Diagnosis of infection with *Lawsonia intracellularis* in swine

By

Jeffrey Paul Knittel

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Jeffrey Paul Knittel

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CHAPTER 1. GENERAL INTRODUCTION

Thesis Organization

The following thesis consists of a general introduction, review of the literature, three separate manuscripts, a general summary, and acknowledgments. The master's candidate, Jeffrey Paul Knittel, is the senior author and principal investigator for all manuscripts.

Introduction

Porcine Proliferative Enteropathy (PPE) is a common disease of swine. The disease is defined as a thickening of the mucosa of the small, and sometimes the large, intestine.[1] Characteristic features of the disease are proliferation of the crypt epithelial cells, thickening of the mucosa of the small intestine and the upper third of the spiral colon, and the presence of small, curved, intracellular organisms in the apical cytoplasm of proliferating cells.[1]

Histopathological lesions of the disease include epithelial hyperplasia, especially in the mucosal crypts. There is also a distinct absence of goblet cells. The proliferating epithelial cells contain intracytoplasmic, slender, curved, rod-shaped bacteria.[2]

The intracellular bacteria associated with PPE were previously and incorrectly referred to as "Campylobacter-like" organisms. The causative agent has now been identified and is considered to be a novel taxonomic genus and species, previously known by the vernacular name Ileal Symbiont (IS) intracellularis.[3] Recently the organism has been given the name *Lawsonia intracellularis*.[4]

Growth of *L. intracellularis* on conventional media has not been successful. The bacteria have been cultivated on tissue culture cells in a reduced oxygen atmosphere of 5.0-8.0% O_2 and 7.0% CO_2.[5,6] Pure cultures of *Lawsonia* (Ileal Symbiont) *intracellularis* were used to satisfy Koch's postulates in pigs in 1993.[7]

Due to the difficulty of culturing the organism, isolation of the bacteria in pure culture is not a realistic method for diagnosing PPE. Diagnosis has usually been based on histopathology and silver staining of tissue sections for the demonstration of the morphologic forms of *L. intracellularis*. These methods, however, require post-mortem tissues from the pig. Newer, more sensitive detection techniques (e.g., polymerase chain reaction [PCR]) can be performed on live pigs by testing for shedding of *L. intracellularis* in the feces.[8,9] A monoclonal antibody specific for an outer membrane component *of L. intracellularis* has also been developed that has been shown to be effective in confirming the presence of the organism in affected tissues using indirect immunofluorescent or immunoperoxidase assays.[10]

Serologic tests employing enzyme-linked immunosorbent (ELISA) and immunofluorescent assays have also been evaluated. In one study the predominant antibody class stimulated by infection with *L. intracellularis* was IgM as determined by an indirect fluorescent antibody test (IFAT).[11] This response persisted for about eight weeks post-inoculation. In another study, an ELISA test was developed to measure the IgG response against *L. intracellularis* in the sera of pigs.[12] Results indicated low and variable antibody titers in inoculated pigs. These findings led to the conclusion that IgG plays a minor role in the immune response to *L. intracellularis* infections.

An indirect fluorescent antibody test (IFAT) has recently been developed that detected IgG in the serum of pigs exposed to *L. intracellularis*. The test employs antigen grown in pure culture which is then stained using serum from test pigs as the primary antibody and fluorescein-labeled antiswine-IgG as the secondary antibody. The test was shown to be more sensitive than PCR for the identification of experimentally exposed pigs.[13]

Economic losses caused by PPE are difficult to estimate. In 1987, losses due to PPE were estimated to be $10 to $20 million annually.[14] More recent estimates are not available. A survey of farms in the United Kingdom indicated that 30% had a diagnosis of chronic PPE in the past three years.[15] In Australia, a survey indicated that 56% of the farms were affected by PPE.[16] A survey of 73 farms in Spain showed that 22% of the farms had pigs infected with *L. intracellularis* as detected by PCR of the feces.[17]

The purposes of the following studies were to obtain a better understanding of the diagnostic tests that are currently available and develop an improved diagnostic test to aid in determining the prevalence and impact of PPE, caused by *Lawsonia intracellularis*.

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CHAPTER 2. *LAWSONIA INTRACELLULARIS* AND ITS RELATIONSHIP WITH PORCINE PROLIFERATIVE ENTEROPATHY

A paper to be submitted for publication in the
Compendium on Continuing Education for the Practicing Veterinarian

Jeffrey P. Knittel

Introduction

Porcine Proliferative Enteropathy (PPE) is a common disease of swine. The disease is characterized by a thickening of the mucosa of the small, and sometimes the large, intestine.[1] Characteristic features of the disease are proliferation of the crypt epithelial cells, thickening of the mucosa of the small intestine and the upper third of the spiral colon, and the presence of small, curved, intracellular organisms which are in the apical cytoplasm of proliferating cells.[1]

Although proliferative enteropathy is most commonly reported in swine it has been described in hamsters,[2] ferrets,[3] rabbits,[4] foxes,[5] dogs,[6] rats,[7] horses,[8] sheep,[9] deer,[10] emus,[11] ostriches,[12] and guinea pigs.[13] Intracellular bacteria were observed in the proliferating epithelium of all of the mentioned species. Evidence suggests that the infectious agent may be common to all animal species affected with proliferative enteropathy.[1]

Lesions

The disease has a wide variety of clinical symptoms along with a variety of descriptive terminologies. Porcine intestinal adenomatosis (PIA) describes the lesion in which the intestinal mucosa is thickened due to epithelial proliferation, similar to a benign tumor of glandular structure, but is relatively free from inflammation, or has only mild

inflammatory lesions on the mucosal surface. PIA occurs most often in pigs 6 to 20 weeks of age and presents with mild clinical signs usually consisting of a failure of the pig to gain weight.[1] Necrotic enteritis (NE) describes deep coagulative necrosis of an adenomatous mucosa. NE is the endstage condition of PIA in which the severe thickening of the ileum or "hose-pipe" ileum occurs, usually accompanied by persistent diarrhea.[1] Proliferative hemorrhagic enteropathy (PHE) descibes massive hemorrhage into the intestinal lumen from proliferated mucosa.[1] Proliferative hemorrhagic enteropathy (PHE) is differentiated from hemorrhagic bowel syndrome (HBS) in which there is no abnormal crypt proliferation and hemorrhage occurs throughout all layers of the intestinal wall. This is most likely due to an entirely different etiology.[14] HBS occurs in growing animals and is an acute syndrome associated with high mortality.[1]

Histopathological lesions common to all forms of PPE include epithelial hyperplasia, especially in the mucosal crypts. The crypts are elongated, enlarged, and lined with crowded immature epithelial cells with mitotic figures. There is also a distinct absence of goblet cells. The proliferating epithelial cells contain intracytoplasmic, slender, curved, rod-shaped bacteria.[15]

Etiology

Presence of the intracellular bacteria with hyperplasia of crypt cells is the definitive feature of PPE.[16] A variety of *Campylobacter* species have been isolated from lesions of PPE. The most common species are *Campylobacter mucosalis*[16] and *C. hyointestinalis,*[17] both of which are morphologically similar to the intracellular bacterium associated with proliferative lesions. It was noted that *C. mucosalis* was isolated from lesions of pigs with

necrotic enteritis but was not present in the alimentary tract of normal pigs.[18] *Campylobacter mucosalis* is also morphologically similar to the intracellular organism (*Lawsonia intracellularis*) by electron microscopy.

Efforts to reproduce PPE with pure cultures of *C. mucosalis* were met with limited success. Pigs inoculated with cultures of *C. mucosalis* remained infected for 40 days and were able to spread the organism to other pigs, but none of the pigs developed lesions of PPE in the ileal mucosa.[19] The only successful reproduction of PPE with *C. mucosalis* was reported by Lomax et al. in which 4 of 10 cesarean derived pigs fed cultures of *Salmonella choleraeesuis* and *C. mucosalis* developed lesions.[20]

Campylobacter hyointestinalis was isolated from 67% of swine with PPE and only 5% of swine with other enteric diseases.[17] *Campylobacter coli* is also often found in swine populations. However, cultures of *C. hyointestinalis* as well as *C. coli* have failed to reproduce the disease despite their ability to colonize the intestine for long periods.[21,22,23,24]

Several experiments have been performed with tissue homogenates from pigs with PPE. Roberts et al. reported reproduction of the disease using freshly homogenized adenomatous mucosa in two orally inoculated pigs.[25] It was later reported that the disease could be reproduced in both specific pathogen free (SPF) pigs[20] and in cesarean-derived colostrum-deprived (CDCD) pigs[26] as well as in four to five-week-old conventional pigs[27] using intestinal homogenates derived from pigs with PPE.

The difficulty encountered in determining the true causative agent of PPE led to a confusing history of names for the etiologic agent. The bacteria were commonly referred

to as *Campylobacter*-like organisms for many years based on their morphological similarity to *Campylobacter* species.

The bacteria which were finally isolated and determined to be the "real" cause of PPE were given the vernacular name Ileal Symbiont intracellularis and were identified as a distinct genus which differed from *Campylobacter* species. DNA sequences of the 16S ribosome from this organism were found to be most similar (91%) to that of a sulfate-reducing proteobacterium, *Desulfovibrio desulfuricans*.[28] The name *Lawsonia intracellularis* was formally given to the organism in 1995 in honor of G. H. K. Lawson, the discoverer of the bacterium.[29] *Lawsonia intracellularis* is described as gram-negative, microaerophilic, obligate intracellular, non-flagellated, non-spore-forming curved or S-shaped bacillus.[30]

Isolation and growth of *Lawsonia intracellularis*

Growth of the bacteria in cell-free media has not been successful. *Lawsonia intracellularis* has been cultivated on tissue culture cells (rat intestinal cells[IEC-18],[30] human fetal intestine [Int 407],[30] rat colonic adenocarcinoma cells,[30] pig kidney [PK-15],[30] piglet intestinal epithelial cells [IPEC-J2][31] and GPC-16[32])when incubated at reduced oxygen of 5.0-8.0% O_2 and 7.0% CO2.[30,32] Isolation of the organism from infected tissues requires homogenization and trypsinization of the tissue, passage of the homogenate through a series of filters down to a pore size of 0.65 μm, and storage of the filtrate containing the organism in a sucrose potassium glutamate solution with 10% FBS at -70° C.[30]

The development of *in vitro* cultivation methods has provided information on the manner in which the bacteria infect cells in the host. It was shown that the bacteria gain entry into the host cell by close association with the cell membrane.[31] The bacteria enter via membrane-bound vacuoles which soon break down and release the bacteria to multiply free in the cytoplasm. It was shown that infection of cells is dependent on cell activity, but not on bacterial viability.[33] A component of the entry process appears to be microfilament dependent but there is also evidence for a non-actin dependent pathway. This is similar to the manner in which *Chlamydia* species enter the host cell. However, *Chlamydia* remain within vacuoles throughout their life cycle.[34] *Lawsonia intracellularis* are often seen in close association with mitochondria and rough endoplasmic reticulum.[35] This mode of entry and growth is similar to some *Rickettsia* species[36,37] however, *Rickettsia* species belong to the alpha subdivision and *L. intracellularis* belongs to the delta subdivision of *Proteobacteria* making them genetically dissimilar.[29,38] Intracellular growth of the bacteria produces little cell morphologic change or lysis.[30,39,40] It was also observed that bacteria were released from infected cells within cytoplasmic protrusions.[31] These findings are similar to what has been observed *in vivo* in host animal studies.[2,35,41]

Reproduction of disease

Koch's postulates were fulfilled in 1993 when PPE was produced in pigs inoculated with pure cultures of *L. intracellularis*.[42] Conventional pigs developed the disease when orally inoculated with *L. intracellularis*, but gnotobiotic pigs did not become colonized or develop the disease. The study also showed that severity of disease is dose dependent further demonstrating that *L. intracellularis* is the primary etiologic

agent of PPE. It was later shown that gnotobiotic pigs would develop lesions if exposed to avirulent strains of *Bacteroides vulgatus or Escherichia coli* prior to inoculation with pure cultures of *L. intracellularis*.[43]

Use of pure culture challenge models has provided information regarding transmission of the organism within herds. It has been demonstrated that pigs given pure culture have been shown to excrete the bacteria up to 10 weeks after inoculation.[44] A seeder-pig sentinel model demonstrated direct transmission of the organism to penmates as well as non-direct transmission to non-penmates located within the same room.[45] This same study also demonstrated that the infective dose of *L. intracellularis* to be low and that the organism is easily transmitted.

