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Challenges with molecular diagnostics (PCR): Ileitis

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Introduction

Proliferative enteropathy (PE) is a common infectious disease that affects weaned animals of various ages[1]. It is of particular economic importance in the pig industry. The disease occurs worldwide in pigs and produces variable clinical manifestations, including a chronic form, termed porcine intestinal adenomatosis, and an acute form, called proliferative hemorrhagic enteropathy.

Both forms of the disease have very unique histologic features, which are proliferation of the intestinal epithelial cells with the presence of many intracellular curved organisms in the apical cytoplasm of these proliferating cells. The etiologic agent of the disease is *Lawsonia intracellularis,* an obligate intracellular bacterium. Identification of this intracellular organism has enabled the recent development of specific assays for use in diagnosis of the disease in affected pigs.

Review of PE diagnostics

Clinical signs

PE is difficult to diagnose because its clinical signs are non-specific, or even lacking, especially with the chronic form. A presumptive diagnosis of PE can be made on the basis of herd history and clinical signs, but neither of these is specific to PE.

Postmortem diagnosis

PE may be diagnosed postmortem based on typical gross lesions, but usually histological confirmation is necessary. Evaluation of proliferation by demonstration of proliferative enterocytes by routine staining may be subjective and only cases with typical severe enterocyte proliferation can be diagnosed. Warthin Starry silver stains may demonstrate the organism within the proliferating epithelial cells, but this method is not specific for *L. intracellularis* and cannot always detect the organism in necrotic debris or in autolyzed tissue.

More specific identification of *L. intracellularis* in these lesions can be achieved by immunohistochemistry staining of fixed tissues. This technique is more sensitive than the silver stain because it reveals organisms within macrophages of the lamina propria during recovery from PE.

Also, extracellular Lawsonia can be identified in either exudate or necrotic debris in the superficial mucosa. In a study comparing diagnostic methods, immunohistochemistry staining detected nearly twice as many pigs with PE lesions as did silver staining of formalinized tissues[2].

If immunohistochemistry is not available, specific identification of *L. intracellularis* in the intestine postmortem can be achieved by PCR of the ileal mucosa. This technique is available in many diagnostic laboratories and, when applied to ileal mucosa rather than feces, is as specific as immunohistochemistry[3].

But how can we diagnose PE in pigs without necropsy? Because of the limitations of postmortem diagnostic procedures, efforts have focused on the development of antemortem testing methods. Antemortem tests are more useful for performing prevalence and epidemiological studies and for monitoring the infection in individual animals over time.

Antemortem diagnosis of PE

Several techniques have been described for the detection of *L. intracellularis* in live pigs. Culture of the organism from feces is not an option because the agent is an obligate intracellular bacterium. Other methods of antemortem diagnosis include detection of *L. intracellularis* in feces by indirect antibody staining[2] or PCR[4] and serological assays for *Lawsonia* antibodies. using either an indirect fluorescent antibody test (IFAT)[3] or an immunoperoxidase monolayer assay (IPMA)[5].

Indirect antibody technique

Demonstration of *L. intracellularis* in feces can be done using a specific monoclonal antibody[6] in an indirect antibody staining technique. It is a standard laboratory technique that is not affected by PCR inhibitors. However, the technique requires a specific monoclonal antibody and some expertise to evaluate results. Also, like PCR, the technique lacks sensitivity for diagnosing subclinically affected animals. In one study using this technique, we were able to detect more pigs with PE lesions than did the fecal PCR test, particularly during the period when clinical signs were most severe[2].

Serological assays

Serological assays are based on the detection of *L. intracellularis* antibodies in serum. Current methods employ *Lawsonia* cultured in an enterocyte monolayer or a preparation of the organism on slides as the antigen. When the cell cultures or slides are examined microscopically, specifically stained bacteria can be distinguished from any background material. Staining of bacteria is either by a fluorescent (indirect fluorescent antibody test, or IFAT) or peroxidase-labeled (immunoperoxidase monolayer assay, or IPMA) secondary antibody. Serology results were similar when using the IFAT or IPMA tests and both were found to be equally sensitive and specific in evaluation of experimentally infected animals[3]. Using IFAT, Knittle et al.[3] detected 90% of inoculated pigs three to four weeks post-inoculation, showing serology to be more sensitive for detecting positive pigs than PCR of fecal samples. A limitation of the serological test is the necessity of *in vitro* maintenance of *L. intracellularis* as a source of antigen for the test. Also, serological results only indicate previous exposure to *L. intracellularis*, not current or previous disease status.

