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Comparative Evaluation of Diagnostic Methods for *Lawsonia intracellularis* Infection in Pigs, with Emphasis on Cases Lacking Characteristic Lesions

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Summary

In this study the following methods for the diagnosis of *Lawsonia intracellularis* infection in pigs were compared in relation to a reference method (examination of ileal mucosal scrapings by the polymerase chain reaction [PCR]): Warthin–Starry (WS) staining of tissue sections, immunohistochemistry (IHC), in-situ hybridization (ISH), and PCR examination of faeces and of paraffin wax-embedded samples of ileum. Of 204 pigs examined, 32 were considered on the basis of the PCR to be infected. Gross and histopathological examination, including the use of WS staining, were of limited value. PCR examination of faeces proved to be the most sensitive (sensitivity 70%) of the methods used but, due to the occurrence of false positives, its specificity (95%) was the lowest. IHC (sensitivity 66%, specificity 99%) and ISH (sensitivity 54%, specificity 100%) were clearly superior to examination of WS-stained sections (sensitivity 34%, specificity 100%) for routine diagnosis; although less sensitive than the PCR, they indicated only cases of clinical relevance and, moreover, were capable of distinguishing different stages and levels of infection. Because examination of paraffin wax-embedded tissue by the PCR was shown to be associated with low sensitivity (41%), IHC was regarded as the method of choice for retrospective studies.

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Introduction

Lawsonia intracellularis is an obligate intracellular bacterium causing porcine proliferative enteropathy (PPE), a transmissible enteric disease mainly affecting growing and finishing pigs. PPE is of particular economic importance in the swine industry worldwide (Lawson and Gebhart, 2000; McOrist, 2005). The infection, which causes diarrhoea and retarded growth or sudden death, or both, affects the aboral small intestine, in particular the ileum. The affected mucosa is characteristically thickened and ridged due to adenomatous proliferation of immature crypt epithelial cells infected by the bacterium (McOrist and Gebhart, 2006).

Classical PPE can be diagnosed *post mortem* by the characteristic gross lesions and by histological examination of tissue sections stained with haematoxylin and eosin (HE); however, such methods do not detect cases in which lesions are resolving and bacteria are mainly confined to the propria and submucosa (Jensen *et al.*, 1997; Guedes *et al.*, 2002). By Warthin–Starry silver stain *L. intracellularis* appears as a curved, rod-shaped organism within the apical cytoplasm of the enterocytes. This staining technique, however, is non-specific and has limitations when applied to necrotic or autolysed samples (Ward and Winkelman, 1990; Jensen *et al.*, 1997). Specific identification of the bacterium can be made by immunohistochemical labelling of paraffin wax-embedded tissue

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sections with *Lawsonia*-specific monoclonal antibodies (McOrist *et al.*, 1987; Guedes and Gebhart, 2003; Boesen *et al.*, 2005). Alternatively, PPE can be diagnosed by molecular methods such as in-situ hybridization (ISH) with *Lawsonia*-specific oligonucleotide probes (Boye *et al.*, 1998; Weissenböck *et al.*, 2007), or the polymerase chain reaction (PCR). Immunohistochemistry (IHC) and ISH are both capable of relating lesions to the presence of the causative bacteria. Moreover, both methods assist in distinguishing different stages of infection on the basis of the distribution and amount of signals. The PCR has been described as a pathogen-specific and sensitive method for the detection of *L. intracellularis* in faeces and tissue samples (Jones *et al.*, 1993; McOrist *et al.*, 1994; Jordan *et al.*, 1999). However, it must be borne in mind that the PCR, which detects the presence of bacterial genome, may give clinically irrelevant positive results in recovering animals that are no longer infected with significant numbers of bacteria. For epidemiological studies, however, the PCR is a powerful diagnostic tool, capable of detecting subclinical infection.