This evidence suggests that weaned pigs can maintain the infection within a herd by fecal excretion of the organisms to penmates, leading to newly infected pigs. The persistence of excretion may lead to some female pigs remaining infected and contagious until their first pregnancy. If this occurs, offspring can be infected in the late suckling period transferring the infection to the next generation.[44]

Diagnosis

Development of diagnostic techniques has been key in understanding the true prevalence of the disease as well as in estimating the economic impact. Until recently the only way to diagnose PPE has been by examination of dead animals for macroscopic and microscopic lesions. Hematoxylin and eosin stains (H & E) of tissue sections allow the diagnostician to evaluate intestine for subtle to dramatic proliferative changes.[46] Silver (Warthin-Starry) stains are used to verify the presence of intracellular, curved, rod-shaped

organisms.[15,1] A modified Ziehl-Neelsen stain on mucosal smears can also provide a simple confirmatory test,[15,1] as *L. intracellularis* will stain red within the cytoplasm of infected cells. These stains are commonly used in most diagnostic laboratories. However, diagnosis using these stains requires post-mortem tissues.

A monoclonal antibody against an outer membrane component *of L. intracellularis* was developed that has been shown to be effective in confirming the presence of the organism in affected tissues through incorporation into an indirect fluorescent antibody or immunoperoxidase assay.[47] The antibody, which has been shown to be specific for *Lawsonia intracellularis,* has been key in identifying the causative agent of proliferative enteropathy of many of the host animal species mentioned earlier. This antibody has also demonstrated that different isolates of *Lawsonia intracellularis* are antigenically similar.[39] The antibody can also be used to confirm shedding of *L. intracellularis* in the feces by staining fecal smears.[47] Use of the antibody has been shown to be a sensitive and specific method of detecting *L. intracellularis* in tissue sections.[48] Again, this requires post-mortem tissues. In order to use as an ante-mortem test on fecal smears, the animal must be actively shedding the organism at fairly high numbers. The antibody is not commercially available at this time.

A hybridization technique was recently developed using DNA probes that were developed from a genomic library of *L. intracellularis* DNA cloned into a plasmid vector.[49] The probes would hybridize *in situ* to test samples from both proliferative hemorrhagic enteropathy (PHE) cases and porcine intestinal adenomatosis (PIA) cases, confirming that the intracellular organisms seen in both the acute and chronic forms of

disease share the same sequences. This demonstrated that the causative agents of the two conditions are identical or genetically similar.

These DNA probes have also been used to develop a polymerase chain reaction (PCR) procedure to produce a highly sensitive, less time consuming assay for the detection of the bacteria in feces from infected animals.[50] The test proved to be a useful tool for detecting infected pigs from field cases as well as from experimentally infected animals.[51] Other studies demonstrated that PCR could identify infected groups of pigs using pooled fecal samples,[50] It also demonstrated that pigs shed *L. intracellularis* in the presence and absence of clinical signs of disease,[48] and that shedding was sporadic and that pigs shed intermittently during an infection.[45,48] DNA primers specific for *Serpulina hyodysenteriae, Salmonella* sp., and *L. intracellularis* have been developed into a multiplex PCR assay that allows the simultaneous detection and identification of each of these agents in swine feces.[53] This test may reduce the time and cost of detecting each of these pathogens.

Serologic tests employing enzyme-linked immunosorbent (ELISA) and immunofluorescent assays also have been evaluated. In one study the predominant antibody class stimulated by infection with *L. intracellularis* was IgM, as determined by an indirect fluorescent antibody test (IFAT).[54] The presence of IgM antibodies directed against *L. intracellularis* was detected for about eight weeks post inoculation. However, for prevalence surveys, detection of an antibody of longer duration, i.e. IgG, would be necessary for accurate results. In another study, an ELISA test was developed to measure the IgG response against *L. intracellularis* in the sera of pigs.[55] The test

animals for this study were either hyperimmunized by intramuscular injection of percoll gradient-purified bacteria from the intestinal mucosa of PHE-affected pigs, or orally inoculated with intestinal homogenates from a proliferative hemorrhagic enteropathy-affected 6-week-old pig. Results indicated low and variable IgG antibody titers in inoculated pigs. These findings led to the conclusion that IgG plays a minor role in the immune response to *L. intracellularis* infections.

An indirect fluorescent antibody test (IFAT) has been developed that detects IgG in *L. intracellularis* exposed pigs.[56] In contrast to the tests previously defined, the test employs antigen grown in pure culture that is then stained using serum from test pigs as the primary antibody and fluorescently labeled antiswine-IgG as the secondary antibody. The test was shown to be more sensitive than PCR for the detection of exposed pigs. It was also shown to be specific for pigs exposed to *L. intracellularis*. A possible reason for the greater success of the IFAT over the ELISA test is that background difficulties were avoided with the IFAT. Each sample was directly observed for fluorescing bacteria by the person performing the test allowing a distinction to be made between nonspecific reactions and the positively reacting bacterial antigen. An ELISA does not allow this distinction.[56] This test can be used as an ante-mortem test to diagnose exposed pigs. Also, the reagents used for the test are less expensive than those used for PCR. However, pigs that are serologically positive are not necessarily infected at the time of testing, but may have been previously exposed to *L. intracellularis*. Seroconversion that can be detected by the IFAT occurs 2 to 4 weeks after exposure.[56] A summary of the diagnostic techniques is given in table 1.

Table 1. Summary of Diagnostic Techniques

Diagnostic Technique	Method	Comment	Limitation
H and E Stain	Histopathological examination for characteristic	Specific	Post-mortem Diagnosis
Silver Stain	Histopathological evaluation for intracellular bacteria	Specific	Post-mortem Diagnosis
Monoclonal antibody	Fluorescent Antibody or Immunoperoxidase stain	Specific	Reagents not commercially available
PCR	Amplification of a DNA fragment of *L. intracellularis*	Specific, shows pigs actively shedding	Possibility of false negative results when testing feces
Serology/ELISA	IgG Response	Variable/Low sensitivity	Test not routinely performed
Serology/IFAT	IgG Response	Sensitive	Demonstrates pigs that have been exposed but not necessarily have an active infection, Seroconversion typically takes 2-4 weeks after exposure.

Epidemiology

With the availability of more sensitive techniques of detecting *L. intracellularis* infected pigs, estimates of the impact of the disease have been revised. Early reports stated a low prevalence of PPE of 0.89% to 2.5% in swine herds.[1] More recent reports have suggested that 5 to 20% of the swine herds had lesions at slaughter with incidence in some herds reaching 40%.[57] Economic losses caused by PPE are difficult to estimate. In 1987, estimates of $10 to $20 million annual losses were made.[58] More recent estimates are not available.

A survey in the United Kingdom indicated that 30% of the farms had a diagnosis of chronic PPE during a three year period.[59] In Australia, a survey indicated that 56% of the farms had a diagnosis of PPE by the producer, consulting veterinarian, or both

between 1988 and 1990.[60] A survey of 73 farms in Spain using PCR indicated that 22% of the farms had pigs infected with *L. intracellularis* as detected by PCR on feces.[61] This estimate is believed to be low, as shedding of *L. intracellularis* in the feces is sporadic as described earlier. A survey of Iowa State University's Veterinary Diagnostic Laboratory swine cases found that 5% of all pigs submitted were positive for *L. intracellularis* by PCR testing of the intestinal mucosa.[62] This study included all swine cases submitted for a period of 6 months (621 total cases) regardless of age or clinical history. The study found that the majority of pigs with *L. intracellularis* were from the nursery and grow-finish pigs. The National Animal Health Monitoring System's national 1995 swine study surveyed a total of 198 swine herds.[56] Results demonstrated that 90% of the herds tested were either serologically positive for PPE in the breeding herd, the growing/finishing herd, or both. In growing/finishing herds, over 50% of the herds had greater than or equal to 40% of the samples positive per herd with anti-*L. intracellularis* antibodies. In breeding herds, 21% of the herds had greater than or equal to 40% of the samples per herd with positive results.[63]

Using pure cultures of *Lawsonia intracellularis* to infect animals, McOrist et. al. demonstrated a 6% to 25% reduction in weight gains in infected pigs.[59] The costs of extra feed and increased time within a facility in the United Kingdom were estimated to be £2 to £7 per pig. Based on the number of pigs in the UK (14.5 million slaughtered annually) and with an estimate of 35% of the pigs affected with PPE,[64] using the market value of a pig at slaughter at the time of the article, the total direct cost of the disease in the UK was estimated to be £2 to £4 million annually.[59] Other investigators have made

similar estimates of the cost of PPE within infected herds. Recent estimates of up to $8.50 per pig have been made in the United States.[65]

Antibiotic treatment/prevention

With the development of pure culture systems, advances have been made in the testing of antimicrobial agents against *L. intracellularis*. Studies using *in vitro* cell culture systems determined the minimal inhibitory concentrations (MIC) of several antibiotics. Penicillin, erythromycin, difloxacin, virginiamycin, and chlortetracycline had the best results with MIC's of < 1 μg per ml. Tiamulin and tilmicosin had MIC's of < 4 μg per ml.[66] These compounds are capable of entering the eukaryotic host cell cytoplasm and blocking prokaryotic protein synthesis.[67] It is cautioned that *in vitro* results may not always correlate well with *in vivo* efficacy because of the pharmacodynamics of the drug involved. For example, the drug would need to be capable of killing microaerobic gram-negative intracellular bacteria located in the epithelium of the lower bowel. Also, some drugs may not readily penetrate into cells or may localize in inappropriate parts of the cell such as in cell lysosomes,[68] while *L. intracellularis* is free within the cytoplasm.[1]

Host animal trials have shown that several antibiotics are effective in the control of PPE. Tiamulin (50 g/ton) as a feed grade medication has been tested in several pure culture trials.[69,70] Results suggest that tiamulin is an effective antibiotic for the treatment and prevention of PPE.

Carbadox (50g/ton), erythromycin (70 g/ton) and chlortetracycline (100 g/ton) were shown to be effective in preventing PPE after gut homogenate challenges.[71,72]

Neomycin-oxytetracycline (150/150 g/ton) and virginiamycin (10 g/ton)[71] were not effective for the control of PPE even though the MIC's of tetracycline and virginiamycin were low.[66]

Tylosin (100 g/ton) was shown to be an effective drug against PPE in controlled field trials.[73] The antibiotic has recently been approved by the Food and Drug Administration (FDA) for the control and treatment of ileitis in the United States.

Conclusion

Porcine proliferative enteropathy is caused by *Lawsonia intracellularis*, a gram negative, obligately intracellular, microaerophilic bacteria. Disease features are hyperplasia of crypt cells in the intestinal mucosa with the absence of goblet cells. Proliferating cells contain intracytoplasmic, curved, rod-shaped bacteria.

The bacteria can be cultivated in tissue culture cells and grown in reduced oxygen atmospheres. Pure cultures have been used to reproduce disease which has helped in the understanding of the disease as well as aided in the development of improved diagnostic techniques. Antibiotic sensitivity testing has shown that the bacteria are susceptible to several antibiotics, some of which aid in the prevention and treatment of PPE. Recent epidemiological surveys have shown that the disease is worldwide with estimates of 30 to 90% of the farms affected. The disease can have a significant impact on performance resulting in costly losses for the producer.

With the tools available, veterinarians and producers are getting better equipped to diagnose PPE. To confirm that a pig is infected, a post-mortem diagnosis using H & E stain, silver stain, monoclonal antibody stain, or PCR of the intestinal mucosa can be

done. To most producers, an ante-mortem test is more desirable. PCR can be used to demonstrate animals that are actively shedding *L. intracellularis* and can also be used to estimate incidence of the organism by testing a percentage of pigs within a herd. The IFAT test on serum can be used as an ante-mortem test to demonstrate pigs that have been exposed to *L. intracellularis*. The test can also be used for surveys to predict incidence of the organisms by testing a percentage of pigs within a herd and estimating how many have been exposed to *L. intracellularis*. The IFAT test may be used to estimate herd shedding/exposure patterns by testing multiple samples over a period of time.