PCR assays

The PCR assay is a very sensitive test that was developed to detect organisms that are present in low numbers in samples or that are difficult to detect by culture, microscopy, or immunodiagnostic techniques. PCR is advantageous in that it is able to detect animals currently infected with *L. intracellularis* and shedding the organism in their feces.

Several PCR assays have been developed for specific detection of *Lawsonia* in feces[4,7,8]. These assays utilize primers that are specific for either a chromosomal[4] or a 16S rRNA gene sequence[8] of *L. intracellularis*. Direct PCR utilizes one or more sets of primers that target a specific area of the chromosome. Amplified products are visualized on ethidium bromide-stained agarose gels.

Nested PCR[7] increases the sensitivity of the direct PCR technique and may overcome any potential problems with non-specific amplification of DNA from fecal samples. However, the increased sensitivity of the nested PCR technique is accompanied by the necessity for increased technical expertise in performing the procedure as any amplicon contamination of samples renders the procedure non-specific.

Use of 16S rRNA sequences as PCR primers generally increases the sensitivity of the technique because of the high copy number of 16S rRNA in many bacteria. In reality, this increased sensitivity has not been realized in PCR assays with *L. intracellularis* 16S rRNA primers[8]. The copy number of 16S rRNA genes in *L. intracellularis* is not known, but is likely to be low.

In conventional PCR, confirmation of PCR products is time-consuming and expensive. A PCR method for the direct detection of amplified product involving enzymatic labelling of the PCR products combined with solid phase detection (enzyme linked oligosorbent assay or ELOSA) has been developed and validated for specific detection of *L. intracellularis* chromosomal DNA from clinical specimens[9]. Comparison of ethidium bromide-stained agarose gel analysis with ELOSA for detection of *L. intracellularis*-specific PCR products from 315 clinical specimens revealed 78% sensitivity and 100% specificity[9].

The sensitivity and specificity of many of these PCR techniques for *L. intracellularis* in fecal samples have been evaluated in many reports, which show variable sensitivity (depending upon which comparisons are made) and consistently high specificity (about 97%). Although PCR is inherently a highly sensitive technique, its sensitivity in feces is limited by a number of factors, including sample quality, the presence of inhibitory factors in feces, and the amount of other competing DNA in the sample. Feces contain proteins and other inhibitory substances that negatively affect the ability of the DNA polymerase enzyme to amplify specific fragments of target DNA and thus inhibit the PCR assay. Also, large amounts of competing non-target DNA, as found in feces, make it difficult for specific PCR primers to anneal to any target DNA present.

Various DNA extraction techniques have been evaluated for the optimal amplification of *L. intracellularis* DNA from feces. Some of these techniques minimize the effect of inhibitory substances. Lysis of feces in a guanidine thiocyanate buffer, followed by extraction of DNA by binding to diatomaceous earth and ethanol precipitation is a widely used technique[4]. Boiling of fecal samples in a lysis buffer and then dilution of samples for direct PCR is a simpler DNA extraction technique[10]. Dilution, however, may lead to reduced sensitivity due to decreased template DNA. Commercial fecal DNA extraction kits, such as Wizard Genomic DNA Purification Kit (Promega Corporation, Madison, WI) and QIAamp DNA Stool Mini Kit (Qiagen, Inc., Valencia, CA) have also been used successfully for extraction of *L. intracellularis* DNA from feces.

Methods for separation of target bacteria or DNA from feces have been evaluated as preliminary steps to PCR to remove target DNA from potential inhibitors. Separation of target bacteria from feces using immunobased methods prior to PCR require a specific and avid antibody to *L. intracellularis*, which is not widely available. A hybridization magnetic capture PCR system was modified to detect *L. intracellularis* from feces[11]. The hybridization capture probe separates target DNA from potential PCR inhibitors found in feces. The initial hybridization capture step also specifically binds *L. intracellularis* DNA and enriches the fecal sample with specific DNA for PCR

amplification. The protocol was evaluated for its ability to minimize problems with inhibitory substances and large quantities of competing DNA in feces and was found to be similar in sensitivity to the nested PCR technique for the detection of *L. intracellularis*. It was effective in detecting *L. intracellularis* shed in the feces of pigs with experimentally-produced PE but did not detect any more pigs shedding *L. intracellularis* than did direct PCR.

