellularis* has been produced, characterized and validated for an immunofluorescence assay (IFA) on pig intestines (Guedes and Gebhart, 2003).

Immunological methods rely on the expression of specific antigens, which may not be constant; phenotypic variation, however, does not pose a problem when rRNA is used as a target. Ribosomal RNA is naturally amplified in growing cells; for example, exponentially growing *Escherichia coli* cells contain 10^4–10^5 copies of 5S, 16S and 23S rRNA per cell. The sensitivity of FISH is related to the amount of rRNA in the target organisms and is therefore strongly influenced by the physiological history and current physiological state of the bacteria; thus, for example, starvation may result in complete lack of detectable hybridization (Moter and Göbel, 2000; Oda *et al.*, 2000). No large-scale investigation of the applicability or sensitivity of in-situ hybridization as a diagnostic test for *L. intracellularis* has been made. Moreover, although post-mortem decomposition often occurs under field conditions, its effect on the accuracy of in-situ detection methods has received little attention. However, it has been shown that the detection of pathogens by immunohistochemical methods may be hampered to a variable degree by autolysis (Pelstring *et al.*, 1991; Brown *et al.*, 1996; Debeer *et al.*, 2001).

The aims of this study were (1) to compare FISH and IFA as diagnostic methods for the detection of *L. intracellularis* in formalin-fixed tissue sections from pigs with naturally acquired PE, and (2) to evaluate the effects of controlled post-mortem intestinal decomposition on the two methods. FISH was performed as described by Boye *et al.* (1998). In the IFA, however, a monoclonal antibody (Law1DK, recognizing a 21 kDa molecule of *L. intracellularis*) recently described by Boesen *et al.* (2005) was used. In addition, the PE lesions were evaluated immunohistochemically.

Materials and Methods

Intestinal Samples

These were obtained from pigs with naturally acquired PE (group 1) and from pigs unlikely to have been exposed to *L. intracellularis* (group 2).

Group 1 samples ($n = 69$) consisted of intestinal tissue submitted for routine diagnosis. The intestines showed gross lesions indicative of PE (mucosal hyperplasia, with ridges; and enterocolitis [superficial fibrinous to severe necrotizing or haemorrhagic]). The intestinal samples were either submitted as such or were obtained from submitted carcasses. The diagnostic material was usually received after overnight transportation, but in some instances arrived as much as 4 days after death of the animal.

Group 2 samples ($n = 50$) consisted of intestinal tissue from healthy pigs, with no history of diarrhoea and shown to be PCR negative for *L. intracellularis* 2 weeks before slaughter. The intestinal samples were received at the laboratory on the day after death. The group 2 animals had been included in a previous study on the infection dynamics of *L. intracellularis* from weaning to slaughter (Stege *et al.*, 2000).

Sampling Procedure

Two samples of tissue taken from each animal consisted of a portion of ileum, 2 cm in length and 10 cm from the ileocaecal valve, and a sample of colon (2×2 cm). After fixation in 10% neutral buffered formalin the samples were embedded in paraffin wax, sectioned (3 μm) and mounted on Super Frost® Plus slides (Menzel-Gläser, Braunschweig, Germany).

Immunohistochemistry for Light Microscopy

After dewaxing in xylene the sections were rehydrated and treated with H_2O_2 0.6% in Tris-buffered saline (TBS; 50 mM Tris, 150 mM NaCl, pH 7.6) for 20 min. They were then washed (3×5 min) in TBS before incubation in a moist chamber for 30 min with the monoclonal antibody (mAb) Law1DK (Boesen *et al.*, 2005), diluted 1 in 1000 in TBS. After further washing in TBS (3×5 min) they were incubated with EnVision+™ (Dako, Glostrup, Denmark) for 30 min. After washing again in TBS (3×5 min) the reaction was developed for 15–20 min with a solution of 3-amino-9-ethylcarbozole (Kementec, Copenhagen, Denmark). The sections were then counterstained with Mayer's haematoxylin and mounted with Glycergel (Dako, Glostrup, Denmark). For negative control purposes, the mAb was absorbed by sonically disintegrated *L. intracellularis* organisms.

Immunofluorescence Assay

After dewaxing in xylene the sections were rehydrated and washed in TBS (3×5 min). They were treated with protease (Sigma, St Louis, MO, USA) 0.07%