As diagnostic tests become more sensitive, detection of the disease will become more efficient. Proper diagnosis of PPE will enable the producer to take the proper course whether it is for prevention or for treatment. As with any diagnostic test, the correct interpretation of the results will be key to making these decisions.

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CHAPTER 3. UNITED STATES ISOLATES OF *LAWSONIA INTRACELLULARIS* FROM PORCINE PROLIFERATIVE ENTEROPATHY RESEMBLE EUROPEAN ISOLATES

Jeffrey Knittel, David Larson, D.L.Hank Harris, Michael Roof, Steven McOrist

Abstract

Three isolates of *Lawsonia intracellularis* (*L. intracellularis*) were isolated from the intestines of American pigs with porcine proliferative enteropathy. The organism was grown and passaged in a rat enterocyte cell line (IEC-18). Growth was not associated with morphological cell change and was monitored by immunostaining of the cells using a monoclonal antibody and by polymerase chain reaction (PCR). The PCR products were evaluated by DNA sequencing and compared to a known sequence from *L. intracellularis*. *Lawsonia intracellularis* is a microaerophilic, gram negative, curved or s-shaped bacillus and is an obligately intracellular organism. These isolates resemble European isolates by PCR, monoclonal antibody reactions, and growth requirements.

Introduction

Porcine proliferative enteropathy (PPE) is a major disease of swine worldwide and continues to be a cause of economic losses in herds including cesarean-derived breeding stock and specific pathogen free (SPF) status pigs. A consistent disease feature of PPE is the thickening of the mucosa of the small and sometimes of the large intestine due to hyperplasia of the crypt enterocytes. The prevalence and incidence of PPE are unknown,

however estimates in the United States have been as high as 20% of the swine herd with losses of $10 to $20 million annually.[1]

The intracellular bacteria associated with PPE were previously and incorrectly referred to as "Campylobacter-like" organisms. The causative agent has now been identified and is considered to be a novel taxonomic genus and species, previously known by the vernacular name Ileal Symbiont (IS) intracellularis.[2] Recently the organism has been given the name *Lawsonia intracellularis* gen., nov., sp. nov.[3] *Lawsonia intracellularis* is an obligate intracellular bacterium and cannot yet be cultivated on conventional cell-free media. Methods for culturing and maintaining *L. intracellularis* have recently been developed in cell cultures.[4] The disease has been reproduced with pure cultures of these bacteria in orally dosed pigs.[5,6] Conventional pigs dosed orally with approximately 3.7×10^6 bacteria passaged six times in cell culture developed severe lesions of proliferative enteropathy and *L. intracellularis* was reisolated from the lesions 21 days after challenge.[5] This study compares *L. intracellularis* successfully cultured from United States pigs to the characteristics previously defined for European isolates.

Materials and Methods

Selection of inoculum samples--Sample N24912 was obtained from a herd in Iowa in which fifteen of 300 five month old finisher pigs were observed to have persistent bloody stools. Penicillin treatment had been attempted. Upon necropsy of the pigs, the mucosa of the ileum was found to be thickened. Histopathological examination with silver stains demonstrated the presence of curved intracellular bacteria and crypt enterocyte hyperplasia confirming the diagnosis of PPE.

Sample N72994 was obtained from a 1.5 year old second-parity SPF sow from a herd in Minnesota. The herd size was between 70-80 sows and antibiotic treatment was unknown. Upon necropsy, the mucosa of the ileum was thickened with some hemorrhage. Giminez staining of the mucosa revealed many curved bacteria.

Sample N101494 was obtained from a 12 week old pig from an Indiana farm with 600 farrow to finish sows. The pig was treated with injectible Tylan® upon the onset of bloody diarrhea, but the pig died soon after treatment.

Preparation of pig derived bacterial inocula--Intestinal samples were kept at -70° C until processing. Bacteria were isolated by methods previously described.[4] The purified bacterial samples were filtered once through filterpaper (Whatman 113V, Whatman Labsales, Hillsboro, OR), then sequentially through 5.0, 1.0 and 0.65 μm membrane filters. Filtrates were aliquoted and frozen at -70° C in 1 ml aliquots. The mucosa from each case was smeared onto a slide for Giminez stain. Separate smears of filtrates were stained by immunofluorescence assay using a specific monoclonal antibody for *L. intracellularis*[7] as the primary antibody and anti-mouse immunoglobulin G-fluorochrome conjugate (fluorescein isothiocyonate, Organon Teknika Corporation, Durham, NC) as the secondary antibody.

Cell Culture--IEC-18 cells (Rat intestinal epithelial cells, ATCC CRL 1589) were grown in Dulbecco's Modified Eagle's Medium (DMEM) (JRH Biosciences, Lenexa, KS) with L-glutamine and 10% FCS and routinely passaged by trypsin weekly. Cell monolayers were grown at 37° C in air with 5% CO_2.[4]

Infecting the cell culture—The IEC-18 cells were infected as previously described.[4] The cultures were placed in anaerobic jars in which the gas was then replaced with H_2 and CO_2 to give a mixture of 8.0% O_2, 10% CO_2 and 82% H_2. The cultures were incubated for 3 hours at 37° C, then refed with DMEM/7% FCS with L-glutamine, Vancomycin (100 µg/ml), Neomycin (50 µg/L), and Amphotericin B (2.0 µg/ml). The cultures were placed back in the anaerobic jars at gas concentrations stated above and incubated for 6 days with media changes every 2 days.[4]

Passage of L. intracellularis--Intracellular bacteria were passed by cell lysis using potassium chloride as described previously[4] then added to fresh IEC-18 monolayers.

Monitoring infection of cell cultures--Infection was monitored by fixing the cells with cold acetone/methanol for 5 min. Staining was carried out by immunofluorescence and immunoperoxidase methods. Both methods employed a mouse monoclonal antibody[7] as the primary antibody and either anti-mouse immunoglobulin G-fluorochrome conjugate (fluorescein isothiocyonate, Organon Teknika Corporation, Durham, NC) or peroxidase conjugate (goat anti-mouse immunoglobulin G, Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD). Bacteria were quantified by counting the numbers of specifically stained bacteria within cells on each slide.

Polymerase chain reaction--Sample inocula and passaged bacteria were incorporated as template DNA into PCR using the sample preparation method and primers as described by Jones et al.[1] The primer set, 5'-TATGGCTGTCAAACACTCCG-3' and 5'-TGAAGGTATTGGTATTCTCC-3' was selected for the DNA amplifications. Cycle parameters were 93° C for 5 minutes, 55° C

for 45 seconds, and 72° C for 45 seconds for the first cycle. Thirty-three cycles were performed at the previously mentioned temperatures for 45 seconds per temperature. The final cycle was 93° C for 45 seconds, 55 for 45 seconds, and 72° C for 2 minutes. Positive inocula only were used to inoculate IEC-18 cells. Polymerase chain reaction was also performed to monitor passage material to confirm infections. DNA produced by PCR was submitted to the Iowa State University Nucleic Acid Facility for sequencing. Results of the sequencing were compared to sequences obtained by Gary Jones.[8]

Results

Selecting inoculum samples--Pig numbers N24912 and N72994 had severe proliferative enteritis with bloody intestinal contents and thickened mucosa. N101494 had severe proliferative enteritis and severe hemorrhage resulting in a large blood clot in the intestinal lumen. Giminez staining of the mucosal smears demonstrated large numbers of curved or S-shaped bacteria. Indirect-fluorescent antibody (IFA) assay stains revealed large numbers of brightly fluorescing bacteria in pig-derived bacterial inocula.

Monitoring infection of cell cultures--Inoculated monolayers were monitored by light microscopy throughout the growth cycle and little morphological change of the cells was observed. Uninfected monolayers grown under reduced oxygen tension (8% O_2) had similar morphology.

Large numbers of curved or S-shaped specifically stained bacteria apparently within cells were observed in immunofluorescence and immunoperoxidase stained infected cultures (Fig. 1). The monolayers did not have confluent infection. Infected cells were often clumped together with infected foci of 1-10 cells. Heavily infected cells

Fig. 1. IEC-18 cells infected with N72994 IFA stained using anti-*L. intracellularis* antibodies. Bar = 10 μm.
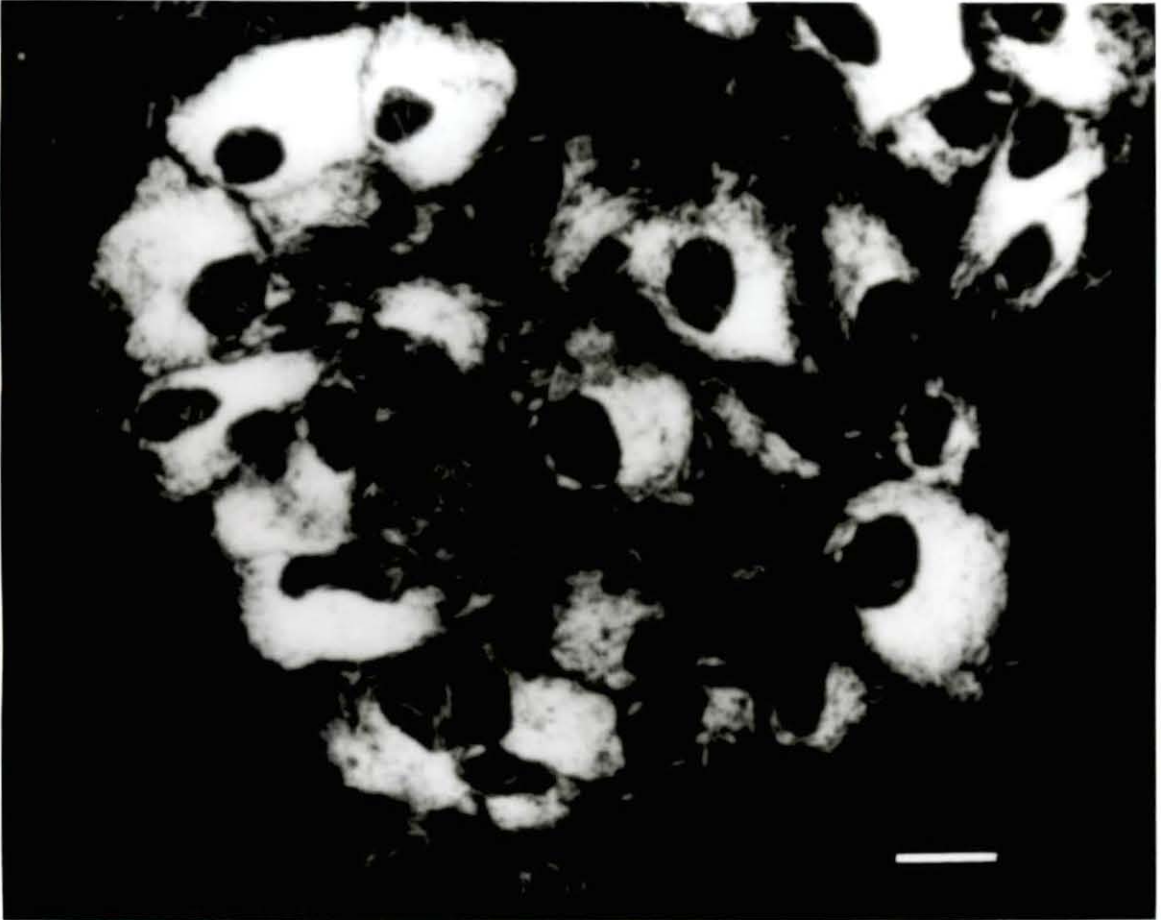
(cells with 30 or more bacteria) were also seen near cells with fewer than 30 bacteria. Bacterial numbers peaked at or about 6 days.

