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Comparison of different methods for diagnosis of porcine proliferative enteropathy

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Proliferative enteropathy (PE) is an intestinal infectious disease caused by the obligate intracellular bacterium, *Lawsonia intracellularis*[1,2]. Although first reported in 1931, research interest was minimal until the early 1970s. This disease has already been described in several species including the rat, guinea pig, rabbit, ferret, emu, pig, monkey, deer, and horse[3]. PE has been best described in swine and it occurs worldwide. The economic losses are caused by two major clinical aspects of the disease: firstly, acute hemorrhagic diarrhea and death; secondly, chronic diarrhea and reduced growth performance. Despite some economic loss estimates present in the literature[4,5], the prevalence of PE in the field around the world remains poorly known. The main explanation for this situation is the lack of availability of accurate methods for diagnosis of the disease.

There are three ways to diagnose PE in live animals:

- serology,
- PCR (polymerase chain reaction) of fecal samples, and
- immunoperoxidase (IPX) of fecal smears.

Lawson et al.[6] and Holyoake et al.[7], using *L. intracellularis* antigen from scraped mucosa of PE affected pigs in an indirect immunofluorescence antibody test (IFA) and enzyme linked-immunosorbent assay (ELISA), respectively, showed results of low and variable antibody titers in inoculated pigs. In contrast, Knittel et al.[8], using an IFA test with pure culture of *L. intracellularis* as antigen, detected a *L. intracellularis*-specific serum titer in 90% of inoculated pigs three to four weeks after challenge.

The sensitivity and specificity of the PCR technique in fecal samples has been evaluated in many reports[9,10]. The specificity is good but there are inhibitory factors within fecal specimens[11] that could hamper the sensitivity of the PCR amplification test. In contrast to the PCR technique, inhibitory factors present in feces do not affect the IPX technique in fecal smears. However, the monoclonal antibody against *L. intracellularis* used in the IPX technique is not widely available.

During post mortem examination of acute or chronically affected animals, the observed macroscopic lesions are frequently not pathognomonic for PE. As a result, formalin fixed samples must be submitted for further histologic examination. Three different staining methods can be used to diagnose PE:

- hematoxylin and eosin (routine histologic stain),
- Warthin Starry silver stain, and
- immunohistochemistry (IHC) using mouse monoclonal antibodies against *L. intracellularis*[12].

Using hematoxylin and eosin, only cases with typical severe enterocyte proliferation can be diagnosed. Using Warthin Starry silver stain the presence of intracellular curved-shaped bacteria can be observed in the cytoplasm of proliferated enterocytes. Finally, using IHC the monoclonal antibodies specifically label antigens of *L. intracellularis* in tissue sections[2]. There is no information in the literature comparing the accuracy of these tests to diagnose PE in experimentally infected animals.

The aim of this study was to compare two different methods of serology tests, three histologic techniques, and two methods to detect shedding of *L.* intracellularisin experimentally infected pigs.

Material and methods

Forty 5-week-old pigs of mixed gender (20 gilts and 20 barrows) and weighing between 20 and 30 pounds were obtained from a herd with no history of PE or any other confounding diseases. All pigs were inoculated with an intestinal homogenate from PE-diseased mucosa via stomach tube on days 0 and 1. All pigs received two doses of 25ml of the inoculum and the total dose given per pig was 3.4×10^9 *Lawsonia intracellularis* organisms. Clinical observations were made on day 7 and daily from 14 to 28 days post-challenge. These observations included fecal consistency and aspect, behavior, appetite, and body condition.

Fecal samples collected at 14, 21, and 28 days post-challenge were analyzed by PCR—using primers as described elsewhere[9]—and by IPX stain, using the method of labeled Streptavidin (DAKO, K765) with mouse monoclonal antibodies[12].

Blood samples were collected at days 0 and 28 post-challenge. Serum samples were tested by an indirect immun-

ofluorescent antibody technique (IFA) using two different antigen preparation methods, tissue culture plates and glass slides. Acetone-fixed 96-well culture plates with McCoy cells highly infected with *L. intracellularis* and 15-well glass slides coated with whole bacteria purified from the supernatant of flasks of *L. intracellularis* pure culture were used for serology. All serum samples were titrated by both methods and analyzed by the Spearman Rank correlation test.