The objectives of the present study were (1) to compare different diagnostic techniques in respect of sensitivity and specificity, and (2) to identify the most suitable methods for routine diagnosis, particularly in *Lawsonia*-positive cases that lack characteristic gross and microscopical lesions.

Materials and Methods

Collection and Preparation of Samples

Pigs ($n = 204$; from 78 herds) submitted to the Clinic for Swine, University of Veterinary Medicine, Vienna for routine diagnosis were used for sample collection. All pigs were aged 1–6 months and had a history of diarrhoea or retarded growth, or both, and 14 animals had been vaccinated against *L. intracellularis*. Sampling was standardized as follows.

At necropsy, a portion (about 10 cm) of the distal ileum was collected. The proximal part (approximately 5 cm) was opened, and carefully rinsed with tap water to remove all visible intestinal contents, thus minimizing potential PCR inhibition by faecal material; from an area of *ca* 3 × 3 cm, the mucosa was then scraped with a sterile scalpel blade into an Eppendorf tube. In addition, a faecal sample was collected from the large intestine. These samples were stored at −20°C until processed for PCR amplification.

The distal part of the ileum sample (the last 5 cm before the ileocaecal valve) was fixed in 7% neutral buffered formalin and a transverse section (0.5 cm thick) from the middle was embedded in paraffin wax. Sections (2 μm) were cut and mounted on glass slides for either haematoxylin and eosin (HE) staining or Warthin–Starry (WS) silver staining according to Bancroft and Cook (1994). Sections in a second set were mounted on Super Frost Plus slides (Menzel Gläser, Braunschweig, Germany) for immunohistochemistry and in-situ hybridization.

For PCR amplification from paraffin wax-embedded tissue, four sections (10 μm) from each block were dewaxed with two washes in xylene for 5 min at room temperature and then washed twice with 95% ethanol. After each washing step the samples were centrifuged for 5 min at 15 000 *g*, resulting in the formation of a visible pellet at the bottom of the tube. This pellet was dried under vacuum for 30 min before the lysis buffer (Nexttec Biotechnologie, Leverkusen, Germany) was added. For DNA extraction from paraffin wax-embedded tissue or mucosal scrapings, a commercial extraction kit was used according to the manufacturer's instructions (Genomic DNA Isolation Kit Tissue & Cells; Nexttec Biotechnologie). DNA from faecal samples was extracted with the QIAamp® DNA Stool Mini Kit (Qiagen, Hilden, Germany).

PCR

The efficacy of DNA extraction from mucosal scrapings and from wax-embedded tissue was controlled by amplification of zinc finger x-encoded and zinc finger y-encoded genes (Aasen and Medrano, 1990).

For specific detection of *L. intracellularis*, the extracted DNA was subjected to PCR with the Eppendorf® HotMasterMix (2.5×) (Eppendorf, Hamburg, Germany), which contains HotMaster Taq DNA Polymerase (50 U/ml), 2.5× HotMaster Taq Buffer pH 8.5 with 6.25 mM $Mg(OAc)_2$, 500 μM of each dNTP and stabilizers, according to the manufacturer's instructions. The primers used (5′-TATGGCTG TCAAACACTCCG-3′ and 5′-TGAAGGTATTGG TATTCTCC-3′) were initially described by Jones *et al.* (1993) and then successfully applied in a number of studies (e.g., Dittmar *et al.*, 2003; Guedes and Gebhart, 2003; Jacobson *et al.*, 2004, 2005). PCR procedures based on these primers are therefore considered to be of high specificity and sensitivity. The PCR was performed in a volume of 25 μl, containing 11 μl H_2O, 10 μl Eppendorf® HotMasterMix (2.5×), 1 μl forward and 1 μl reverse primer, and 2 μl DNA template. The thermocycling was performed in a GeneAmp® PCR system 9700 (Applied Biosystems, Vienna, Austria), as follows: initial denaturation at 94°C for 2 min, followed by 40 cycles with denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 1 min. The final

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