Infection was dependent on specific growth conditions. A gas mixture of 8% O_2 and 10% CO_2 was necessary to support growth (other gas mixtures were attempted but not successful-data not shown). The bacteria were successfully passaged by the cell lysis procedure.[4]

Polymerase chain reaction—Polymerase chain reaction of chromosomal DNA generated a 319 bp fragment (including primers) from all isolates (Fig. 2). A fragment of appropriate size was visually compared to strain 916/91 (NCTC 12657), a known positive sample generated by McOrist using PCR. Sequence analysis of the PCR products of N24912, N72994, and N101494 confirmed greater than 99% homology to the p78 sequence determined by Gary Jones.[8]

Discussion

This study reports the successful cultivation of isolates of *L. intracellularis* from United States pigs. The current understanding of PPE has been hampered by the fastidious growth requirements of *L. intracellularis*. Porcine proliferative enteropathy has been experimentally reproduced in pigs dosed orally with homogenized adenomatous mucosa originating from United States pigs.[9] This has been effective in causing disease; however, it is difficult to determine the infectious dose or whether other organisms were involved in causing lesions. Reproducing the disease with pure cultures of *L. intracellularis* using European isolates was demonstrated by McOrist et. al.[3] Investigators have been able to reproduce PPE in pigs from a pure culture of the United

Fig 2. Agarose gel of PCR products from isolates grown in IEC-18 cells showing 319 bp

fragments. Lane 1, N101494; Lane 2, N72994; Lane 3, N24912; Lane 4, X174 Hae III

marker.
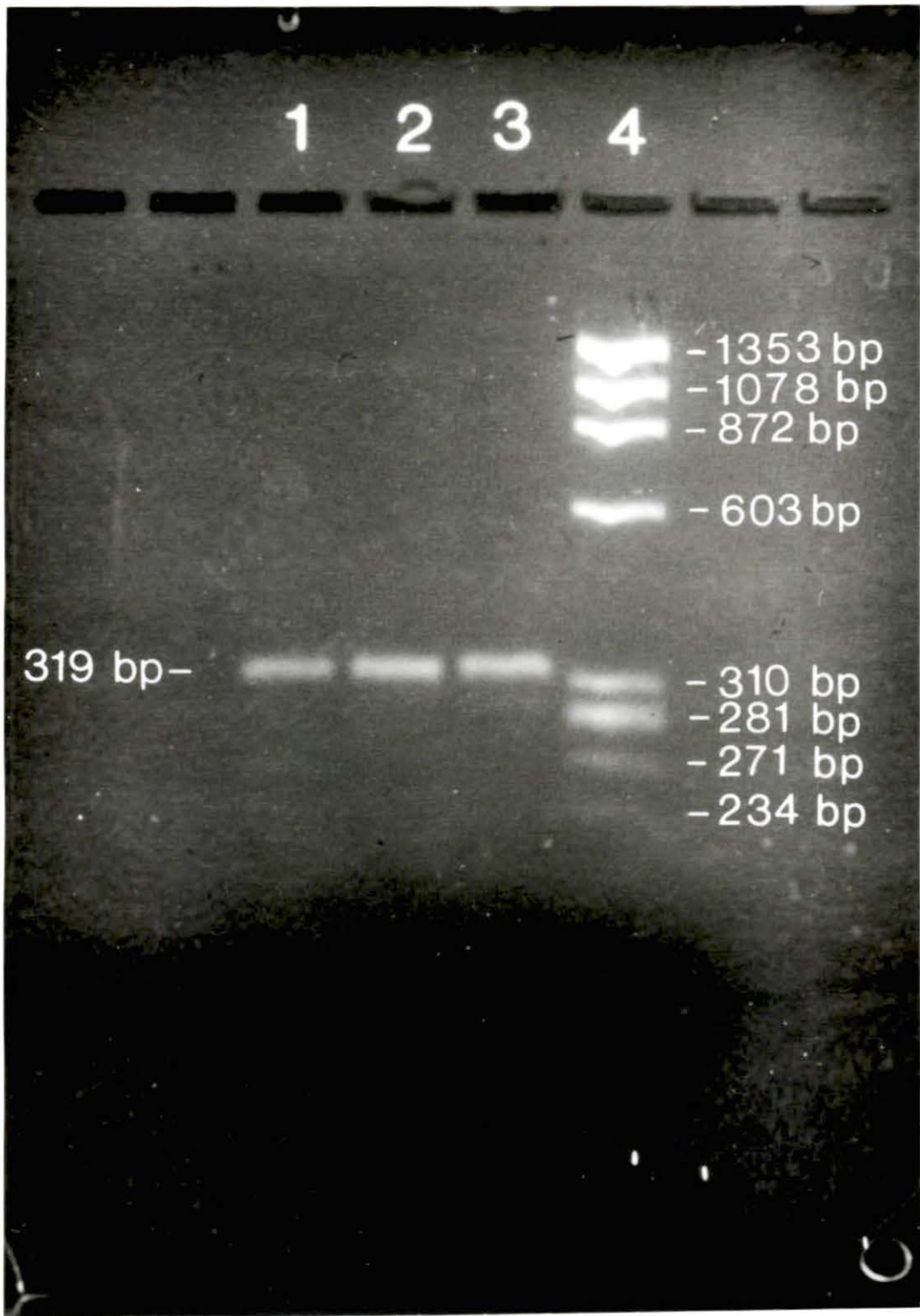
States strain N72994.[6] This demonstrated that the organism in pure culture could cause disease in pigs after passage in cell culture.

Clinical signs of PPE are often difficult to identify in a field situation. Early clinical signs are often associated with reduced weight gain, most often occurring between 5 and 12 weeks of age.[10] Previous epidemiological studies have had limited success due to the difficulty of culturing the organism and limited success of serum testing.

IgA, IgM,[11] IgG[12] antibodies have been detected in infected animals; however, titers are low or short lived. Lawson et al. reported that pig sera of known disease status had no IgA response if there was no IgM response.[11] Those that did have an IgA response showed low titers. The IgM response to the bacteria was short-lived and persisted for about eight weeks.[11] As reported by Holyoake, et al., pigs experimentally infected with intestinal homogenates produced low IgG titers in response to infection of *L. intracellularis,* suggesting that IgG plays a minor role in preventing PPE in pigs.[12] To date, serological diagnosis has not been effective due to low antibody titers and short duration. Polymerase chain reaction techniques have been established, allowing a more sensitive detection of *L. intracellularis* in PPE cases.[1,13] Culturing *L. intracellularis* required specialized tissue culture techniques, including a reduced O_2 tension and the use of intestinal epithelial tissue culture cells. The bacteria did not produce cytopathic effects on the monolayers, including cells that were heavily infected. These growth characteristics are similar to the growth characteristics of European isolates reported by Lawson et. al.[4]

Conditions and storage of the intestinal samples were also critical. Ileal sections selected for culture should show severe lesions with gross thickening of the gut. Due to the fragile nature of the bacteria, samples were stored frozen and maintained at -70° C as quickly as possible after necropsy. Samples stored at higher temperatures resulted in reduced infection or no infectivity. If the intestine could not be frozen at -70° C immediately, then it was stored on ice until it was delivered to a facility capable of long term storage.

The ability to grow isolates of L. intracellularis from the United States is a major advance in the understanding of PPE that will allow us to test for virulence and develop a challenge model as well as provide an opportunity to study the pathogenesis of *L. intracellularis* in vitro.

Acknowledgments

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CHAPTER 4. DIAGNOSIS OF PORCINE PROLIFERATIVE ENTERITIS

Jeffrey P. Knittel, Kent J. Schwartz, Steven McOrist,

Michael B. Roof, Dianna M. Jordan, D.L. Harris

Abstract

Lawsonia intracellularis, an obligately intracellular bacterium, is the causative

agent of Porcine Proliferative Enteritis. Thirty-one pigs at 6 weeks of age were randomly

divided into 4 groups and the groups were penned separately. Groups 1, 2, and 3 with 8

pigs in each group were dosed with a pure culture of *L. intracellularis* strain N72994

derived from a 1.5 year old second parity SPF sow. Group 1 was dosed with 3.5×10^6

$TCID_{50}$/pig. Group 2 was dosed with 3.5×10^5 $TCID_{50}$/pig. Group 3 was dosed with

3.5×10^4 $TCID_{50}$/pig. Group 4 had 7 pigs and were dosed with non-infected tissue

culture. Fecal shedding of *Lawsonia intracellularis* was monitored using PCR and was

shown to peak 21 days after inoculation. PCR testing of the ileal mucosa 24 days after

challenge revealed the presence of *L. intracellularis* in 100% of the animals in Group 1,

75% of the animals in Group 2, and 63% of the animals in Group 3. Histopathology

results indicated an increase of mononuclear cells in the lamina propria and submucosa of

88% of the animals in Group 1, 75% of the animals in Group 2, and 88% of the animals

in Group 3. Crypt cell hyperplasia was observed in 50% of the animals in Group 1, 63%

of the animals in Group 2, and 50% of the animals in Group 3. Specific fluorescent

antibody (FA) staining revealed *L. intracellularis* in tissue sections of the ileum in 88%

of the animals in Group 1, 63% of the animals in Group 2, and 63% of the animals in Group 3. Group 4 animals were negative for the presence of *L. intracellularis* via PCR, FA, and silver stains. In conclusion, a pure culture of the U.S. isolate, *L. intracellularis* strain N72994, was able to colonize and produce disease in swine that appears to be dose dependent. PCR detection was found to be more sensitive on mucosas than silver stains and more specific than hematoxylin-eosin stains.

Introduction

Porcine Proliferative Enteritis (PPE) is a major disease of swine worldwide. It continues to be a cause of economic losses, even when swine producers use breeding stock delivered by cesarean section and specific pathogen free (SPF) pigs. Clinical cases of PPE are observed most commonly in pigs between 6 and 20 weeks of age.[1] Early reports stating a low prevalence of PPE of 0.7% to 1.63%.[2-4] were derived from slaughterhouse pigs of a wide age range. More recent reports have suggested that 5% to 20% of the swine herds had lesions at slaughter; prevalence in some herds reaching 40%.[5] Economic losses are difficult to estimate. The most recent estimates, from 1987, of $10 to $20 million annually were made.[6]

Pathology

Consistent features of PPE are thickening of the mucosa of the small, and sometimes the large intestine as a result of hyperplasia of crypt enterocytes along with a decrease in goblet cells.[1] A further consistent feature is the presence of curved intracellular bacteria within the cytoplasm of crypt cells.

The intracellular bacterium associated with PPE was previously called "Ileal Symbiont (IS) intracellularis".[7] The bacterium has since been assigned to a novel taxonomic genus and species (*Lawsonia intracellularis* gen., nov., sp. nov.).[8] It is an obligate intracellular bacterium and cannot yet be cultivated in conventional cell-free

media. Methods for culturing and maintaining *L. intracellularis* in cell cultures have been developed.[9]   The disease has been reproduced by feeding pure cultures of these bacteria to pigs.[10] Conventional pigs fed approximately 3.7 x 10^6 bacteria passed six times in cell culture developed severe lesions of PE. *L. intracellularis* was reisolated from the lesions 21 days after challenge.[10]

Diagnosis

Due to the difficulty of culturing the organism, isolation of the bacteria in pure culture is not a realistic method for diagnosing PPE. Diagnosis has usually been based on histopathology and silver stain. These methods, however, necessitate killing the pig. Newer, more sensitive detection techniques (e.g., polymerase chin reaction [PCR]) can be performed on live pigs and will allow better estimates of incidence.[11-13]

A PCR test of feces can be used to determine whether a pig is shedding the infective agent. This test may, however, yield false-negative results for *L. intracellularis* infection. Infected animals do not always shed the organism Whether a fecal PCR for an infected pig is positive may depend on the stage or severity of infection. The best way to confirm the diagnosis of PPE has been postmortem histopathology involving H & E and Warthin-Starry silver stains.

Better diagnostic technology is essential for developing an effective treatment regimen. To compare the currently available diagnostic techniques, we infected 6 week old pigs with three different dilutions of a U. S.-derived isolate of *L. intracellularis* grown in pure culture and used PCR, Warthin-Starry silver stain, hematoxylin and eosin stain, and immunofluorescent antibody stain for the diagnosis of PPE.