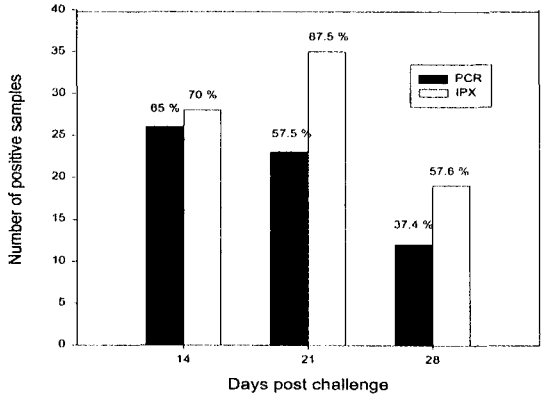
All pigs were euthanized 28 days after inoculation. Samples of ileum were fixed by immersion in 10% neutral buffered formalin and processed routinely for histology. One section was hematoxilin and eosin stained, one Warthin Starry silver stained, and one section was stained using the IHC method of peroxidase labeled Streptavidin (DAKO, K765) with mouse monoclonal antibodies to *L. intracellularis*[12].

Results

By day 7 post-challenge, 9 of the 40 pigs (22.5%) had semi-solid diarrhea with no sign of blood. By day 14 post-challenge, 21 pigs (52.5%) had diarrhea; 13 of them had semi-solid diarrhea and 8 watery diarrhea without any sign of blood. Days 17–22 were the most prevalent period of diarrhea with up to 29 animals affected, some (6 animals) with blood-tinged feces. Up to 15 of the animals with diarrhea were lethargic between day 20 and 25 post-challenge. In general, the animals tended to recover from this critical period until day 28, when all the pigs were humanely euthanized. Four pigs had to be euthanized during the experiment, on days 21, 23, 24, and 26 post challenge, owing to very poor clinical conditions. Each of these four pigs had severe watery diarrhea for four or more days.

The results of immunoperoxidase and PCR of fecal samples are shown in **Figure 1**.

Figure 1: Comparison between the results of the PCR and immunoperoxidase (IPX) techniques in fecal samples from days 14, 21, and 28 post-challenge



All serum samples were negative using both serology tests on day 0. Thirty-six serum samples were tested on day 28 post-challenge owing to the death of four pigs. As shown in **Table 1**, 31 samples were positive and 3 were negative in both serological tests (94.4% agreement). Only two samples had different results between the tests. The titers of the samples tested by both test methods ranged from 1:30 to 1:960 and were highly correlated (r=0.72).

Upon histological examination of ileum sections stained by hematoxylin and eosin, 14 out of 40 challenge pigs (35.0%) had evident immature enterocyte proliferation typical of PE. Using Warthin Starry silver stain it was possible to detect 19 out of 40 challenge pigs (47.5%), including those 14 diagnosed by hematoxylin and eosin. And using IHC, 33 out of 40 challenge pigs (82.5%) were diagnosed PE-positive, including those diagnosed by Warthin Starry silver stain and hematoxylin and eosin.

Conclusions

The IPX technique in fecal smears detected more positive pigs among the challenged animals than did the fecal PCR test throughout the experiment. The difference between these two tests was more evident on days 21 and 28 post-challenge, primarily on day 21, when the clinical signs were more severe.

Using the IHC stain in tissue samples, it was possible to diagnose PE in almost twice as many pigs as by Warthin Starry silver stain (82.7% and 47.5%, respectively). It was also possible to diagnose more than twice as many pigs by IHC than by hematoxylin and eosin (82.7% vs. 35.0%). The great advantage of IHC over Warthin Starry silver stain and hematoxylin and eosin was the ability to detect *L. intracellularis* antigen even when only present in the cytoplasm of macrophages in the lamina propria, or in exudate or necrotic debris in the superficial mucosa. These results show the importance of the monoclonal antibody for diagnosing PE in fecal smears and formalin fixed tissue samples.

Serology IFA showed similar results when using either cell culture and glass slides. Serology IFA tests have been shown to be sensitive and specific in evaluation of ex-

Table 1: Serology results using tissue culture plate and glass slide IFA methods on day 28 post-challenge.

	Slide positive	Slide negative	Subtotal
Culture plate positive	31	1	32
Culture plate negative	1	3	4
Subtotal	32	4	36

perimentally infected animals[8]. Both cell culture IFA and glass slide IFA seem to be good options for epidemiological studies in the field.

In conclusion, these results indicate the importance of the immune-based tests for diagnosing PE.

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