None of the PCR techniques in use are able to detect *L. intracellularis* in feces of pigs up to one week post-inoculation. Pigs affected with PE shed more than 2 (10^5 *L. intracellularis* organisms per gram of feces at certain timepoints post-exposure[7,12] and, since the sensitivity of PCR for detection of fecal shedding has been reported to be from 10^2 to 10^4 *L. intracellularis* per gram of feces[4,7], the sensitivity of the technique should be adequate during active shedding periods. Shedding of *L. intracellularis*, however, occurs in cyclical patterns when monitored in feces collected at weekly intervals[3]. Detection of *L. intracellularis* over a number of sequential fecal samplings increases the certainty of detecting shedding of individual pigs.

In spite of its lack of sensitivity, fecal PCR has been used to monitor *L. intracellularis* infection in individual pigs, to do epidemiological studies on infected pigs, and to estimate the prevalence of *L. intracellularis* in pig herds worldwide. The sensitivity of the technique is adequate to demonstrate *L. intracellularis* in the feces of clinically affected pigs but not sensitive enough to detect the organism in the feces of subclinically affected animals.

Comparison of different methods for the diagnosis of PE

We conducted a comparative study to test the sensitivity of various PE diagnostics, including serology, three histologic techniques, and fecal PCR and IPX in pigs experimentally infected with *L. intracellularis*.

Forty pigs were inoculated with intestinal homogenate from PE-diseased pigs on day 0. Clinical evaluation was done on day 7 and daily from days 14 to 28 post-inoculation. Fecal shedding of *L. intracellularis* was monitored by use of direct PCR analysis at 7-day intervals. Serum was obtained on days 0 and 28 for serological testing by IFAT. At euthanasia on day 28, ileum samples were collected for histologic analyses, which included staining by hematoxylin and eosin, Warthin Starry silver stain, and immunohistochemistry.

The peak of the disease occurred at the end of the third week when up to 29 of 40 pigs had diarrhea. **Table 1** summarizes the sensitivity results obtained for each diagnostic test. Immunoperoxidase staining of fecal smears was more sensitive than PCR for detecting fecal shedding, especially on day 21 (89.5% versus 60.5%, respectively) and day 28 (59.4% versus 37.5%, respectively) post-inoculation. The immunohistochemistry stain was much more sensitive for detecting infection than the hematoxylin and eosin and Warthin Starry silver stains.

Based on the challenge model, we can conclude that serology seems to be an appropriate technique for detecting *L. intracellularis*-infected pigs, that PCR of fecal samples has low sensitivity and that immunohistochemistry is the best diagnostic tool for formalin-fixed samples.

Summary

To summarize antemortem diagnostics, PCR detection of Lawsonia in feces is an adequate test for PE when necropsy is not possible. It can detect pigs currently infected with *L. intracellularis*, but the sensitivity is limited. The indirect antibody detection of *Lawsonia* in feces appears to be more sensitive because it is not affected by PCR inhibitors, but its specificity is not known. Serology is a sensitive test for detection of pigs that have been exposed to Lawsonia, and is specific when using a pure culture antigen along with an IFAT or IPMA detection

Table 1: Sensitivity of *L. intracellularis* diagnostic tests based on the number of positive animals detected by the test divided by the number of affected pigs determined by the pre-defined criteria (animals positive by fecal PCR at any collection date and/or positive by immunohistochemistry)

Diagnostic test	Sensitivity (%)
IFAT serology	91.2
Fecal PCR (day 14 post-challenge)	71.1
Fecal PCR (day 21 post-challenge)	60.5
Fecal PCR (day 28 post-challenge)	37.5
Fecal IPX (day 14 post-challenge)	76.3
Fecal IPX (day 21 post-challenge)	89.5
Fecal IPX (day 28 post-challenge)	59.4
Hematoxylin & eosin	36.8
Warthin Starry silver stain	50.0
Immunohistochemistry	86.8

system. However, serology detects previous exposure rather than current disease. A combination of serology and fecal PCR may be used for cross-sectional or serial disease profiling to determine the timing of exposure of pigs to Lawsonia. This information will enable development of more strategic methods of prevention and control of the disease. Serology profiles can be related to clinical disease or fecal shedding of *L. intracellularis* on pig farms and these results can be used to confirm diagnoses, detect subclinical PE, and determine the timing of exposure to *L.intracellularis*.

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