Thirty-one conventional pigs were delivered from SPF sows from a herd with no recorded cases of PPE. The piglets were weaned at 21 days. At day 0 (6 weeks of age) of the experiment, the pigs were randomly divided by weight into four groups and fecal swabs were collected for PCR testing. Group 1 pigs then received undiluted inoculum. Groups 2 and 3 received different dilutions of the inoculum. Five of the seven pigs in group 4 received noninfected tissue culture; the other two received nothing.

The pigs were subsequently weighed on days 10, 17 and 24. Clinical signs were evaluated on days 0, 3, 7, 10, 14, 17, and 20 (Table I). Fecal swabs were collected on days 0, 7, 14, 21, and 24. All pigs were euthanized 24 days after dosing. Samples of jejunum, ileum, and colon were immersed in 10% buffered formalin for fixation. Sections were stained by routine hematoxylin and eosin (H & E) stains and Warthin-Starry silver stains for light microscopy. Sections were also stained by an indirect immunofluorescent assay that incorporated a monoclonal antibody against *L. intracellularis*.[14]

Table I.   Incidence of Diarrhea

		Positive Animals/Total Animals (%)						
Group	Day 0	Day 3	Day 7	Day 10	Day 14	Day 17	Day 20
Group 1	0/7 (0)[a]	3/8 (38)[a]	4/8 (50)[a]	5/8 (63)[a]	6/8 (75)[a]	1/8 (13)[a]	4/8 (50)[a]
Group 2	0/7 (0)[a]	0/7 (0)[b]	2/8 (25)[ab]	4/8 (50)[ab]	2/8 (25)[ab]	0/7 (0)[a]	0/7 (0)[b]
Group 3	0/7 (0)[a]	0/7 (0)[b]	1/8 (13)[ab]	1/8 (13)[bc]	3/8 (38)[b]	0/7 (0)[a]	1/8 (13)[b]
Group 4	0/7 (0)[a]	0/7 (0)[b]	0/7 (0)[b]	0/7 (0)[c]	0/7 (0)[b]	0/7 (0)[a]	0/7 (0)[b]

Groups with same letter are not significantly different (P>0.05).

Results

At necropsy, gross lesions were found in two pigs from group 1 (which received the undiluted inoculum). Approximately 1 m of the small intestine had severe thickening in both and the intestine was necrotic in one of these pigs. No gross lesions were found in the other pigs in the experiment.

Microscopic lesions consistent with PPE included an increase of mononuclear cells in the lamina propria and submucosa (H&E stain), crypt enterocyte hyperplasia (H&E stain), and the presence of intracellular organisms within affected enterocytes (Warthin-Starry silver stain and FA stain). These lesions were evident in pigs from groups 1, 2, and 3 (Table II). Intracellular organisms were always associated with inflammatory exudates in the crypts. Cells that contained bacteria were generally quite immature. One of the seven pigs in the control group had increased numbers of mononuclear cells in the lamina propria and submucosa, with no enterocyte proliferation. All control animals were free of *L. intracellularis* as determined by silver stain.

Table II. Microscopic Evaluations

| | Positive Animals/Total Animals (%) | | | |
| | H&E Stain | | | |
	Mononuclear infiltration	Crypt Hyperplasia	Silver Stain	FA Stain
Group 1	7/8 (88)[a]	4/8 (50)[a]	7/8 (88)[a]	7/8 (88)[a]
Group 2	6/8 (75)[a]	5/8 (63)[a]	5/8 (63)[a]	5/8 (63)[a]
Group 3	7/8 (88)[a]	4/8 (50)[a]	7/8 (88)[a]	5/8 (63)[a]
Group 4	1/7 (14)[b]	0/7 (0)[b]	0/7 (0)[b]	0/7 (0)[b]

Groups with same letter are not significantly different ($P>0.05$).

Immunofluorescent Antibody stains

Immunostaining of affected intestinal sections with monoclonal antibody against *L. intracellularis* showed specifically stained bacteria in the apical cytoplasm of 88% of the pigs in group 1, and 63% pigs in groups 2 and 3 (Figure 1). No bacteria were observed in the group 4 pigs.

Polymerase Chain Reaction

Samples that were positive by PCR produced a 319 bp product, as detected by gel electrophoresis, when amplified with primers specific for *L. intracellularis* (Figure 2). Fecal shedding was first observed at day 14 in one pig from group 1 (Table III). Three pigs from group 1 were positive by PCR at day 21. No pigs from the other groups were positive at day 14 or 21. At day 24, two pigs in group 1, no pigs from group 2, and one pig from group 3 were positive by PCR. Pigs that shed during the experiment were also the most heavily infected, as determined by post-mortem FA stain. At necropsy, PCR testing of mucosal scrapings of the ileum for *L. intracellularis* resulted in 100% of group 1, 75% of group 2, and 63% of group 3. No positive PCR results for control pigs were observed during the experiment.

Table III.   Polymerase Chain Reaction results

Group	Day 0 Fecal	Day 7Fecal	Day 14Fecal	Day 21Fecal	Day 24Fecal	Day 24 Mucosa
			Positive Animals/Total Animals (%)			
1	$0/7\ (0)^a$	$0/7\ (0)^a$	$1/8\ (13)^a$	$3/8\ (38)^a$	$2/8\ (25)^a$	$8/8\ (100)^a$
2	$0/7\ (0)^a$	$0/7\ (0)^a$	$0/7\ (0)^a$	$0/7\ (0)^b$	$0/7\ (0)^a$	$6/8\ (75)^{ab}$
3	$0/7\ (0)^a$	$0/7\ (0)^a$	$0/7\ (0)^a$	$0/7\ (0)^b$	$1/8\ (13)^a$	$5/8\ (63)^b$
4	$0/7\ (0)^a$	$0/7\ (0)^a$	$0/7\ (0)^a$	$0/7\ (0)^b$	$0/7\ (0)^a$	$0/7\ (0)^c$

Groups with same letter are not significantly different (P>0.05).

Fig 1. Section of ileal mucosa stained with monoclonal antibody to *Lawsonia intracellularis*. The crypt cells are heavily infected. (Bar = 10 μm)
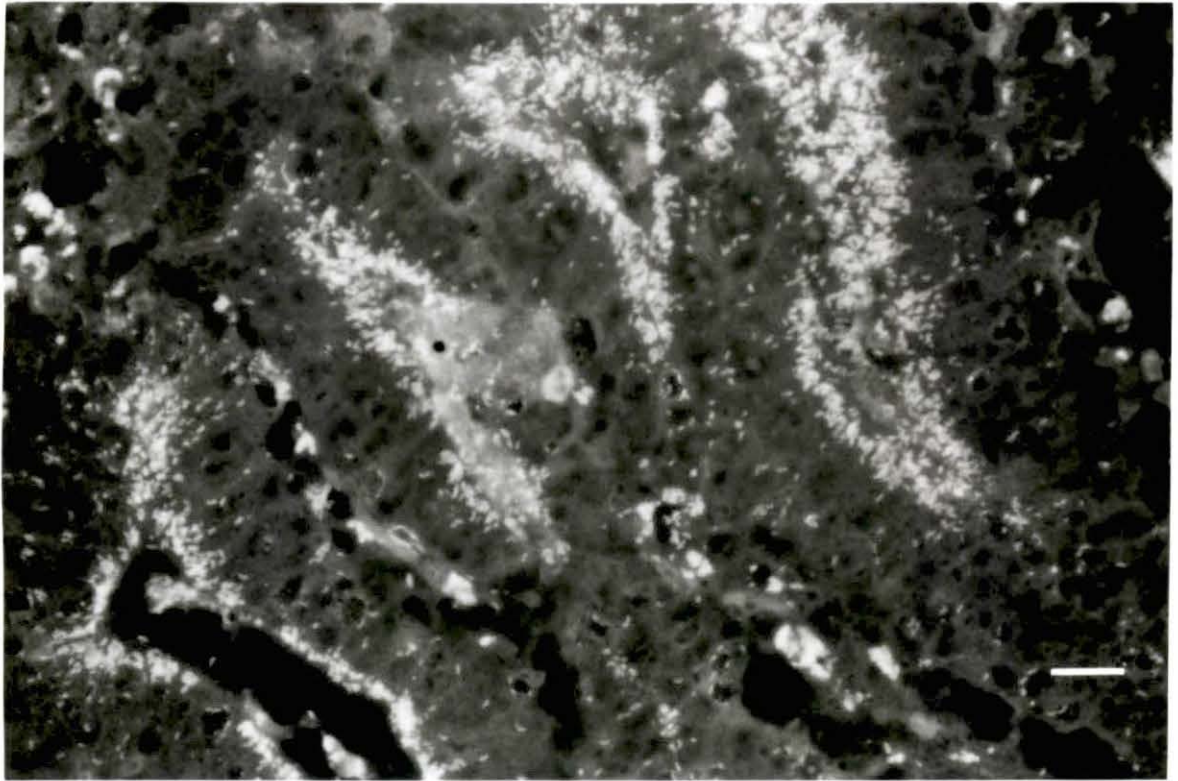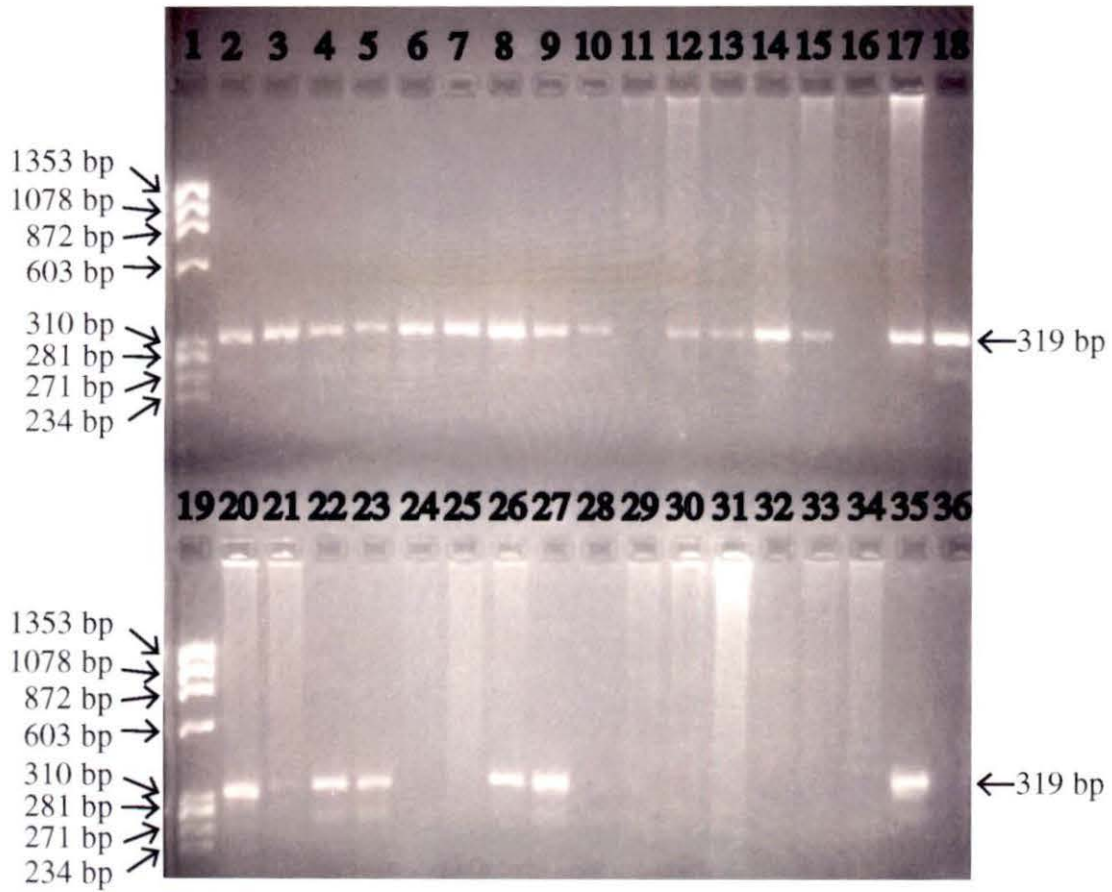
Fig 2. Extraction and amplification of DNA from mucosa of pigs on day 24 . Lanes 1 and 19 are φX174/*Hae* III markers. Lanes 18 and 35 are positive controls from a pure culture of *L. intracellularis*. Lanes 2-9 are from Group 1, Lanes 10-17 are from Group 2, Lanes 20-27 for group 3, and Lanes 28-34 are from group 4.

Weight gains

Table IV shows the average daily weight gains for each group. Group 1 had least weight gain compared with the other groups. Average daily gain was inversely related to the dose of inoculum.

Table IV.	Weight Gains
Group	Average Daily Weight Gains (kg)
1	0.399^{ab}
2	0.485^{a}
3	0.503^{b}
4	0.481^{ab}

Groups with same letter are not significantly different ($P>0.05$).

Discussion

This study demonstrated the use of various techniques to diagnose PPE. Only two animals had gross lesions, but many more were colonized by *L. intracellularis*. As expected, the animals with gross lesions were heavily infected as determined by silver and FA stains. One pig in the high dose group shed the organism (as determined by fecal PCR), but had no gross lesions, even though silver and FA stains showed that the ileum was heavily colonized. Also, one pig that shed the organism on day 24 had low levels of infection and no gross lesions. Shedding of the bacteria depended on the dose of inoculum as well as on the amount of colonization, as determined via silver stains and FA stains.

Although only two of the pigs that received a high dose of inoculum had gross lesions, PCR testing showed that the small intestinal mucosa of all these pigs was

infected. Pigs that received smaller doses of the same culture had a lower rate of infection.

Detection of infection by FA staining was also dose related: Positive results were obtained in 88% of the high-dose group and 63% of the medium- and low-dose groups.

The PCR tests of mucosa yielded more positive results than did FA testing in the high- and medium-dose groups but fewer positive results in the low-dose group. The H & E, silver, and FA stains had identical results for the high dose group; but H&E and silver stains yielded more positive results than did PCR and FA in the medium- and low-dose groups. These results may have been due to the lower sensitivity of H & E and silver stains: The control pig with suggestive microscopic lesions was negative for the presence of *L. intracellularis* by silver stain, FA stain, and PCR.

Clinical signs consisting of diarrhea were more apparent in the high-dose group. The incidence of diarrhea was consistently higher in the high-dose group. Nevertheless, looseness of the stools began to subside by day 14 in all affected groups. Average daily gains were lower in the high-dose group than in the other groups. However, these results were not statistically significant because of the low number of pigs in each group. Further studies involving larger numbers of animals inoculated with pure cultures may give a more accurate picture of reduced weight gains resulting from *L. intracellularis* infection.

Pure cultures have many obvious advantages over intestinal homogenate for inducing experimental PPE. Intestinal homogenates may contain other agents that can

induce microscopic lesions or clinical signs (e.g., diarrhea and weight loss). Pure cultures can also provide a consistent inoculum for this disease model unlike the variable intestinal homogenates.

Because of the difficulty in culturing the organism, diagnostic laboratories must rely on other methods for diagnosing the infection. PCR is the most specific test and appears to have the highest sensitivity when testing the ileal mucosa. Fecal PCR can be used as an ante-mortem test to screen for animals that are actively shedding. However, infected animals do not always shed the organism; fecal PCR results may depend on the stage or severity of infection. The H&E and Warthin-Starry silver stain are currently the methods used by most diagnostic laboratories for the detection of PPE but require sacrificing animals for testing.

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CHAPTER 5. EVALUATION OF ANTE-MORTEM POLYMERASE CHAIN REACTION AND SEROLOGICAL METHODS FOR THE DETECTION OF LAWSONIA INTRACELLULARIS EXPOSED PIGS

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Jeffrey P. Knittel, Dianna M. Jordan, Kent J. Schwartz, Bruce H. Janke,

Michael B. Roof, Steven McOrist, D.L. Harris

Abstract

Objective--Evaluate polymerase chain reaction (PCR) for the detection of *Lawsonia intracellularis* DNA in feces and an indirect fluorescent antibody test (IFAT) detecting serum IgG antibodies for the detection of pigs exposed to *Lawsonia intracellularis*.

Design—Three experiments were run on 7 week old and 3 week old pigs that were inoculated with pure cultures of *Lawsonia intracellularis*.

Animals--Twenty-three pigs were inoculated with a pure culture of *Lawsonia intracellularis*, 31 pigs were used as non-inoculated control pigs and 3 pigs were used as sentinels.

Procedure--Fecal shedding of *L. intracellularis* was monitored using PCR at 7 day intervals. Ilea were collected at termination for PCR and histopathology. Sera was collected at 7 day intervals and tested by IFAT.

Results--PCR detected *L. intracellularis* DNA in the feces of 39% of the inoculated pigs during the study. Ninety percent of the inoculated pigs developed IgG antibodies by IFAT at 21 to 28 days post inoculation. No *L. intracellularis* DNA or IgG antibodies were detected in any of the non-inoculated control pigs at termination. Sera from pigs inoculated with enteric pathogens other than *L. intracellularis* did not have detectable antibodies that reacted with *L. intracellularis* by IFAT.

Conclusion--The results of this study indicate the IFAT for anti-*L. intracellularis* IgG antibody detection appears to be a more sensitive ante-mortem test for detecting pigs experimentally infected with *L. intracellularis* than a PCR method for direct detection of the organism in the feces.

Clinical Relevance—Not all animals that are infected with *L. intracellularis* shed the organism at detectable levels in the feces. Only pigs that are actively shedding *L. intracellularis* are detected. (*Compendium* 1997;19:S26-S29.)

Introduction

Porcine proliferative enteropathy is a disease of worldwide distribution in pigs with variable clinical symptoms but common underlying pathologic lesions.[1] Similar lesions have been observed in many other species.[1] The subacute and chronic form of the disease reduces growth rates and causes diarrhea, occurring most often in pigs 6 to 20 weeks of age. The acute disease consists of severe diarrhea and acute deaths in pigs from 12 to 30 weeks of age or older. The characteristic pathological lesions consist of marked hyperplasia of enterocytes within crypts of the ileum, or, occasionally, jejunum, cecum or colon as well. A consistent feature is the presence of intracellular bacteria within the apical cytoplasm of proliferating crypt enterocytes.[2] The causative agent has been identified and assigned to a novel taxonomic genus, *Lawsonia intracellularis* gen., nov., sp. nov.[3], previously known by the vernacular name Ileal Symbiont (IS) intracellularis.[4]

Lawsonia intracellularis is a gram negative, obligately intracellular bacterium that must be grown in a reduced oxygen atmosphere. To date the organism has not been successfully grown on conventional cell-free media. The bacterium is co-cultivated in actively growing tissue culture cells at 8% oxygen with a growth time of about 6 days before passage to new cells.[5] Cells that are infected do not exhibit cytopathic effect (CPE), and infected cells are detected by a fluorescent antibody stain or an immunoperoxidase stain using antibodies specific for *L. intracellularis*.[6]

A consistent and sensitive ante-mortem diagnostic test for animals infected with *L. intracellularis* would be helpful to determine the actual prevalence of exposure. Direct culture from the feces is not a practical option due to the fastidious growth requirements

of the bacteria along with a high number of contaminating organisms commonly found in the intestine of pigs.

A test employing polymerase chain reaction (PCR) for amplification of the 16s ribosomal DNA of *L. intracellularis* offers both specificity and sensitivity for the detection of the bacteria.[7] By PCR amplification of a 319 base pair fragment of *L. intracellularis*, as few as 10^3 bacteria/g of feces have been detected successfully in experimentally inoculated animals.[8] However, in another study, animals experimentally infected with *L. intracellularis* did not consistently shed the bacteria in sufficient quantity in the feces to be detected by PCR even though the ileal mucosa was colonized.[9]

Serologic tests employing enzyme-linked immunosorbent (ELISA) and immunofluorescent assays also have been evaluated. In one study the predominant antibody class stimulated by infection with *L. intracellularis* was IgM as determined by IFAT.[10] This response persisted for about eight weeks. In another study, an ELISA test was developed to measure the IgG response against *L. intracellularis* in the sera of pigs.[11] The test animals for this study were either hyperimmunized by intramuscular injection of percoll gradient-purified bacteria from intestinal mucosa of PHE-affected pigs, or orally inoculated with intestinal homogenates from a PHE-affected 6-week-old pig. Results indicated low and variable antibody titers in inoculated pigs. These findings led to the conclusion that IgG plays a minor role in the immune response to *L. intracellularis* infections.

The goal of our study was to develop a more sensitive serologic test that would provide a method of ante-mortem diagnosis to facilitate epidemiological investigations.

The IgG response in pigs was compared to the detection of *Lawsonia intracellularis* DNA by PCR of the feces of pigs that were given pure cultures *L. intracellularis*. The IgG response was chosen over the other immunoglobulins because of the long lasting nature of the antibody which is necessary for epidemiological studies.

Materials and Methods

Pigs

In experiment 1, 15 pigs were obtained from a herd with no history or recorded cases of porcine proliferative enteropathy. The pigs were weaned at 18 days of age. At 7 weeks of age, the pigs divided into 2 groups of 10 pigs (inoculated) and 5 pigs (control) with similar weight ranges represented in each group. The 2 groups were housed in separate rooms with 5 pigs in a pen throughout the experiment and fed identical corn-soy meal diets.

In experiment 2, 24 pigs were obtained from a herd, other than the herd used in experiment 1, with no history or recorded cases of porcine proliferative enteropathy. The piglets were weaned at 18 days of age. At 3 weeks of age, the pigs were divided into 2 groups by evenly distributing the pigs between groups according to weight. The inoculated group had 8 pigs and the non-inoculated (control) group had 16 pigs. The groups were housed in separate rooms with all of the pigs from each group in the same pen. More pigs were incorporated into the non-inoculated group to better establish and confirm that the pig source was negative for *L. intracellularis*.

In experiment 3, 18 pigs, 3 weeks of age were acquired from a herd, other than the herds used in experiments 1 and 2, with no history or recorded cases of porcine

proliferative enteropathy. The piglets were weaned at 18 days of age. The pigs were housed in a similar fashion as experiment 1 and at 3 weeks of age were divided by evenly distributing the pigs according to weight into 3 groups. The inoculated group had 5 pigs. Two control groups with 5 pigs in each group were used. Three sentinel pigs were added to the inoculated group 7 days after inoculation. The groups were kept in separate rooms with all of the pigs in each room kept in the same pen.

All three studies were conducted in a double-blind fashion in which the identities of the pigs were not revealed to the investigators until laboratory testing was completed.

Origin of bacterial inocula

Lawsonia intracellularis strain N343 was obtained from the ileum of a 1.5 year old sow from a farm in Minnesota. The herd size was between 70-80 sows. At necropsy, the mucosa of the ileum was thickened with some hemorrhage. Giminez staining of the mucosa demonstrated many curved bacteria within intestinal epithelial cells.

Preparation of pig-derived *Lawsonia intracellularis* inocula

The intestinal sample was kept at -70° C until processing. Isolation of the bacteria was performed as previously described.[5] The sample was aliquoted and frozen at -70° C in 1 ml aliquots.

Preparation of inoculum

Co-cultivation of the bacteria was carried out as previously described.[5] Bacteria in the co-cultures were harvested after 8 to 10 passages by centrifuging the infected cells and media at 3000 x g for 20 minutes. The pellet was resuspended in sucrose-potassium-

glutamate[12] with 10% fetal bovine serum (FBS) (JRH Biosciences, Lenexa, KS) and passed 4 times through a 25 G needle.

Control inoculum

The inoculum for the negative control pigs consisted of SPG with 10% FBS containing non-infected tissue culture cells.

Inoculation of pigs

On day 0 of the 3 experiments, the pigs were weighed, fecal swabs were collected for PCR testing, and serum samples were collected. The inoculated pigs received a pure culture of *L.* intracellularis via stomach tube. The control pigs received non-infected tissue culture. Each pig was dosed with 2 ml inoculum intragastrically in experiment 1 and 10 ml inoculum intragastrically in experiment 2. In experiment 3, each pig was dosed with 14 ml inoculum via stomach tube. All inoculated pigs and 5 control pigs were injected with 4 mg dexamethasone intramuscularly on days 0, 3, 6, and 9 of the experiment. Three pigs were co-mingled with the inoculated pigs 7 days after initiation and received no dexamethasone treatment. The dexamethasone was administered to attempt to immunocompromise the pigs, and increase the likelihood of infection, and exacerbate clinical disease. A control group without dexamethasone treatment was included to compare clinical signs between conventional and immunocompromised pigs.

Fecal swabs and sera were collected on days 0, 7, 14, and 21 of experiment 1. Fecal swabs and sera were collected on days 0, 7, 14, 21, 28, 35, and 42 of experiment 2. Fecal swabs and sera were collected on days 0, 7, 14, 21, and 28 of experiment 3.

Quantitation of inocula

Quantitation of the Tissue Culture Infectious Dose 50 percent $(TCID_{50})$[13] of viable *L. intracellularis* was accomplished by making serial 1:10 dilutions of the inoculum in Dulbecco's Modified Eagle's Medium (DMEM)(JRH Biosciences, Lenexa, KS) with 5% FBS. The dilutions were added to a 96 well microtiter plate with 0.1 ml/well and 12 wells for each dilution. The microtiter plates were seeded with tissue culture cells at 1250 cells/well and grown 18-24 hours prior to infection. The plate was incubated for 6 days in atmospheric concentrations of 8.0% O_2, 8.8% CO_2 and 83.2% N_2. The cells were fixed with cold 50% acetone and 50% methanol for 2 minutes. To the wells, 0.03 ml/well of anti-*L. intracellularis* monoclonal antibody[6] diluted 1:2000 in PBS was added. The plate was incubated for 30 minutes at 37° C and then washed 3 times with PBS. Anti-mouse immunoglobulin G-fluorochrome conjugate (fluorescein isothiocyonate) diluted 1:30 in PBS was added at 0.03 ml/well and incubated 30 minutes at 37° C. The plate was washed 3 times with ddH_2O and allowed to dry. Samples were observed on a fluorescent microscope and the $TCID_{50}$/ml of bacteria was calculated using the Reed-Muench method.[13]

Inoculated pigs in experiments 1, 2 and 3 received an inoculum of $TCID_{50}$ $10^{5.7}$, $TCID_{50}$ $10^{6.7}$, and $TCID_{50}$ $10^{6.7}$ respectively on day 0 of the experiments.

Clinical observations

Daily health observations for all 3 experiments were made including stool consistency, behavior, appetite, body condition, and hair coat. Any symptoms that were abnormal were recorded.

Necropsy and collection of samples

All pigs in experiment 1 were euthanized 21 days after inoculation. Samples of ileum and colon were fixed by immersion in 10% buffered formalin. Sections were processed routinely, stained by hematoxylin and eosin (H & E) and Warthin-Starry silver stains, and examined by light microscopy. Sections were also examined using an indirect immunofluorescence assay, incorporating a monoclonal antibody against *L. intracellularis*, as described previously.[6]

In experiment 2, 4 control pigs were randomly selected for necropsy at 21, 28, 35, and 42 days post-inoculation. Four pigs from the inoculated group were necropsied at 21 days post-inoculation and the remaining 4 pigs were necropsied at 42 days post-inoculation.

In experiment 3, all pigs except the 3 sentinels were necropsied 21 days post-inoculation. The sentinels were necropsied on day 28 of the study, 21 days after co-mingling.

Polymerase chain reaction

Fecal samples collected weekly as well as ileal mucosal scrapings obtained at necropsy were analyzed by PCR using primers as previously described.[7] The colons from experiment 3 animals were also tested by PCR. DNA was extracted using a guanidine thiocyanate nucleic acid extraction kit. The primer set, 5'-TATGGCTGTCAAACACTCCG-3' and 5'-TGAAGGTATTGGTATTCTCC-3' was selected for the DNA amplifications. Cycle parameters were 93°C for 5 minutes, 55°C for 45 seconds, and 72°C for 45 seconds for the first cycle. Thirty-three cycles were

performed at the previously mentioned temperatures for 45 seconds per temperature. The final cycle was performed at 93°C for 45 seconds, 55° for 45 seconds, and 72°C for 2 minutes. PCR positive samples produced a 319 bp product detected by gel electrophoresis.

Serologic test

Plates for serology testing were prepared by seeding 72 well Terisaki plates (Nunc, Denmark) with tissue culture cells at 125 cells/well and grown 18-24 hours prior to infection. Pure cultures of *L. intracellularis* strain N343 were added to Dulbecco's Modified Eagle's Medium (DMEM)/5% FBS at a dilution of approximately 3000-5000 bacteria/ml and 10 μl was added to each well. The plates were incubated for 6 days at gas concentrations of 8.0% O_2, 8.8% CO_2 and 83.2% N_2. The cells were fixed with cold 50% acetone and 50% methanol for 2 minutes. Serum samples were diluted 1:30 in PBS and 10 μl was added to the test well. The plates were incubated for 30 minutes at 37° C and then washed 3 times with PBS. Anti-swine immunoglobulin G-fluorochrome conjugate (fluorescein isothiocyonate, Organon Teknika Corporation, Durham, NC), diluted 1:10 in PBS, was added at 10 μl/well and incubated 30 minutes at 37° C. The plate was washed 5 times with ddH$_2$O and allowed to dry. Samples were observed on a fluorescent microscope and wells with fluorescing bacteria were labeled as serologically positive.

Specificity testing

Specificity of the test was determined by reacting serum from pigs experimentally exposed to the following enteric pathogens and known to have antibodies

against them: *Serpulina hyodysenteriae* (Provided by Dr. Michael Wannemueller at Iowa State University), *Salmonella typhimurium* and *Salmonella choleraesuis* (Provided by Dr. Michael Roof at NOBL Laboratories, Inc.), *Campylobacter mucosalis* and *Campylobacter hyointestinalis* (Provided by Dr. Connie Gebhart at the University of Minnesota) and *Serpulina pilosicoli,* and *Serpulina innocens* (Provided by Gerald Duhamel at the University of Nebraska).

Positive control serum was derived by hyperimmunizing 4 week-old pigs with *L. intracellularis* derived from a pure culture and inactivated in formalin. The bacteria were administered intramuscularly in Fruend's incomplete adjuvant 3 times at 2 week intervals. Negative control serum was obtained from a two-week old gnotobiotic pig.

Statistical analysis

The clinical scores for the 3 experiments were analyzed using the F-test in a repeated measures ANOVA. A Student's t-test was used for comparison of average daily weight gains in the 3 experiments.

Results

PCR testing

In experiment 1, fecal shedding was first observed at 7 days post-inoculation in 1 of 10 (10%) pigs from the inoculated group. Fecal shedding was detected in 3 (30%) pigs from the inoculated group by PCR at 14 days post-inoculation. These 3 pigs were different from the pig that was positive at day 7. No pigs (0%) from the inoculated or control groups were shedding at detectable amounts at 21 days post-inoculation. (Figure 1) PCR of the ilea from 4 of 10 (40%) inoculated pigs demonstrated the presence of *L.*

intracellularis. The ileal mucosa from the control pigs were all negative (0%) at day 21 by PCR.

In experiment 2, no fecal shedding of *L. intracellularis* was detected throughout the study. (Figure 2) PCR of the ilea did not demonstrate the presence of the bacteria in any of the pigs.

In experiment 3, PCR testing did not detect shedding of L. intracellularis in any of the inoculated or control pigs at 7 or 14 days post inoculation. PCR testing demonstrated *L. intracellularis* in feces and ilea of 5 of 5 pigs (100%) at 21 days post-inoculation. PCR testing did not detect *L. intracellularis* DNA in the feces or ilea of the control animals given dexamethasone (0 of 5, 0%) or no dexamethasone (0 of 5, 0%). (Figure 3) PCR testing did not detect *L. intracellularis* DNA in the feces or ilea of the sentinel animals. However, the organism was detected in the colon from one sentinel animal.

Clinical scores and average daily gains

Clinical observations for the 3 experiments essentially showed no differences between the inoculated and control groups in all experiments.

No significant ($P < 0.05$) differences in average daily weight gains were demonstrated between treatment groups in the 3 experiments. In experiment 1, inoculated pigs had an average daily gain of 0.5 kg and control pigs had an average daily gain of 0.48 kg. In experiment 2, the inoculated pigs had an average daily gain of 0.34 kg and control pigs had an average daily gain of 0.42 kg. And, in experiment 3, inoculated pigs had an average daily gain of 0.35 kg, control pigs given dexamethasone

had an average daily gain of 0.39 kg and control pigs not given dexamethasone had an average daily gain of 0.47 kg.

Indirect fluorescent antibody test

In experiment 1, IgG antibodies against *L. intracellularis* were first observed 14 days after inoculation in 3 of 10 animals (30%). At 21 days post-inoculation, 9 of 10 (90%) inoculated pigs had IgG antibodies against *L. intracellularis*. No control animals seroconverted. (Figure 1)

In experiment 2, IgG antibodies against *L. intracellularis* were first observed at 14 days after inoculation in 2 of 8 (25%) animals. At 21 days post-inoculation, 7 of 8 (88%) inoculated pigs had IgG antibodies against *L. intracellularis*. One of fifteen (7%) pigs in the negative control group had a positive response at 21 days post-inoculation, but was not positive at later samplings. (Figure 2)

In experiment 3, IgG antibodies against *L. intracellularis* were first observed 21 days after inoculation, when 5 of 5 (100%) inoculated pigs had IgG antibodies against *L. intracellularis*. No pigs in the control groups seroconverted. (Figure 3) One sentinel pig (33%) that was co-mingled with the inoculated group seroconverted 21 days after co-mingling. This was the same sentinel animal with a PCR positive colon.

Ninety percent of the animals exposed to *L. intracellularis* in these 3 experiments developed an IgG antibody response.

Histopathology

In experiment 1, light microscopy examination of intestinal sections of all pigs demonstrated the presence of mild lymphoplasmacytic atrophic enteritis and mild crypt

hyperplasia. This lesion was consistent amongst all pigs. Silver stains demonstrated hyperplastic crypt epithelium to frequently contain granular material in the apical portions of enterocytes. Within this dark staining material, curved rod-shaped bacteria could not be discerned. Four of 10 (40%) inoculated pigs had moderate crypt hyperplasia typical for PPE, in which intracellular bacteria were identified. Immunostaining of intestinal sections with *L. intracellularis* specific monoclonal antibody demonstrated specifically stained bacteria in the apical cytoplasm in 7 of 10 (70%) pigs from group 1. No pigs were positive by IFA stains in the control group.

In experiment 2, histopathological findings did not reveal any pigs to have lesions. IFA staining of intestinal sections did not demonstrate any pigs infected with *L. intracellularis*.

In experiment 3, light microscopy examination of intestinal sections revealed lesions typical for PPE in 5 of 5 (100%) inoculated pigs and in one (33%) pig from the sentinel group that was co-mingled with the inoculated pigs (same pig that was PCR positive and serologically positive). Within these sections, moderate to severe crypt hyperplasia was observed. Silver stains consistently demonstrated the abundant presence of curved intracellular bacteria within hyperplastic crypt enterocytes. IFA staining of sections from the animals demonstrated that intestinal epithelial cells from all pigs inoculated were moderately to heavily infected and two sentinel pigs co-mingled with the inoculated pigs were mildly to moderately infected with *L. intracellularis*. All negative control animals showed no abnormalities by H and E stains and silver stains. No specifically stained bacteria were detected by IFA in the control group.

Specificity testing of Serologic test

Porcine anti-serum against *Serpulina hyodysenteriae*, *Serpulina innocens*, *Serpulina pilosicoli*, *Salmonella typhimurium*, *S. choleraesuis*, *Campylobacter mucosalis*, and *C. hyointestinalis*, did not react in the IFA test.

No non-specific reactions were observed in the gnotobiotic serum. Dilutions of the hyperimunized serum, to determine the endpoint titration, demonstrated that antibodies were detected beyond titers of 1000.

Discussion

In this study, a serologic test was compared to PCR on feces and ileum to identify pigs exposed to *L. intracellularis*. Both tests can be used for ante-mortem diagnosis, however, PCR of feces has been shown to have limited sensitivity for the detection DNA of *L. intracellularis*.[8,9]

Combining the results of all three experiments, 39 percent of the experimentally infected pigs shed detectable levels of *L. intracellularis* DNA in the feces. PCR of the ileal mucosa at necropsy demonstrated 47 percent positive for *L. intracellularis*. In contrast, ninety percent of the animals exposed to *L. intracellularis* in these 3 experiments developed an IgG antibody response. Comparisons of fecal PCR and IFAT suggest this serological assay may be more sensitive for the detection of pigs infected with *L. intracellularis*.

PCR of the feces and ileum did not completely agree in experiment 1 as only one pig that shed in the feces was positive when testing the ileal mucosa. Three pigs that shed were negative in the ileum. Three pigs with PCR positive ilea did not shed during

the experiment. These results may be due to the low infectivity levels. Because of the absence of gross lesions, areas where colonization may have occurred may not have been collected for testing, which may account for the inconsistent results.

The variability of the infection or colonization may be due to several factors. The pigs for each experiment were derived from different herds. Also, there were age differences as experiment 1 used pigs 7 weeks of age and experiments 2 and 3 used pigs 3 weeks of age. Experiment 3 also used dexamethasone to attempt to increase the infection and exacerbate clinical disease. Infection frequently occurs in the absence of clinical disease. The serological data only indicates previous exposure to the organism rather than current or previous disease. Titers were not determined because of the subjectivity that could be encountered with this type of test especially when multiple people are interpreting the results.

Previous serological studies of pigs experimentally infected with *L. intracellularis* have shown that IgG responses using an ELISA were variable[11] and IgA and IgM responses using an IFAT were weak.[10] Those studies used antigens prepared from the intestines of infected pigs rather than antigen prepared from a pure culture. Sera from pigs may have reacted with other gut-associated antigens used for the tests. Evaluation of an ELISA using antigen derived from pure cultures produce results similar to those stated in previous studies (data not shown). In contrast, this investigation used a pure culture as antigen for both inoculating the pigs, as well as for the IFAT detection assay. A possible reason for the greater success of the IFAT over the ELISA test is that

IFAT background difficulties were avoided since the test requires identification of fluorescing bacteria over background. An ELISA does not allow this distinction.

PCR testing has value as a diagnostic tool. Holyoake, et. al., showed that PCR could be used to confirm the presence of *L. intracellularis* on farms using the pooled fecal samples from pens.[14] This allowed the investigators to estimate the age and location of exposure in the production facility. PCR testing, however, is quite expensive and labor intensive and may not be the most desirable method for ante-mortem screening of large numbers of pigs. Also, multiple sampling may be necessary in order to detect actively shedding pigs. McOrist, et. al., reported that only animals with active lesions excreted sufficient organisms for detection.[15] This was confirmed in another study which demonstrated that shedding appears to be cyclic and that pigs may not be shedding the organism even though the ileum is colonized.[9] This makes diagnosis difficult in a field situation since finding animals with active lesions may require many animals sampled at various time periods. The presence or absence of clinical symptoms is also not an accurate method to diagnose exposure. Treatment groups had little differences based on the clinical symptoms observed in the three experiments, yet differences in active shedding, colonization or serological response were seen between groups.

Further investigation is warranted to determine the duration of the immune response after exposure and the duration of maternal antibodies in endemic herds. Maternal antibodies appear to be detected using the IFAT for at least 5 to 6 weeks of age (unpublished data). Serologic testing would allow a much more economical and efficient method of screening pigs exposed to *L. intracellularis*. The test could aid in

determining the time and location within a production facility at which pigs are exposed to *L. intracellularis*. This would provide information to the practitioner on when and where preventative treatments should be given. As with most serological assays, pigs that are serologically positive are not necessarily infected with the organism. The duration of the serological response is yet to be determined and the length of *L. intracellularis* persistence has been variable in the pure culture model.

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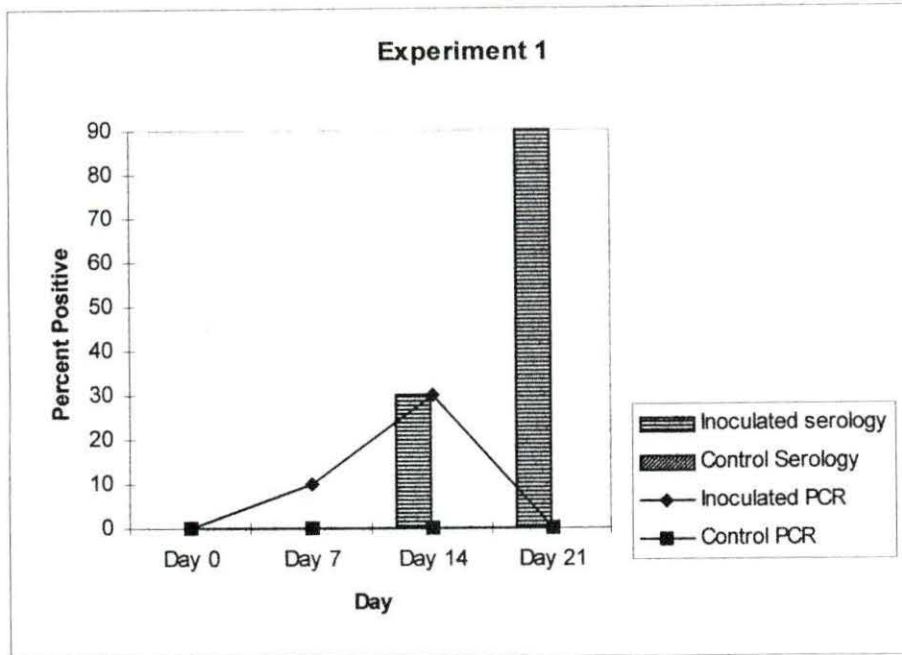
Figure 1--Percentage of pigs positive by serological IFAT and PCR in experiment 1.
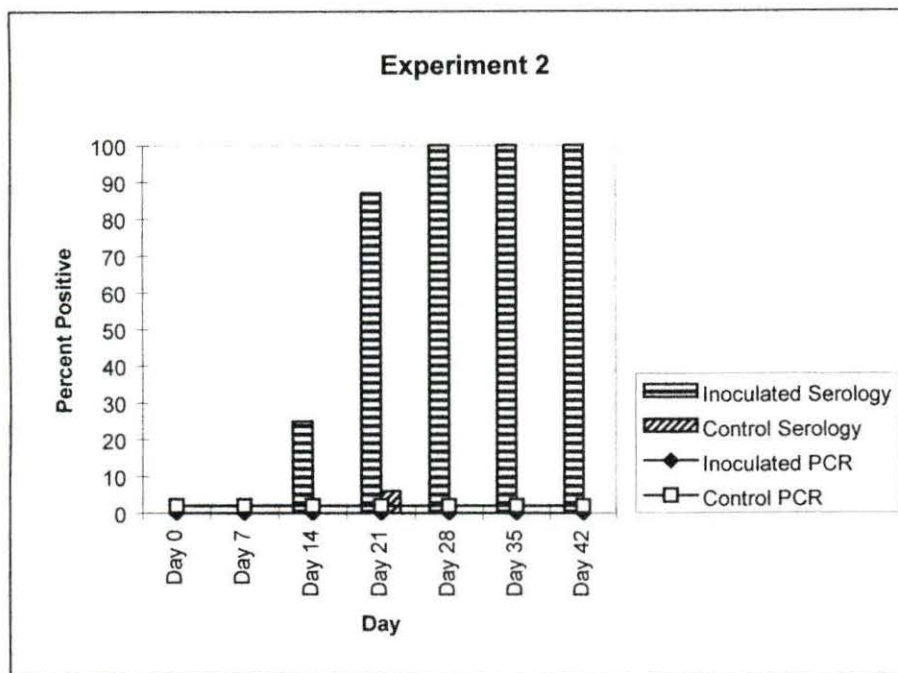

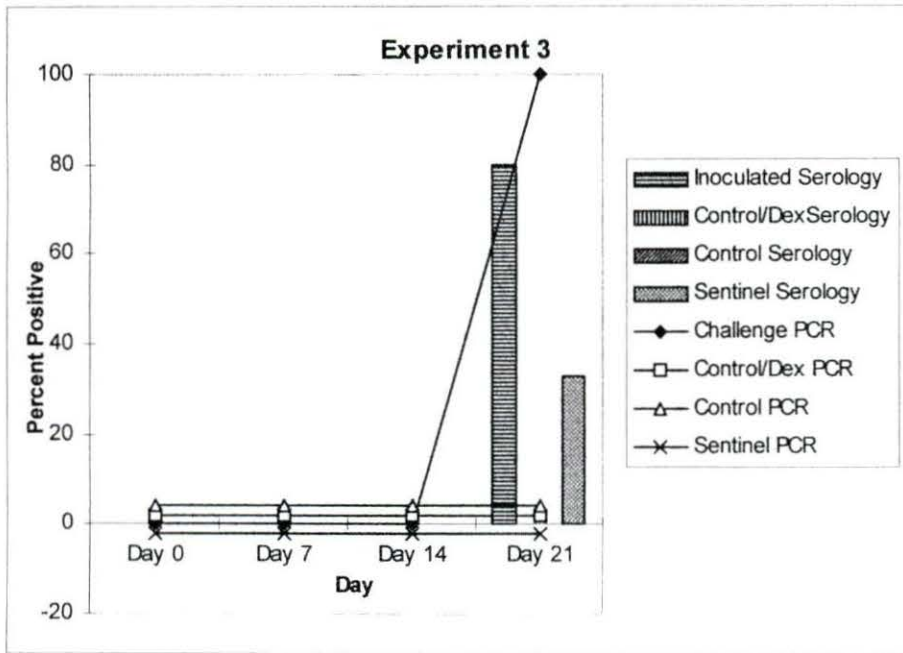Figure 2--Percentage of pigs positive by serological IFAT and PCR in experiment 2.

Figure 3--Percentage of pigs positive by serological IFAT and PCR in experiment 3.

CHAPTER 6. SUMMARY AND CONCLUSIONS

In the studies described herein, isolates of *Lawsonia intracellularis* from United States swine were isolated and cultivated *in vitro* to develop a better understanding of the organism and the symptoms and lesions of PPE. The isolates were compared to the characteristics of European isolates, for the development of challenge models, and for improvement of diagnostic techniques.

Results showed that isolates from U.S. swine were similar to European isolates in their growth requirements, phenotypic characteristics and DNA sequences of the 319 bp PCR product. They were antigenically similar based on their reactions with a monoclonal antibody prepared from an European isolate. Also, the PCR products were virtually identical in all strains tested.

The development of a challenge model enabled the comparison of several diagnostic techniques for identifying pigs with *L. intracellularis* infections. Pigs were infected with a pure culture of *L. intracellularis* and several diagnostic techniques were compared as to their value for the identification of infected pigs. The experiment showed that pigs intermittently shed the organism in their feces and that PCR as an ante-mortem technique may result in false negative results depending on the severity or stage of infection. PCR demonstrated to be the most sensitive and specific test when used on tissue samples.

PCR and IFAT were compared to determine their value as ante-mortem diagnostic tools for identifying pigs that were exposed to *L. intracellularis*. It was determined that the IFAT was a reliable, specific, and sensitive test for detecting infected or exposed pigs.

Comparisons to fecal PCR results showed that the IFAT was more sensitive in detecting exposed pigs than PCR of the feces. The serology test can be used to show previous exposure, incidence, and prevalence of *L intracellularis* and help estimate the time of exposure. PCR can be used to show which pigs are actively shedding and help determine shedding patterns within a herd.

The results from the described experiments may be helpful in understanding the usefulness of the various techniques that are available for diagnosing PPE. As these techniques improve, the true impact of the disease will be revealed. Also, diagnosis is extremely helpful to the producer as the correct method of treatment or prevention depends on identifying when and where the disease occurs.

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